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In vitro study of heat shock protein 60 expression and mitochondrial morphology in response to mitochondrial stress in HeLa cells

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Abstract

Diabetes mellitus (DM) is a metabolic disorder and affecting worldwide as stated by WHO that diabetes will be seventh leading cause of death in 2030. Diabetes mellitus is characterized by hyperglycaemia and the production of reactive oxygen species that damages proteins, lipids and DNA. This further elicits mitochondrial specific stress, resulting in increased production of HSP60 which is a mitochondrial stress protein. The aim of this study was to investigate the mitochondrial morphology changes and heat shock protein responses of HeLa cells subjected to physiological levels of hyperglycaemia (25mM).

HeLa cell growth was found to be slightly reduced with increasing glucose concentrations (10mM – 25mM) as compared to the control (5mM). Furthermore, high glucose conditions (25mM) also affected mitochondrial activity as statistically significantly decreased mitochondrial dehydrogenase activity was observed as compared to the control. These results altogether conclude that high glucose (25mM) acted as a mitochondrial stressor.

Mitochondria is a dynamic organelle that fuses and divides according to environmental stimuli and energy requirements. As a result the shape of mitochondria varies from small ovals to tubules and reticular networks. These events are mainly controlled by fission and fusion proteins and regulatory machinery. Therefore mitochondrial morphology was also analyzed by using confocal microscopy to determine the effects of high glucose (25mM) on mitochondrial morphology as compared to the control. It was found that the

majority of mitochondria in the control (5mM) was tubular as compared to high glucose which showed increased mitochondrial fragmentation suggesting that growing cells in the presence of hyperglycemic conditions lead to mitochondrial stress.

Mitochondrial specific stress results in selective induction of molecular chaperone, HSP60 which is mainly localized in the matrix of mitochondria. So furthermore , the expression of HSP60 was investigated. Consistent western blots results showed upregulation of HSP60 with heat shocked protein set as a positive control but not with high glucose concentration (25mM). Interestingly, HSP70 expression was found to be upregulated with high glucose (25mM) as compared to control therefore it can be concluded that there were more general cellular stress as compared to mitochondrial specific stress.

This study concluded that high glucose (25mM) effects HeLa cell growth and mitochondrial activity as well as mitochondrial morphology . There are various evidence which links oxidative stress and mitochondrial dynamics and observed increased production of ROS along with short and fragmented mitochondria in high glucose conditions.

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List of abbreviations

˚C	degree Celsius
ADP	Adenosine diphosphate
AGE	Advanced glycated end product
T1D	type 1 diabetes
T2D	type 2 diabetes
WHO	world health organization
FADH	Flavin adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide
DAG	diacyl glycerol
mPTP	mitochondrial permeability transition pore
CypD	Cyclophilin D
ROS	reactive oxygen species
UCP2	uncoupling protein 2
mtDNA	mitochondrial DNA
OXPHOS	oxidative phosphorylation
Drp-1	dynamin-related protein 1
Mff1 and Mff 2	mitofusin 1 and 2
Opa 1	optic atrophy
GTP	Guanosine-triphosphate
ATP	adenosine triphosphate
MID	Middle
GED	GTPase effector domain

MiD 49 and MiD 51	mitochondrial dynamics proteins 49 and 51
ER	endoplasmic reticulum
CDK1/cyclin	cyclin dependent kinase1
CaMK1α	Ca^{2+}/calmodulin dependent protein kinase 1α
ROCK1	rho-associated coiled coil containing protein kinase 1
PKA	protein kinase
MAPL	mitochondrial anchored protein kinase
SENP 5	sentrin-specific protease 5
MIS	mitochondrial import sequence
TM	transmembrane helix
MPP	mitochondrial processing peptidase
IMS	Inner rmembrane space
HR	heptad repeat regions
DN	diabetic neuropathy
DRG	dorsal root ganglia
Pd	Parkinson disease
MPT	mitochondrial permeability transition
SIMH	stress induced mitochondrial hyperfusion
HSP	heat shock protein
HSE	heat shock elements
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
TBS	Tris buffered saline
DMEM	dulbecco's modified eagle media
FBS	fetal bovine serum
LSCM	laser scanning confocal microscope

PMSF	phenylmethylsulfonyl
DMSO	dimethyl sulfoxide
BCA	bicinchoninic acid
BSA	bovine serum albumin
TBS	tris buffered saline
TBST	tris buffered saline tween 20
SDS	sodium dodecyl sulphate

Chapter 1

Literature review: Introduction

This chapter provides an introduction to diabetes and its pathophysiology. The main mitochondrial factors involved in the pathophysiology of diabetes which have been reviewed in literature include mitochondrial structure and function, mitochondrial dynamics, as well as mitochondrial stress protein heat shock protein 60 (Hsp60) and general stress protein heat shock protein 70 (Hsp70).

1.1 Diabetes

1.1.1 Statistics

Diabetes mellitus is an abundant metabolic disorder that is caused due to high blood sugar (hyperglycemia). The World Health Organization (2012) diabetes factsheet states that 347 million people worldwide have diabetes and will be the seventh leading cause of death in 2030. The disease is mainly divided into two forms: type 1 diabetes (T1D) which develops due to a deficiency of insulin and type 2 diabetes(T2D) which is caused when the body cells do not respond to insulin or develop insulin resistance. T2D is the most common metabolic disease and WHO states that T2D related deaths will double between 2005 and 2030. Global estimates of diabetes prevalence suggested that in 2013, 382 million people had diabetes and this number is expected to rise to 592 million by 2035. The risk of diabetes is high and rising in every country and found to be the result of unhealthy lifestyle, obesity and societal influences. It has been found that diabetes is one of major public health problem in South Asia and diabetes

prevalence is higher in urban areas as compared to rural areas(Gupta & Kumar, 2008). Other finding in this area is an increase in the prevalence of diabetes in young children mainly below the age of 5 (Long & Bingley, 2013). The main factors found to be associated with increased risk of diabetes in all cases are obesity, changed lifestyle, diet, family history of diabetes and environmental factors. Further the biggest problem is that the diabetes is not a single disease, as many other complications are associated with this disease such as cardiovascular problems, atherosclerosis, retinopathy, neuropathy, stroke and many more which increases the mortality rate to high extent.

1.1.2 Pathophysiology of diabetes

Normal blood glucose level is maintained by a complex interplay between glucose absorption, production and utilization. Regulation of glucose is carried out by many hormones, mainly insulin, which is secreted by the pancreatic beta cells. Pancreatic beta cells secrete insulin on detection of high blood glucose. Insulin then maintains blood glucose level by facilitating various processes such as gluconeogenesis and glycogenesis. It is well established that defects in the insulin secretion by pancreatic beta cells results in the progression of hyperglycemia.

Normal glucose stimulated insulin secretion from the pancreatic beta cells is controlled by mitochondrial metabolism (**figure 1.1).** Various steps have to be completed inside and outside the mitochondria before the secretion of insulin. Primarily by glucose transporters (GLUT1) glucose is transported across the beta cell membrane. Glucose is converted to pyruvate by glycolysis of which 90% moves into the mitochondria where TCA cycle further breaks down the pyruvate into acetyl CoA .This leads to the production of ATP, along with reducing equivalents such as FADH and NADH. Next, through the electron transport chain, more ATP is generated. So as the processing of glucose increases , the ATP/ADP

ratio also increases. This increase causes the ATP-sensitive K channels to close and the depolarization of voltage gated Ca channels which causes the influx of calcium ions. Finally the insulin vesicles dock to the membrane and expel the insulin out of the cells (Kaufman *et al.*, 2015).

Besides glucose beta cells respond to other nutrients such as amino acids and fatty acids that can also stimulate insulin release. Leucine along with glutamine can enhance insulin secretion from beta cells. Leucine activates glutamate dehydrogenase which converts glutamate to a ketoglutarate which enters TCA cycle and enhances ATP production and hence insulin secretion. Synthesis of long chain acetyl CoA and DAG from free fatty acids has also been found to enhance insulin secretion from the beta cells (Fu et al, 2013).

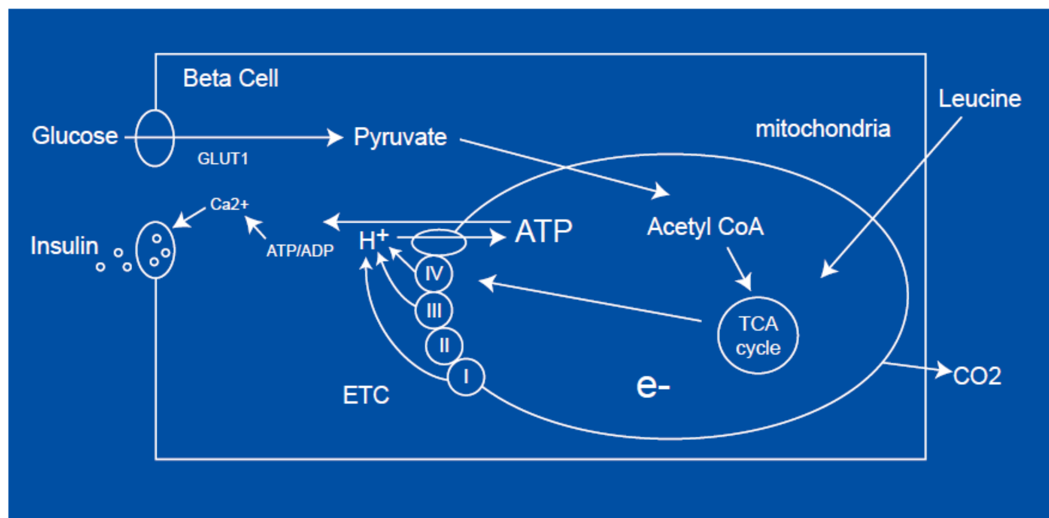

Figure 1.1: Beta cell mitochondrial system showing glucose sensing(Adapted from (Plaza, 2002)

Various insulin responsive organs such as skeletal muscles, liver and adipose tissue sense the insulin signals and respond according to it by maintaining the accurate amount of glucose in the body. When the insulin responsive organs do not respond to insulin or require a high amount of insulin for glucose sensing, this gives rise to a condition called insulin resistance which further results in the

failure of glycogen synthesis and inhibition of glucose production in the liver. This abruptly increases the levels of glucose in the body and is termed hyperglycemia.

Hyperglycemia is found to be one of the major causes for the altered mitochondrial function in diabetic persons. Hence, mitochondrial dysfunction is found to be associated with T2D (Lowell, 2005) . It is found that mitochondrial dysfunction results in insulin resistance and pancreatic beta cell dysfunction. Beta cell dysfunction is also found to be associated with elevated circulating serum fatty acids levels which trigger the synthesis of toxic derivatives such as diacylglycerol, triacylglycerol and reactive oxygen species. This leads to undesirable metabolic changes such as impaired autophagy and mitochondrial dysfunction which collectively lead to type 2 diabetes (Supale *et al.*, 2012; Cernea & Dobreanu, 2013). Opening of the mitochondrial permeability transition pore (mPTP) is also found to link mitochondrial dysfunction to insulin resistance in skeletal muscles (Taddeo *et al.*, 2014). The mPTP allows the exchange of molecules between the cytoplasm and mitochondrial matrix and maintains homeostasis. The inhibition of mPTP opening with CypD inhibitor cyclosporine A in vitro has been found to prevent insulin resistance (Taddeo*, et al.*, 2014)
Mitochondria as the power house of the cell plays an important role in the production of ATP through metabolic process such as tricarboxylic acid cycle, the electron transport chain and oxidative phosphorylation which also results in balanced production of reactive oxygen species (ROS). In different physiological states such as high blood glucose, various mitochondrial metabolic processes are disturbed leading to decreased ATP and increased ROS production which completely causes damage to all mitochondrial components. These defects in the

mitochondrial function have been found to play important role in causing insulin resistance and type 2 diabetes(Blake & Trounce, 2014). Some studies also suggest that mitochondrial dysfunction is caused by decrease in mitochondrial fatty acid oxidation which increases the level of diacylglycerol and intracellular fatty acyl CoA inducing insulin resistance in skeletal muscles. In 3T3-L1 adipocytes , mitochondrial dysfunction is found to be induced by high levels of glucose and free fatty acids(Gao *et al.*, 2010). Mitochondrial dysfunction is also found as a possible cause of accelerated senescence of mesothelial cells when exposed to high glucose(Ksiazek *et al.*, 2008).

The other factor that causes mitochondrial dysfunction is the change in function and expression of uncoupling protein-2(UCP2) present on the inner membrane of mitochondria. Studies suggest that UCP2 decreases glucose stimulated insulin secretion as well as decrease the yield of ATP from glucose. It was found that genetic deficiency of UCP2 greatly improves B cell function in rodents model of diabetes. Many studies together suggest that UCP2 plays important role in type 2 diabetes (Lamson et al, 2002).

Mutations in the mitochondrial DNA have also been found to be associated with type 1 and type 2 diabetes. Mitochondria contains its own DNA(mtDNA) which is double stranded, supercoiled , circular and present within the inner membrane in unprotected forms. Studies have shown that mtDNA mutations, mtDNA polymorphisms and polymorphisms in nuclear genes regulating mitochondrial function are associated with type 2 diabetes conditions.(Cho *et al.*, 2007)

Mitochondria are dynamic organelles, hence mitochondrial dynamics such as fusion and fission events found to play an important in maintaining mitochondrial shape and function. It was found that high glucose conditions causes alterations in mitochondrial morphology and dynamics. One study reported that 3T3-L1

adipocytes treated with high glucose and free fatty acids shows more compact and smaller mitochondria. Levels of mitofusion protein mfn 1 found to be decreased and levels of the mitofission protein Drp1 increased as compared to control one in high glucose treated 3T3-L1 adipocytes (Gao*, et al.*, 2010). It is also suggested that key molecules that mediates the autophagy pathways such as LC3B, Beclin1 and Drp1 also found to be associated with mitochondrial dysfunction in diabetic animal model- Goto Kakizaki rats (Yan et al, 2012).

Hence there are many evidences that states that mitochondria plays an important role in the pathopysiology of diabetes. As this thesis work is mainly related to study of changes in mitochondrial morphology in normal and hyperglycemic conditions, the structure and function of mitochondria as well as mitochondrial dynamics and morphology will be reviewed in detail.

1.2 Mitochondria: Structure and Function

1.2.1 Mitochondria

Mitochondria is a double membrane enclosed organelle and present in most of eukaryotic cells. There are two theories regarding the origin of mitochondria: endosymbiotic and autogenous. The endosymbiotic theory states mitochondria ancestors are prokaryotic cells and later became part of eukaryotic cells through endosymbiosis. Autogenous theory states that at the time of divergence with prokaryotes some portion of DNA from the nucleus split off and became enclosed by membranes giving rise to mitochondria. But the most established theory at this time is the endosymbiotic one. Mitochondria range from 0.5 to 10μm in diameter and are considered as the power house of the cells because most of the energy is generated in this organelle in the form of ATP through the process of oxidative phosphorylation.

1.2.2 Mitochondria Structure

From the structural point of view mitochondria is unusual as it contains two membranes which divides the entire organelle into four compartments: i) the outer membrane ii) the intermembrane space iii) the inner membrane and iv) the matrix. The outer membrane covers the entire organelle and forms a smooth boundary that separates it from the cytosol. It allows molecules that are 5000 Daltons or less in molecular weight to pass from one side of membrane to the other through porins. Large proteins pass through carrier proteins using selective mechanisms. The outer membranes also contains different enzymes for different kinds of functions. The outer membrane is important as disruption leads to leakage of proteins into the cytosol and ultimately death of the cells. The inner membrane as compared to outer membrane is highly impermeable and transport mostly all ions and molecules in and out of the matrix through specific channels and carrier proteins. The inner membranes folds into invaginations called cristae that floats in the matrix. It also contains enzymes and performs different functions. The complex and sophisticated machinery is also located in the inner membrane responsible for the production of ATP in the process of oxidative phosphorylation(OXPHOS) (Sherratt, 1991; Plaza, 2002).

1.2.3 Mitochondria function

Mitochondrial respiratory chains and ATP synthesis: ATP production in the mitochondria involves three steps: i) combustion of acetyl in tricarboxylic cycle(TCA) ii) the electron transport chain iii) ATP synthase action.

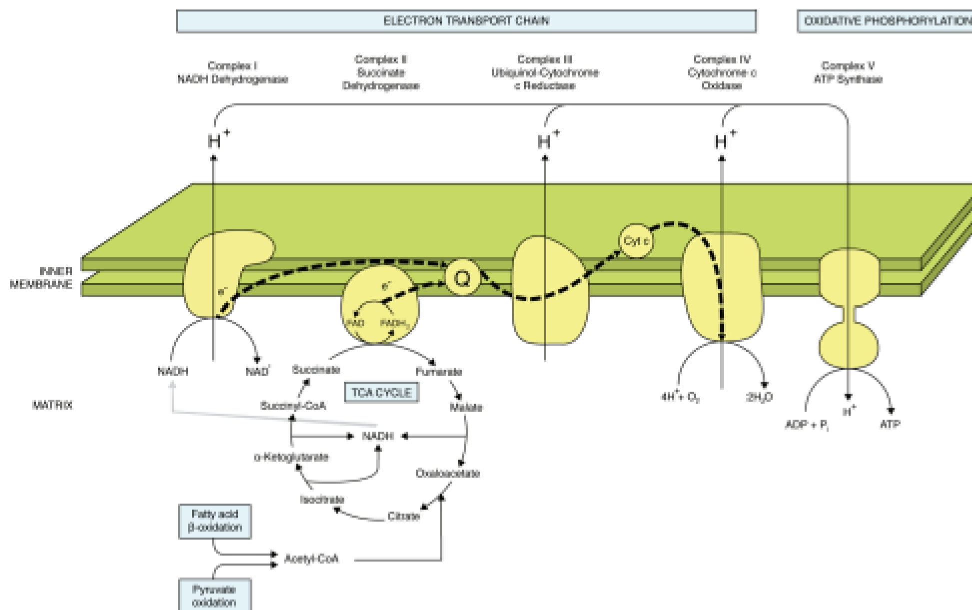

Figure 1.2: The general mechanisms leading to oxidative phosphorylation (Adapted from (Labieniec-Watala *et al.*, 2012)

ATP production is mainly carried by series of five complexes(I-V). Complex I which is NADH dehydrogenase carries out the oxidation of NADH and complex II that is succinate dehydrogenase responsible for the oxidation of FADH. Electrons from both complexes are transferred to ubiquinone, a small mobile molecule in the inner membrane which transfers the electrons further to complex III (ubiquinol-cytochrome c reductase) which further shift the electrons to cytochrome c placed in the intermembrane space and finally to the complex IV(cytochrome c reductase) which reduces the oxygen to water. The oxidation of NADH and FADH provides energy and transport the protons from the matrix to the intermembrane space which is responsible to create electrochemical proton gradient across the membrane and also termed as mitochondrial transmembrane potential **(figure 1.2).** The entry of protons back to the matrix through complex V (ATP synthase) generates energy that phosphorylates ADP into ATP. This process is called oxidative phosphorylation (OXPHOS) (Sherratt, 1991; Plaza, 2002).

1.2.4 Mitochondrial Networks

The numbers of mitochondria in a cell vary according to the energy demands of the specific tissue cells. Somatic cells contain few dozens to several thousand mitochondria per cell but the organs that are more metabolic active such as the brain, liver, heart and muscles contains large numbers of mitochondria. It is also found that like numbers of mitochondria, the dynamics and morphology of mitochondria is also cell or tissue specific. Generally a separate mitochondria shows large tubular structures called mitochondrial networks. Mitochondria changes its morphology from elongated, tubular structures to a condensed form according to the cell type, and changes in the surrounding circumstances. So this change in mitochondrial shape is mainly mediated by fission and fusion events , which is termed as mitochondrial dynamics (Zorzano *et al.*, 2009; Youle & van der Bliek, 2012). During fusion, two membranes fuse with each other whereas fission yields smaller mitochondria. The balance between these two events is very important as they control the shape, length and number of mitochondria, according to the demands of the cells. It has been reported by various studies that defects in fission and fusion give rise to various human diseases(Archer, 2013). In this theses work the morphology of mitochondria is studied mainly in hyperglycaemic and normal glucose levels conditions. It has shown that in high glucose conditions the cells mainly shows condensed mitochondria. So it makes important to review the process of fission and fusion events, molecular mechanisms and its regulation in details.

1.3 Mitochondrial Dynamics

1.3.1 Mitochondrial dynamics: Fission and fusion

Mitochondria is a dynamic organelle, mitochondrial morphology have a vast spectrum ,according to the demands and types of cells and tissues. Its form varies from small ovals to short tubules to elongated tubules and reticular networks. Mitochondria maintain their homeostasis through several processes and one of them is constant fission and fusion events. The fusion process allows the exchange of material between two healthy mitochondria and the fission process plays a role in the segregation of damaged mitochondria from the healthy ones and finally the removal of damaged mitochondria from the cell through mitophagy (autophagy) process(Ni *et al.*, 2015). There are several proteins and components that participate in the regulation and maintainance of mitochondrial morphology. Mitochondrial dynamics is mainly mediated by different forms of dynamin related GTPases which are highly conserved among mammals, yeast and flies. Dynamin-related protein(Drp-1) is the master fission mediator and fusion is mainly mediated by Mitofusin 1 and 2 (Mff1 and Mff2) involved in fusion of outer membranes and Optic atrophy (Opa1) that involved in fusion of inner mitochondrial membrane(Hall *et al.*, 2014).

1.3.2 Mitochondrial Fission Component: Drp1

Drp1 is the master fission mediator protein which is cytosolic protein and is recruited to the mitochondria during a fission event. It is an -80kDa protein mainly termed as dynamin related protein 1 in mammals, Dnm1 in yeast and DRP3A/B in *Arabidopsis thaliana.* Mutations in Dnm1 changes the mitochondrial compartment into a net of interconnected tubules and Dnm1 is also found to be localized on the cytoplasmic face of the constricted mitochondrial tubules by

immunogold labelling(Bleazard *et al.*, 1999) The domain structure shows high similarity to dynamin 1 protein as it contains GTPase domain at amino terminus, a middle (MID), a variable domain and GED (GTPase effector domain) at C-terminus(**figure 1.3).** It also contains two splicing regions: one in the GTPase domain and the other in the variable domain that results in the production

of approximately 12 different splice variants. The MID and GED domain forms a stalk which acts as an interface for interaction with adjacent Drp1. In this way the Drp1 molecule, which first appears as ring like structures, oligomerises itself and forms a tube or spirals itself around the mitochondria at fission site. The fission site is where GTP hydrolyses, performs constriction and finally divides the mitochondria(Smirnova *et al.*, 2001). During GTP hydrolysis the outer diameter of lipid tubule changes from -120nm to -60nm which releases drp1 from the lipid tubules. It was observed that dynamin 1 properly interact with lipids bilayer but Drp1 does not directly interact with lipid bilayer which shows that it requires additional cofactors or receptors on the mitochondria for its recruitement to the mitochondria (Galloway *et al.*, 2012; Elgass *et al.*, 2013).

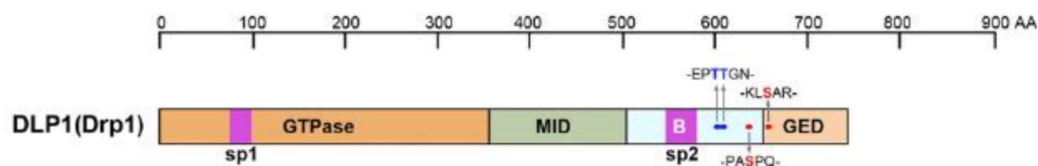

Figure 1.3: Molecular structure of dynamin-related GTPases DLP1(drp1) involved in fission (Adapted from (Galloway, *et al.*, 2012)

1.3.3 Additional factors in mitochondrial fission

Drp1 is recruited to the mitochondria by different kind of receptors. Fis 1 , a small protein present on the outer mitochondrial membrane was initially regarded as a receptor for Drp1. Knockdown of this protein was found to result in fragmented mitochondria and overexpression leads to condensed mitochondria. Mitochondrial fission factor(Mff) was identified as another receptor for the Drp1 and is also

present in the outer mitochondrial membrane . Both of these proteins anchor themselves to the membrane with their C terminal end with the rest of the protein projecting in the cytosol (Elgass, *et al.*, 2013). Both are found to recruit Drp1 protein but some contradictory results are also related to them. Knockdown of Mff was found to release Drp1 from the outer mitochondrial membrane resulting in a network type mitochondrial morphology whereas overexpression of Mff stimulate recruitment of Drp1 and mitochondrial fragmentation. It is also demonstrated that Mff dependent fission is independent of Fis1(Otera *et al.*, 2010). The two additional proteins that act as receptors for Drp1 are mitochondrial dynamics proteins- MiD49 and MiD51 , which are also present in the outer membrane anchored with the N terminal domain and majority of protein present in the cytosol(Elgass, *et al.*, 2013).

The endoplasmic reticulum has also been found to play a role in mitochondria fission.The ER and mitochondria fuses with each other and exchange materials such as protein and lipids between them. This fusion site is also recognised as future fission site and sites that colocalizes the Drp1 protein .However this event has been found to occur independent of this protein.

Other accessory proteins such as MTP18 located in the inner membrane and GDAP1 located in the outer membrane are also involved in the mitochondrial fission proteins (Palmer *et al.*, 2011; Elgass, *et al.*, 2013).

1.3.4 Regulation of mitochondrial fission

Drp1 protein undergoes several post translation modifications such as phosphorylation, S-nitrosylation, ubiquitination and SUMOylation which affect the dynamics, activity and localization of Drp1**(figure 1.4).**

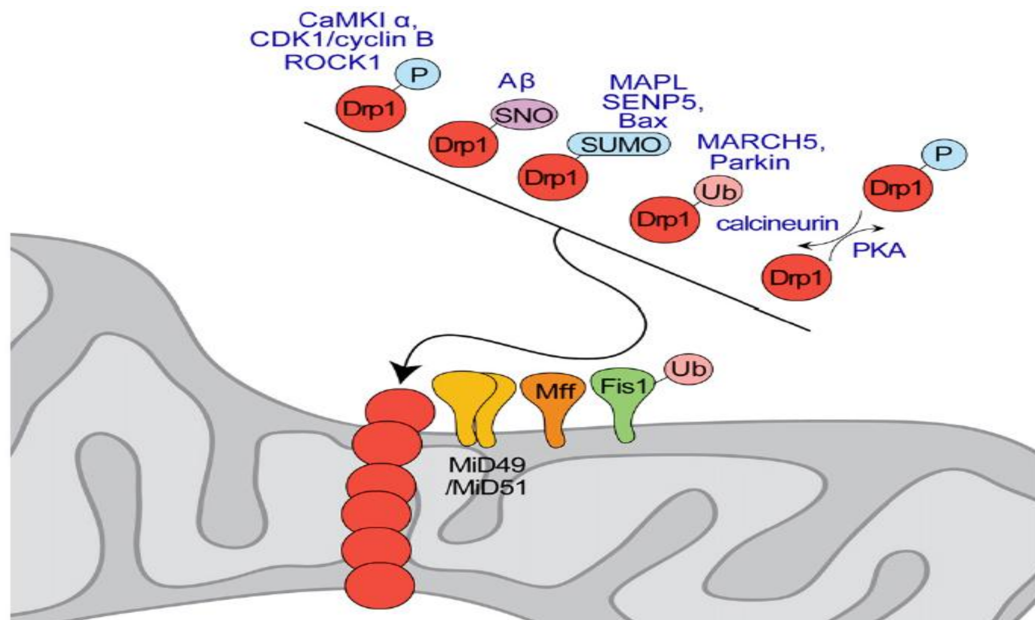

Figure 1.4: Regulation of mitochondrial fission via post translational modification of Drp1 (Adapted from (Elgass*, et al.*, 2013)

During mitosis, adequate segregation of mitochondria between two daughter cells are required which is facilitated by mitochondrial fission activity. Phosphorylation of Drp1 at Serine600 residue by cyclin dependent kinase 1(CDK1/cyclin B) stimulates the mitochondrial fission at the onset of mitosis. Similar to this phosphorylation by Ca2+/calmodulin dependent protein kinase 1a(CaMKI a) and Rho- associated coiled coil containing protein kinase 1(ROCK1) at the same serine 600 residue also stimulates mitochondrial fission by recruiting Drp1 to the membrane and interaction with Fis1. In contrast phosphorylation of Drp1 at the same ser600 residue by cAMP-dependent protein kinase (PKA) inhibits mitochondrial fission. Dephosphorylation of Drp1 by calcineurin increases mitochondrial fission by enhancing Drp1 translocation and association with Fis 1. Aurora A is a mitotic kinase associated with centrosome that phosphorylates small Ras like GTPase. Along with its effector RALBP1 relocalizes from the plasma membrane to mitochondria, enhancing Drp1 recruitment and the fission process(Elgass*, et al.*, 2013).

Mitochondrial fission is further stimulated by SUMOylation of Drp1 by mitochondrial- anchored protein ligase(MAPL) and Bax, whereas deSUMOlytion of Drp1 by sentrin- specific protease 5(SENP 5) also stimulate mitochondrial fission. MAPL is characterized as the first mitochondrial anchored SUMO-E3 ligase by using biochemical assays, overexpression and RNAi experiments. It has been stated that there is direct link between MAPL and fission machinery as Drp1 act as a substrate for MAPL(Braschi *et al.*, 2009). Ubiquitination of Drp1 and Fis1 by MARCH5 mainly facilitates Drp1 binding to mitochondria correct scission sites and enhances fission . MARCH mutants and MARCH RNAi was found to inhibit mitochondrial division and induce elongation of mitochondria so it is identified as a critical regulator of fission process(Karbowski *et al.*, 2007). Parkin(Parkinson associated cytosolic E3 ligase) was also found to ubiquinate Drp1 and target it for proteosomal degradation . Studies have revealed that mutations or knockdown of Parkin protein inhibits ubiquitination and degradation of Drp1 which increases the level of Drp1 and mitochondrial fragmentation(Wang *et al.*, 2011).

Drp1 in apoptosis: Cell death or apoptosis is mainly characterized by conversion of elongated mitochondria forms to small swelling or fragmentation, depolarization of membrane potential and release of cytochrome c. Drp1 as a mediator of mitochondrial fission also founds to play a novel role in apoptosis as it is found that Drp1 redistributes to mitochondrial membranes during apoptosis inducing fission and swelling of mitochondria. Inhibition of Drp1 functions founds to block apoptotic cell death(Frank *et al.*, 2001).

1.3.5 Mitochondrial fusion proteins

Elongated tubules of mitochondria is mainly mediated by two forms of proteins: t Opa1 and two isoforms of Mfn(Mfn1 and Mfn2). Both are dynamin related GTPases proteins. Opa 1 is responsible for the fusion of the inner membranes as they are anchored with their N- terminal domain in the inner membrane and the rest of the protein body in the intermembrane space. Opa 1 structure like the dynamin protein consists of three conserved regions : GTPase domain, middle region and GED domain**(figure 1.5).** Before the GTPase domain , the mitochondrial import sequence(MIS) is present followed by transmembrane helix(TM1) and hydrophobic segments(TM2a and TM2b) . This entire portion is mainly a region of alternative splicing that generates eight splice variants of Opa1. MIS targets Opa1 to the mitochondria and on reaching at its target site this sequence is cleaved by Mitochondrial processing peptidase(MPP) generating long forms of OPA1(l- Opa1) and further processing of opa1 generates short forms of opa1 . l-Opa1 is anchored in the inner membrane whereas s-opa1 is peripherally attached to inner membrane and rest of proteins are in the IMS (Belenguer & Pellegrini, 2013).

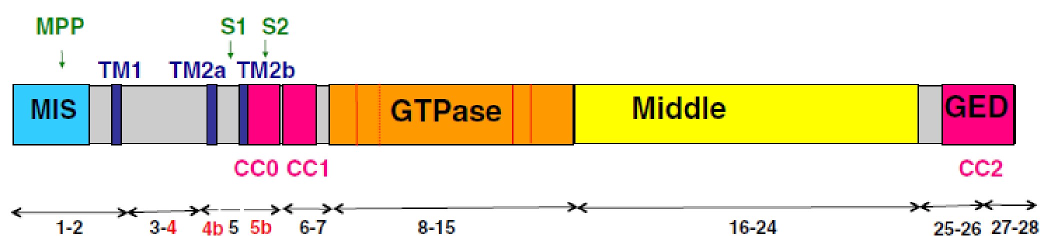

Figure 1.5: Schematic representation of OPA 1 structure (Adapted from (Belenguer & Pellegrini, 2013)

Two isoforms of mitofusin (Mfn): Mfn1 and Mfn2 are the fusion proteins that are responsible for the fusion of the outer membranes in mammals. In yeast and flies, Fzo(fuzzy onions) is mitochondrial transmembrane GTPases that mediates mitochondrial fusion. Mfn also contains a GTPase domain at the N terminal and

two transmembrane domains in the C terminal which are flanked by Heptad repeat regions (HR regions)**(figure 1.6)** . The two transmembrane domains present in the outer membrane orientate rest of the Mfn molecule in the cytosol(Galloway*, et al.*, 2012). It was found that Mfn1 and Mfn2 both regulate mitochondrial fusion and are essential for embryonic development. Embryonic fibroblasts that lack these proteins show fragmentation of mitochondria(Chen *et al.*, 2003).

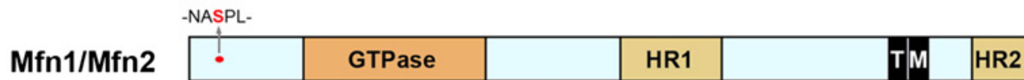

Figure 1.6: Schematic representation of Mfn 1/Mfn2 involved in fusion (Adapted from (Galloway, *et al.*, 2012)

1.3.6 Disrupted fission / fusion and mitochondrial function

Disruptions of mitochondrial fission , fusion and mitophagy are linked to mitochondrial dysfunction, cell death and several diseases. It is suggested that cells with mutations in Mfn1 and Mfn2 show low levels of mitochondria fusion and also less cellular defects. But cells that are deficient of Mfn1 and Mfn2 completely lacks mitochondrial fusion and show severe cellular defects such as poor cell growth, decreased cell respiration and heterogeneity in the membrane potential. The same results were observed with the disruption of Opa1 by RNAi(Chen *et al.*, 2005b). Mutations in the fusion proteins Mfn1 and Opa1, cause oxidative stress resulting in the disruption of morphology, producing dysfunctional mitochondria mentioned as an early event in neurodegeneration (Knott *et al.*, 2008). In hyperglycaemic conditions, Drp1 in pancreatic beta cells were found to be increased drastically and it has also been reported that Drp1 plays an important role in high glucose induced beta cell apoptosis. It is also reported that by induction of Drp1 wild type mitochondrial fission, cytochrome c release, caspase3 activation and decreased membrane potential all are

enhanced(Men *et al.*, 2009). Diabetic neuropathy(DN) shows degeneration of dorsal root ganglia(DRG) neurons and mitochondrial damage as an in vitro model of DN exposed to high glucose exhibited an increased level of fission protein DRP1 and pro apoptotic proteins, Bim and Bax, These proteins promote mitochondria fragmentation and apoptosis which suggests a role of mitochondrial fission in DN (Leinninger *et al.*, 2006). Altered mitochondrial dynamics in diabetes has been observed which shows increased mitochondrial fragmentation and increased production of reactive oxygen species. When cultured human aortic endothelial cells are exposed to high glucose condition it exhibits increased expression of Fis1 and Drp1 and also a loss of mitochondrial networks which links altered mitochondrial dynamics to endothelial dysfunction in diabetes(Shenouda *et al.*, 2011). Mitofusin 2(Mfn2) proteins in cultured cells were found to regulate mitochondrial function by stimulating respiration, substrate oxidation and expression of subunits of respiratory complexes . Alterations in the activity of Mfn2 were found to reduce the mitochondrial function present in skeletal muscle in obesity and type 2 diabetes (Zorzano*, et al.*, 2009). Parkinsons disease(PD) , a movement disorder is also been linked with impaired mitochondrial dynamics in which mutations of PINK1 and Parkin makes animals more susceptible to ROS stress and mitochondrial toxins leading to mitochondrial dysfunction and also defective apoptosis processess (Büeler, 2009). Pathogenic mutations in Opa1 alters its functions and causes autosomal dominant optic atrophy and mutations in mfn 1 and Mfn2 cause axonal charcot – marie-tooth disease type 2A(Cartoni & Martinou, 2009; Burte *et al.*, 2015). Abnormal mitochondrial dynamics and oxidative stress has also been reported in Alzheimer disease in which mitochondrial were found to be fragmented and fission protein Dlp1 levels also found to be increased (Wang *et al.*, 2014).

1.3.7 Stress signalling of mitochondrial fission and fusion

Mitochondrial morphology is found to be highly linked to the bioenergetic status of the cells. The balanced fission and fusion process is required so that the cell surpasses various stress conditions such as redox state, nutrition state and toxicity load. Mitochondria first respond to these stress conditions by fusing into long hyper fused mitochondria, but if the mitochondrial damage is too high and stress prevails then the mitochondria undergoes under fragmentation to meet the high energetic demands of the cell. This finally lead to autophagy of the dysfunctional mitochondria or the cells from the organism. So its shown that during starvation mitochondria elongate and spare itself from the autophagy. This stress induced mitochondrial hyper fusion(SIMH) was found to rely upon Opa-l forms which is protected from proteolytic cleavage by hypoxia induced gene domain protein 1a (Higd-1a) bound to Opa1(Westermann, 2012). There are various studies linking oxidative stress and mitochondrial dynamics. Cells incubated in high glucose conditions show increased levels of ROS, which causes mitochondrial permeability transition(MPT) and cell death along with short and fragmented mitochondria mediated by Dlp1 (Yu *et al.*, 2008). Inhibition of mitochondrial fission prevents MPT and ROS overproduction, which all together demonstrate that mitochondrial fission is an important component for the increased level of oxidative stress. However, some studies state that oxidative stress initiate fission, so this process is still debatable. Dynamic change of mitochondrial morphology was also found to be linked or controlling insulin secretion in pancreatic β cells. It was observed that upon glucose stimulation (20mM) insulin secretion in INS-1E cell lines require mitochondrial function and induces short and fragmented mitochondria (Jhun *et al.*, 2013). Diet is also found to have its impact on mitochondrial dynamics . Different fat sources affect differently on mitochondrial

dynamics as omega 3 polyunsaturated fatty acids founds to improve mitochondrial function and induce fusion process in contrast to saturated fatty acids. Studies also demonstrate that altered mitochondrial function and an increased level of fission were found in liver and skeletal muscles in case of diet induced obesity(Putti *et al.*, 2015).

1.4 Mitochondrial stress and heat shock proteins

1.4.1 Heat shock proteins

Heat shock proteins belong to a superfamily of stress proteins expressed in response to various environmental or pathological conditions. Heat shock proteins(HSPs) were first discovered by Ferrucio Ritossa in 1962 who was studying the chromosomal patterns in puffs of salivary glands of drosophila. By mistake, the high temperature of the incubator resulted in a different puffing pattern which later on this was associated with the stress response and expression of HSPs(Ritossa, 1996). HSPs intracellularly function as molecular chaperons which play an important role in the correct folding of protein, transportation to their localized cell organelles and degradation of aged proteins. Six major families of HSPs include hsp 40, hsp60, hsp70, hsp90 and hsp110 and the small HSPs. All these HSPs are found to be localized in different specific cell compartments. These molecular chaperons functions to maintain proteins homeostasis as they sense any change in proteins levels and in the folding states of proteins. The expression levels of these proteins increases during a variety of stress conditions such as during high temperature, exposure of cells to heavy metals, oxidants and amino acids analogue, glucose analogue, microbial infections, hormones or antibiotics (Habich & Burkart, 2007). There is growing evidences which suggest

that under stressful conditions HSPs are released from the cells into the extracellular space. The extracellular HSPs act as danger signals to the immune system ((Pockley *et al.*, 2008).

1.4.2 Heat shock protein 60

Heat shock proteins 60 are the proteins with the molecular weight of approximately 60 kDa. They are also termed as molecular chaperons as they assist in folding and assembly of proteins. These proteins are present in all bacteria and eukaryotic cell organelles such as mitochondria as well as in the cytoplasm and chloroplasts in plants and shows high sequence homology. HSP60 is found to be localized in the matrix of mitochondria. It was found that when mitochondrial specific stress was applied in form of a loss of mitochondrial DNA resulted in the selective induction of other molecular chaperones, chaperonins(cpn)60 and cpn10(Martinus et al, 1996). HSP60 was found to be a mitochondrial specific protein assisting the correct folding and degradation of misfolded mitochondrial proteins.

The structural information for Hsp60 is mainly deducted from the bacterial homologue of HSP60, *Escheichia coli* GroEL which folds the protein in an ATP dependent manner along with co-chaperonin GroES also termed as hsp10. The crystal structure reveals that GROEL consists of 14 identical subunits arranged as two seven membered rings stacked back to back. A single subunit in each groel ring consists of three domains: apical domain which binds to the substrate as well as to the co chaperonin hsp10 (GroES), equatorial domains which bind ATP and intermediate domain which connects these two domains covalently and act as a hinge. When the co – chaperonin hsp10 which is a single heptameric ring when binds to hsp60 it produces a conformational changes which increases the size of

central gravity and make it available for the polypeptide to fit (Chaudhuri *et al.*, 2009).

The sequence characterstics of HSP60 shows that the C terminal ends of hsp60 contains one or more repeats of the sequence GGM or GGGM. Also the N terminal leader sequence is characteristic with basic and hydroxylated amino acids such as Arg, Lys, Ser and Thr important for the import of proteins into the mitochondria. In contrast of this no unique sequence features regarding hsp10 that have been identified (Gupta, 1995; Haldar *et al.*, 2015) .

1.4.3 Heat shock protein 70

Hsp70 is the heat shock protein with molecular weight of 70 kDa and it is the most highly conserved protein in evolution. It is present in all organisms from archaebacteria to plants and humans and it is approximately 50% identical to the prokaryotic homologue DnaK. There are at least eight different gene products of Hsp70 which all different from each other in localization, expression level and amino sequence. Hsp70-9 (mtHSP70 or GRP75) is located in mitochondria and Hsp70-5(Bip or GRP-78) is localized in endoplasmic reticulum. All other six gene products are located in cytosol or in the nucleus. The highly conserved structure of Hsp70 mainly consists of three domains: the N terminal or ATPase domain , the peptide binding domain the binds the unfolded or folded proteins or the C terminal domain with highly conserved sequence EEVD through which it binds to other co chaperones or HSPs. Some of the HSP70 members also have a localization signal at the N terminal that directs them to their particular locations or compartments and Bip that resides in ER also possess a retention sequence at their C termini. The HSP 70 family functionally act as molecular chaperones and assist in the .translocation of unfolded proteins across membranes of mitochondria

and ER which is caught by HSP60 and leads to protein folding(Kiang & Tsokos, 1998; Daugaard *et al.*, 2007)

1.4.4 Regulations of heat shock proteins

For the regulation of HSPs and for the activation of HSP gene expression under stress conditions, Heat shock factor1(HSF1) is found to be essential. HSF1 resides in the cytosol in an inactive form and an active form bound by HSP. Under stress conditions, HSF1 separates from HSP becomes phosphorylated by protein kinases and is converted from the inactive monomer to the active homotrimer DNA binding state and translocated to nucleus . During nuclear translocation, HSF1 results in HSP gene transcription by binding to HSE(heat shock elements) situated upstream of heat shock responsive genes (Ahn & Thiele, 2003). After transcription , mRNA HSP leaves the nucleus and is translated to HSP in the cytosol where it is again bound by HSF. This is how HSPs are regulated in stress conditions.

HSP60 is found to be found to be associated with type 2 diabetes mellitus (Imatoh *et al.*, 2009). In T2D , hyperglycaemia and production of reactive oxygen species were found to elicit mitochondria specific cell stress and hence increase production of HSP60 and HSP70 observed (Hall & Martinus, 2013) . There is evidence stating that heat and hydrogen peroxide activates HSF1 which lead to activation of HSP gene expression (Ahn and Thiele,2002). The increased induction of HSPs in response to various physiological, pathological and environmental stress , their immunoregulatory properties and their association with various diseases have raised their importance in the discovery field so that improved therapeutic approaches against various diseases conditions can be developed.

1.5 Aims and Objectives

According to world health organisations, diabetes will be the seventh leading cause of deaths in 2030. Diabetes mellitus is affecting people worldwide and results in various complications such as atherosclerosis and cardiovascular diseases. There are many studies and investigations that have shown that there are various mitochondrial factors involved in the pathophysiology of diabetes. This has created awareness regarding the role of mitochondria and opened pathways for new research and discoveries. So the aim of this thesis work was to study the role of mitochondria specifically mitochondrial morphology in high glucose conditions as well as the expression level of mitochondrial stress specific protein Hsp60 and general stress protein Hsp70 was also studied.

First i) to study the effect of high glucose on HeLa cell growth , a dose response curve will be prepared by growing HeLa cells in the presence of 5mM (control), 10mM, 15mM and 25mM as glucose level . ii) Next the mitochondrial function will also be analyzed using MTT assay. iii) To determine if there was any alterations to mitochondrial morphology during growth in high glucose levels live cell imaging of HeLa cell mitochondria will be carried out after staining the mitochondria with mitotracker red cmx ros by confocal microscopy.

iv) Finally, in order to determine if HeLa cells grown in the presence of high glucose was also able to elicit mitochondrial stress, the expression level of stress proteins Hsp60 and Hsp70 will be measured by western blotting.

Chapter 2

Materials and methods

2.1 Cell culturing

2.1.1 HeLa cell culture

HeLa cells were grown in the dishes in DMEM (Dulbecco's modified eagle medium) media containing 10% fetal bovine serum (FBS) at $37^{\circ}C$, 5%CO_2 in a humidified incubator. To maintain healthy growing condition media was changed on the 3rd day and cells were passaged every 7th day. During cells passage, media was removed and discarded. 5ml of PBS was used to wash the cells for 5min. After that trypsin (2ml) was added and placed in the incubator for 5 minutes and checked under microscope for cell loosening. Once the cells loosens completely it was neutralized by adding DMEM media (8mls) and centrifuged for 5 min at 1000rcf. The pellet was dissolved in fresh pre warmed DMEM media and divided further into different flasks for further cells culturing.

2.1.2 Dose response experiments for glucose treatment

Dose response experiment was performed to examine the growth of HeLa cells with different glucose concentrations (5mM, 10mM, 15mM and 25mM). Cells were seeded in 24 well plates at density of approximately 60,000 cells/ml and the growth was examined after every 24 hours for 7 days. Each well contained one ml of cell suspension and at least three wells were taken into account for one specific range of glucose. Cells counting were done by using tryphan blue exclusion

method and haemocytometer. Three wells with one specific range of glucose was counted and then averaged to find out the total number of cells/ml.

2.2 MTT Assay

MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] is a tetrazolium dye and MTT assay is used to assess the cell metabolic activity. MTT is reduced to purple colour formazon by NAD (P) H dependent cellular oxidoreductases enzymes. The coloured formazon crystals are dissolved into the coloured solution and absorbance is measured using a spectrometer which will be indicative of mitochondrial dehydrogenase activity. MTT dye was prepared by dissolving in DMEM at a concentration of 5mg/ml. Cells were seeded in 96 well plate in 100µl of DMEM media supplemented with different glucose concentration. The plate was placed in the incubator for three days. After 72 hours when the cells would be in the exponential phase, the media was removed and replaced with fresh media and also 20µl of reconstituted MTT was added and placed in the incubator for 2 hours. After 2 hours the media was carefully removed and discarded without disturbing the cell pellet and 100µl of MTT solubilisation solution was added and placed on the minishaker for 10 minutes. When the cell pellet became completely mixed up, the absorbance was read by using a dual filter one at 570nm and other at 650nm. Then the averaged sample readings were subtracted from the averaged blank reading as per equation 2.2

$$\text{Absorbance(corrected)} = \text{Sample averaged reading- blank averaged reading}$$

Equation 2.2 – Calculating corrected MTT absorbance.

Then the percentage of mitochondrial dehydrogenase activity is measured as per equation 2.3

Dehydrogenase activity (%) = 100% x Experimental sample absorbance (corrected) / Control sample absorbance (corrected)

Equation 2.3 – Calculating mitochondrial dehydrogenase activity.

2.3 Mitotracker RED CMX Ros Staining of HeLa Cells

To determine the effect of high glucose on mitochondrial morphology, HeLa cells were grown in the fluorodishes in normal (5mM) glucose level and high glucose (25mM) for three days. On the third day the cells were labelled with mitotracker red CMX ros and visualized under confocal microscope and images were captured.

2.3.1 Mitotracker red CMX ros

The vial containing 50µg of the dye was warmed to at the room temperature, then 94µl DMSO was added to make 1mM cmx ros stock solution. After that 10µl of 1mM cmx ros was taken out in separate tubes and 9.99 ml of DMEM was added to make 10ml of 1µm cmx ros. This 1µM cmx ros was stored in the freezer below -20°C and used as a stock solution.

2.3.2 Labelling with mitotracker

From the 1µM cmx ros solution 100nM cmx ros solution was prepared and used to label the cells. To label the cells, the media was removed from the fluorodishes and the cells were washed with DMEM media 3 times. After that 200µl of prewarmed working solution was added in the dishes and these dishes was incubated for 15 minutes at 37°C. Followed by the removal of labelling solution and washed with DMEM media 3 times and again the prewarmed DMEM media was added and observed under the confocal microscope. An Olympus FV1000 laser scanning confocal microscope (LSCM) was used to collect higher resolution

images using the Olympus FluroView software. When the imaging was performed using confocal microscope, the fluorodishes was placed in the incubator at 37°C and 5% CO_2 that was build around the confocal microscope.

2.4 Quantification of HSP expression

2.4.1 Heat shock control cells

Some HeLa cells were heat shocked so that can be used as a positive control. To prepare the heat shock samples, HeLa cells were grown in the cells culture flask for 3 days at standard incubation conditions and with normal glucose level. When the cells at exponential growth stage, media was replaced with media pre warmed at 45°C and placed in the water bath at temperature of 45°C for 30 minutes. Again the media was replaced with pre warmed DMEM media at 37°C and placed in the incubator at the standard conditions for 12 hours. After that cells were harvested for protein extraction.

2.4.2 Protein extraction

To extract the proteins, the cells in the culture flask at exponential growth stage were trypsinized and after centrifugation the pellets were resuspended in TENT buffer with freshly added 0.4mM phenylmethylsulfonyl (PMSF) as a protease inhibitor. After the addition of 1ml of TENT buffer samples were ruptured by vortexing and were placed in the fridge at 4°C for 30 minutes. After this time period, centrifuge the samples at 10,000rcf for 5 min at 4°C. Cloudy suspension were visible as a cell debris discard it and separate the whole protein and transferred to a new tube and stored at -20°C. Following the same procedure protein was extracted from culture flask with different glucose concentration and incubated at -20°C.

2.4.3 Protein estimation using BCA assay

Protein concentration was estimated using the Pierce BCA protein assay kit. The instructions were followed and albumin (BSA) standards and BCA (bicinchoninic acid) working reagents (WR) were prepared. BCA working reagents were prepared by mixing 50 parts of BCA reagent 1 with 1 part of BCA reagent B (50:1). 10µL of each standard and unknown sample were pipetted out and put in the micro plate wells in replicates. 200µl of the WR was added to each well and plate mixed thoroughly on the plate shaker for 30seconds. The plate was covered and incubated at 37°C for 30 minutes. The plate was cooled and absorbance was measured at 570nm using Bio-Rad micro plate reader. The concentration of the sample was calculated by plotting standard curve.

2.4.4 Protein separation and transfer

To separate the total protein, 10% polyacrylamide discontinuous gel was prepared then samples were loaded onto the gel and run in a Bio-Rad mini protean 3 cell gel tank. Samples were prepared by taking 20µg of the protein and made it up to 20µl by adding loading buffer. Samples were placed in the boiling water for 5 minutes to denature the protein. After denaturation, proteins were all loaded into the wells. Molecular standards (See blue pre-stained standard (1x)) were also run in each gel in separate lanes. As the gel was electrophoresed at 200v for 50min, the bands ran through the stacking cells and then resolving gel. At a time two gels can be prepared by using Bio-Rad mini protean 3 cell gel tank and for a reference one gel can be stained with coomassie blue dye to check the proper transfer of all bands throughout the gel.

After the protein separation was completed, proteins were transferred from the gel to the nitrocellulose membrane using eBlot protein transfer system. The

instructions were followed to perform fast semi dry electro blotting of proteins from mini polyacrylamide gels to membrane. First eBlot anode pad was removed out of the package and placed on the anode plate then the nitrocellulose membrane was soaked in the equilibration buffer for one minute and the membrane was placed on the eBlot anode pad. The air bubbles were removed gently with the small shovel. The gel that contained the protein samples was removed and washed with distilled water and placed on the membrane and again the air bubbles were removed. The appropriate gel window that completely covers the margin of the membrane was chosen and placed properly. After all this setup the cathode pad was removed and placed on the gel and lid was closed. The timing is set according the size of the protein; for heat shock protein 7 min was set. After the transfer of protein, the membrane was rinsed in distilled water and stained with ponceau red stain for 15 sec. The membrane was stained to check the proper transfer of proteins on the membrane and then washed the membrane with distilled water to remove the stain off.

2.4.5 Western blotting

The membrane was blocked in 10% skim milk in TBST at 4°C overnight. The next day the membrane was washed with TBST 6 times and for 5 min each time on a gyro rocker followed by incubation with primary polyclonal rabbit hsp60 (1:250) in 5%skim milk in TBST at 4°C in a humidified chamber for overnight. The following day the membrane was again washed with TBST six times for 5 min each time on gyro rocker. Then the membrane was incubated with secondary antibody Peroxidise conjugated affinipure goat anti-rabbit IgG 1:1000 dilution in 5% skim milk in TBST and placed in a humidified chamber on gyro rocker for 5 hours at room temperature. After 5 hours the membrane was washed with TBST 6 times and 5 min each time on a gyro rocker. After that the membrane was

incubated with Thermo scientific super signal west pico chemilumiscent (1:1) dilution for 1 minute on a glass plate. After 5 min the excess was removed and the membrane was placed in the plastic sheet and visualized using the LAS-100 plus gel documentation.

2.5 Media, reagents and common solution preparations

Table 2.1: Media, reagents and common solution preparations

Name	Composition
Full DMEM media	1mL penicillin/streptomycin (100X). 5mL FBS Bring volume to 50mL with DMEM medium then filter through a 0.2µm filter. Store at 4˚C.
HeLa media absent of glucose	1mL penicillin/streptomycin (100X). 5mL FBS Bring volume to 50mL with DMEM medium containing NO glucose then filter through a 0.2µm filter. Store at 4˚C.
Phosphate buffered saline (PBS)	8g NaCl (137mM). 0.2g KCl (2.7mM). 1.44g Na2HPO4 (4.0mM). 0.24g KH2PO4 (1.7mM). Dissolve in 800mL ddH2O and adjust to pH 7, bring to 1L.
1M Glucose	1.80g Glucose. Dissolve in 10mL ddH2O Prepared 5mM, 10mM, 15mM and 25mM glucose concentrations mixing right amount of 1M glucose with HeLa media absent of glucose
TENT buffer(Protein Extraction)	1.51g Tris (50 mM). 10.96g NaCl (150 mM). 0.37g EDTA (5 mM). 0.75 mL TritonX-100 (0.25%). Dissolve in 200 mL ddH2O, adjust to pH 7.4 and bring to 250mL. Supplement with 0.4 mM phenylmethylsulfonyl fluoride fresh.
1M Tris-HCL pH 6.8	30.285g Tris Dissolve in 200mL dd H_2O, adjust to pH 6.8 and then add H_2O upto 250mL.

1.5M Tris-HCL pH 8.8	45.42g Tris Dissolve in 200mL dd H_2O, adjust to pH 8.8 and then add H_2O upto 250mL.
0.5M Tris-HCL pH 6.8	15.14g Tris Dissolve in 200mL dd H_2O, adjust to pH 6.8 and then add H_2O upto 250mL.
10% SDS	5g SDS Make up to 50mL with ddH2O water.
10%APS(ammonium persulphate)	1g of APS make upto 10 ml of H_2O.
5x Electrode buffer	15g Tris (124mM). 72g glycine (959mM). 50 mL of 10% SDS (0.5%). Make up to 1L with ddH2O.
10x Tris buffered saline (TBS)	60.6g Tris (0.1M). 87.66g NaCl (1.5M). Dissolve in 800mL ddH_2O, adjust to pH 7.7 and bring to 1L.
TBS-Tween (TBST)	100mL 10x TBS. 0.5mL Tween20 (0.05%). Make up to 1 L with ddH2O.
Protein loading buffer	3mL 10% SDS. 1mL 1M Tris pH6.8. 0.2mL 2% bromophenol blue. 4mL glycerol. 0.8mL ddH2O. Mix well and store in 1mL aliquots at -20˚C.
2% bromophenol blue	1g in 50mL of dd H_2O.
Ponceau S.	Add in the order: 10mL dd water. 0.3mL glacial acetic acid. 33mg Ponceau S. Bring to 30mL with dd water and store at RT.
5% skim milk in TBST	12.5 g of skim milk dissolved in 250mL of TBST.
10% skim milk in TBST	25g of skim milk dissolved in 250mL of TBST.
Membrane stripping buffer	15g glycine. 1g SDS. 10mL Tween20. Dissolve in 800mL ddH_2O, adjust to pH 2.2 and bring to 1L.
10% Resolving gel	4.1mL dd H_2O 3.3mL 30% acrylamide bis stock(bio-rad) 2.5mL 1.5mL Tris(pH8.8) 100µl of 10% SDS 50µl freshly prepared 10% APS and 10µl TEMED before casting added in the fumehood A layer of dd H_2O overlaid left for 30min.

5% stacking gel	6.1mL dd H_2O 1.3mL 30% acrylamide bis stock(bio-rad) 2.5mL 0.5mL Tris(pH6.8) 100µl of 10% SDS 50µl freshly prepared 10% APS and 10µl TEMED before casting added in the fumehood left for 30min.
1 µM Mitotracker CMX Ros (CMX Ros) stock solution	50 µg CMX Ros Dissolve in 94 µl DMSO to make 1 mM CMX Ros Take 10 µl of 1 mM CMX Ros Add with 9.99 ml to make 10 ml 1 µM CMX Ros Store both solutions below -20oC
100 nM Mitotracker CMX Ros (CMX Ros) working solution	100 µl 1 µM CMX Ros Add with 900 µl HeLa DMEM media to make 100 nM CMX Ros Use immediately
Membrane stripping buffer	15g glycine 1g SDS 10ml Tween 20 Dissolve in 800ml dd H_2O and adjust to pH 2.2 and bring the volume to 1L.

2.6 Statistical analysis

In this work, Microsoft excel was used for all statistical analysis that was calculated. The mean values of the data were calculated where necessary and standard error of mean (S.E.M) was also calculated using equation displayed below:

S.E.M. = STDEV (A1:A2) / SQRT (COUNT (A1:A2))

Equation 2.6: Calculating standard error of the mean in Excel

To compare and determine significance of the data sets two tailored t-test was used . The accepted level of significance was $p< 0.05$ and denoted by “*”.

Chapter 3

Effect of glucose on HeLa cell growth

3.1 Introduction

High glucose or hyperglycaemia and oxidative stress are associated with type 2 diabetes. It is also been investigated by various studies that high glucose conditions impair mitochondrial function and also lead to the overproduction of reactive oxygen species. The first task of this project is to study the effect of glucose on HeLa cell growth. Different concentrations of glucose mainly the concentrations that are valid at a physiological level were used. The glucose concentrations used were 5mM (control), 10mM, 15mM and 25mM (high glucose level) and a dose response glucose curve was plotted. The methodology used for this task is described below.

3.2 Methods

Cell culture and dose response curves: To prepare the different glucose concentrations (5mM, 10mM, 15mM and 25mM), stock solution of 1mM glucose was prepared as in chapter2. Cells were seeded in 24 well plate at density of approximately 60,000 cells/ml containing 1ml of glucose free HeLa media supplemented with different concentration of glucose that is 5, 10, 15 and 25mM glucose in triplicate and the growth was examined after every 24 hours for 7 days. Cells counting were done by using tryphan blue exclusion method and haemocytometer.

3.3 Result

Dose response for glucose treatments: Cells that were treated with different glucose concentration such as 10mM, 15mM and 25mM showed slightly reduced growth rate as compared to controls as shown in **(fig 3.1).** But a considerable difference can be seen between control (5mM) and high glucose (25mM) **(fig 3.1).**

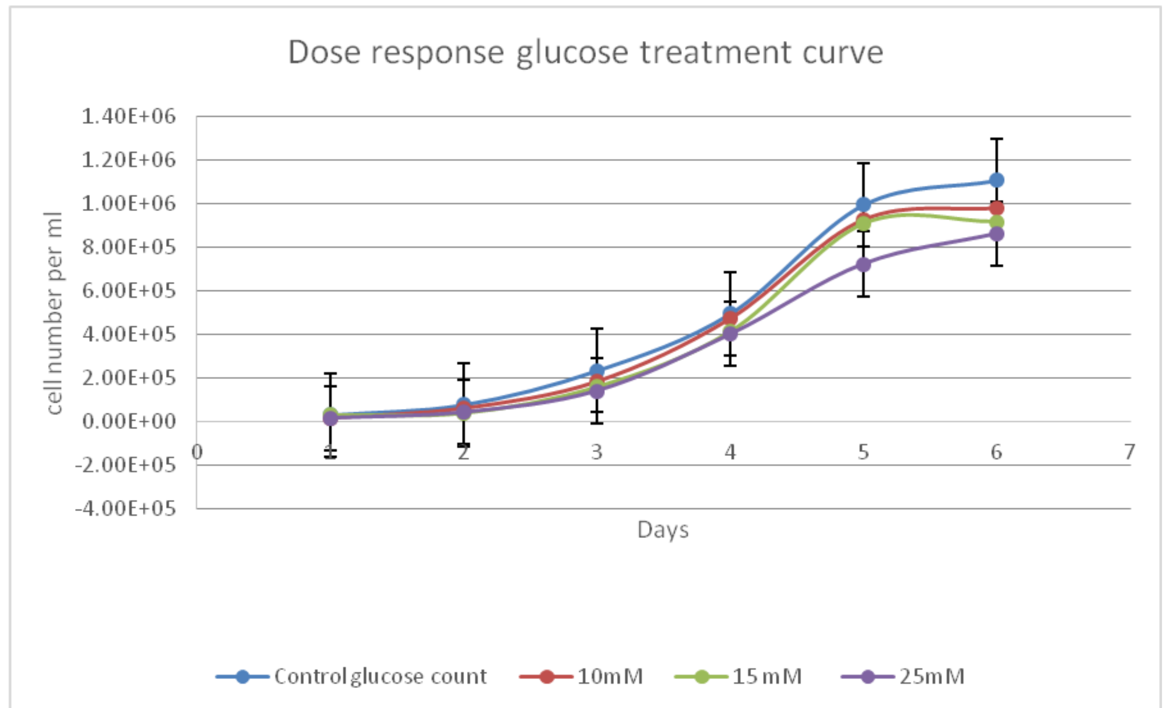

Figure 3.1: Cell growth in presence of various glucose levels.

3.4 Discussion

There are many evidences which states decreased cell growth in high glucose conditions. One study regarding the acute effect of high glucose on exposure to human proximal tubule epithelial cells (hPTEC) demonstrated dual effect that is early cell proliferation and late apoptosis. This study shows that early cell proliferation was due to MAP kinases being activated and late apoptosis was result of reactive oxygen species (ROS) being induced by high glucose (Samikkanu *et al.*, 2006). In cultured mesanglial cells it has also been shown that

high glucose levels cause an increase in the accumulation of extracellular matrix proteins such as collagen and fibronectin as well as increase in growth factors such as transforming growth factor-b1 (TGF-β1). It has also been suggested that this high glucose induced TGF-β1 production in porcine glomerular mesanglial cells is mediated by hexosamine biosynthetic pathways (Kolm-Litty *et al.*, 1998). High glucose induced endothelial cell growth inhibition, which is also found to be associated with the secretion of TGF-β1(Mather *et al.*, 2009). Culturing endothelial cells in constant high glucose and lipid exposure (up to 12 days long) was found to cause alteration in gene expression and cellular senescence (Meln *et al.*, 2012) Human umbilical endothelial cells, when exposed to intermittent high glucose, were found to stimulate the production of ROS which lead to cell apoptosis(Quagliaro *et al.*, 2003). Various studies have demonstrated apoptosis and decreased cell proliferation in high glucose levels and also the overproduction of ROS which further links high glucose conditions to mitochondrial damage as mitochondria are the main source of ROS in mammalian cells. Metabolism of Chinese hamster ovary (CHO) in cell culture was studied and characterized by high glucose uptake and increased production of ammonium and lactate which have inhibitory effects on cell growth and protein production. So strategies such as using limited glucose can combat this negative effect(Lao & Toth, 1997).

Chapter 4

Effect of high glucose on HeLa cell function

4.1 Introduction

MTT is an assay that is used to determine the mitochondrial dehydrogenase activity. The dose response glucose curve showed that growth was slightly impaired in case of high glucose conditions (25mM glucose concentration). Now the question is that is this impaired growth is due to necrotic cell death or any mitochondrial damage. So this MTT assay was used to determine if the impaired cell growth was due to any effects on mitochondrial activity. This assay is widely used and applied to determine the relationships between cell growth , cell differentiation and cell metabolism mainly when there are changes in cell culture environments such as high glucose conditions(Marshall *et al.*, 1995).

4.2 Method

MTT Assay:

MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] is a tetrazolium dye and is used to assess the cell metabolic activity. MTT is reduced to a purple colour formazan by NAD(P)H dependent cellular oxidoreductases enzymes. The coloured formazon crystals are dissolved into the coloured solution and the absorbance is measured using a spectrometer which will be indicative of mitochondrial dehydrogenase activity. MTT dye was prepared by dissolving in DMEM at a concentration of 5mg/ml. Cells were seeded in a 96 well plate in 100µl of DMEM media supplemented with different glucose concentrations. The

plate was placed in the incubator for three days. After 72 hours when the cells would be in the exponential phase, the media was removed and replaced with fresh media and also 20µl of reconstituted MTT was added and placed in the incubator for 2 hours. After 2 hours the media was carefully removed and discarded without disturbing the cell pellet and 100µl of MTT solubilisation solution was added and placed on the minishaker for 10 minutes .When the cell pellet was completely mixed up ,the absorbance was readied by using dual filter one at 570nm and other at 650nm. Then the averaged sample readings were subtracted from the averaged blank reading as per equation 2.2. The percentage of mitochondrial dehydrogenase activity was measured as per equation 2.3.

4.3 Results

The MTT assay was performed using the methodology described above. It was found that all glucose concentrations (10mM, 15mM and 25mM) showed a decrease mitochondrial dehydrogenase activity as compared to control (5mM) **(fig 4.1).** But only 25mM glucose concentration showed a statistically significant ($p< 0.05$) difference as compared to control which proved that some mitochondrial damage was there **(fig 4.1).** 25mM glucose concentration showed 30% decreased mitochondrial dehydrogenase activity as compared to control.

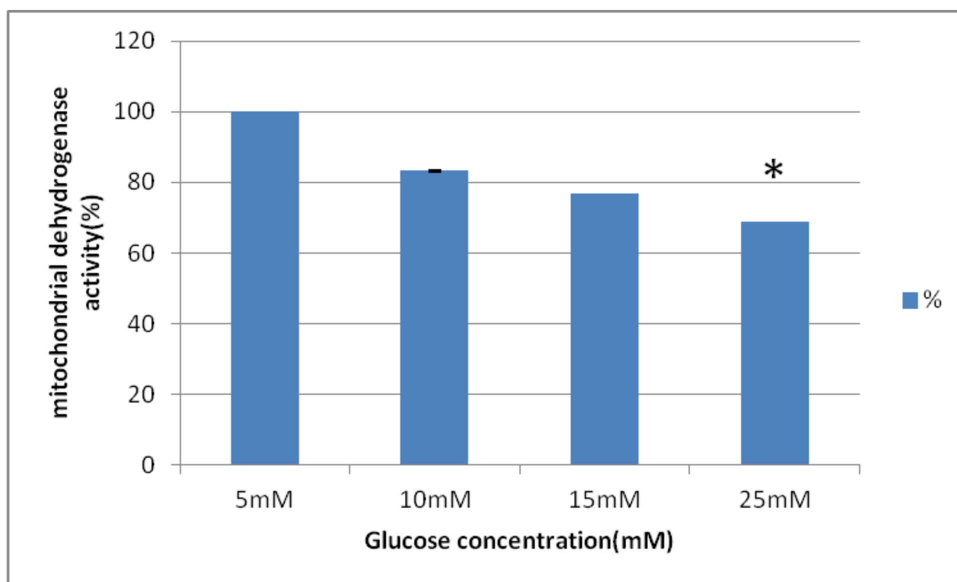

Figure 4.1: Mitochondrial dehydrogenase activity in the presence of glucose. Bar graph showing the mean ± S.E.M. * = $p < 0.05$ (n=3).

4.4 Discussion

The MTT assay results showed that high glucose conditions (25mM) has reduced mitochondrial activity. In diabetic conditions, hyperglycaemia is considered to be a pathogenic factor for many complications associated with diabetes and altered mitochondrial function is one of them. There are various studies linking hyperglycaemia to mitochondrial dysfunction. One of the main factors considered for mitochondrial dysfunction is hyperglycaemia induced oxidative stress, as well as the

formation of advanced glycation products (AGPs) (Rolo & Palmeira, 2006). Mitochondria are considered as a major site for ROS generation. During OXPHOS superoxide is released in small amounts on a routine basis by complex I and complex III of mitochondria. But in high glucose conditions it is proposed that an increase substrate availability increases flux by OXPHOS which in turn increases the production of superoxide, further causing various complications associated with diabetes. There are many mechanisms that are activated in hyperglycaemic conditions such as polyol pathways, Hexosamine pathways, Protein kinase C pathway , pentose phosphate pathways and advanced glycation end products pathways which play a role in the development of various complications(Blake & Trounce, 2014). High glucose and free fatty acid stimulates the production of ROS observed through protein kinase C dependent activation of NAD (P)H activation(Inoguchi *et al.*, 2000; Inoguchi *et al.*, 2003).One experimental study in axons of diabetic neurons in high glucose conditions observed that polyol pathways contribute to elevated level of ROS(Akude *et al.*, 2011). In vitro study found that high glucose increases the level of calcium in mitochondria results in increase production of reactive oxygen and nitrogen species led to mitochondrial dysfunction and apoptosis(Kumar *et al.*, 2012). In dorsal root ganglion(DRG) , high glucose was found to induce oxidative stress and mitochondrial dysfunction in terms of altered (Russell *et al.*, 2002). This mitochondrial reactive oxygen species contributes in the progression of diabetes by reducing insulin secretion by pancreatic beta cells(Sakai *et al.*, 2003). One study on human vascular endothelial cells concluded that high glucose levels had an effect on cell viability, induced cell apoptosis as well as increased levels of reactive oxygen species (Hou *et al.*, 2015). In 3T3-L1 adipocytes, mitochondrial dysfunction is found to caused by high levels of glucose and free fatty acids(Gao*, et al.*, 2010). So there are many studies that all together links hyperglycaemic conditions to the mitochondrial dysfunction and overproduction of ROS. High glucose also found to have a profound

effect on mitochondrial morphology and dynamics. Rat retinal endothelial cells grown in high glucose conditions showed disrupted mitochondrial morphology as well as increased mitochondrial fragmentation (Trudeau *et al.*, 2010). It is also postulated that in high glucose conditions, the dynamic change of mitochondrial morphology contributes to ROS overproduction and also highlights the importance of mitochondrial fission/fusion machinery in hyperglycaemic conditions(Yu *et al.*, 2006; Yu, *et al.*, 2008).

Chapter 5

Effect of high glucose on mitochondrial morphology

Dose response glucose curves and MTT assay have demonstrated that high glucose affects the HeLa cell growth (by showing reduced growth rate) and also affects HeLa cell function (MTT assay showed 30% reduced mitochondrial dehydrogenase activity in high glucose as compared to control) respectively. Therefore the next step was to check if there was any changes produced in mitochondria at the morphological level.

5.1 Introduction

Mitochondria were labelled using a fluorescent dye , Mitotracker RED CMX Ros that produced a strong red fluorescence and has peak absorption at 578 nm and a peak emission at 599 nm. The fluorescence of Mitotracker CMX Ros is dependent on actively respiring mitochondria where it then becomes oxidised and binds to the mitochondria.

5.2 Methods

To determine the effect of high glucose on mitochondrial morphology, Hela cells were grown in the fluorodishes in normal (5mM) glucose level and high glucose (25mM) for three days. On the third day the cells were labelled with mitotracker red CMX ros and visualized under confocal microscope and images were captured.

Mitotracker red CMX ros working solution and labelling : The vial containing 50µg of the dye was warmed at the room temperature then 94µl DMSO was added to make 1mM cmx ros stock solution. From the 1mM cmx ros solution 1uM and finally 100nM cmx ros working solution was prepared as described in chapter 2.4. To label the cells, the media was removed from the fluorodishes and the cells were washed with DMEM media 3 times. After that 200µl of prewarmed working solution was added in the dishes and these dishes were incubated for 15 minutes at 37°C. Followed by the removal of labelling solution, the cells were washed with DMEM media 3 times and again the prewarmed DMEM media was added and observed under the confocal microscope. An Olympus FV1000 laser scanning microscope (LSCM) was used to collect higher resolution images using the Olympus FluroView software.

5.3 Results

The cells viewed under the confocal microscope showed significant mitochondrial damage at the mitochondrial morphology level. Mitotracker staining showed that the mitochondrial morphology of HeLa cells in the control glucose level are mostly tubular and filamentous **(figure 5.1)**, whereas the mitochondria of the cells incubated in high glucose concentration (25mM) were mostly smaller and in condensed form which showed that high glucose act as mitochondrial stressor and results in mitochondrial fragmentation (**figure 5.2).** Both kind of mitochondrial morphology states that are tubular and condensed and were observed to be present in control as well as in high glucose conditions. **Figure 5.3 B** showed more of the condensed mitochondria along with some

tubular mitochondria in cells in high glucose. **Figure 5.4** also showing some of the stressed cells as well as tubular mitochondria in high glucose conditions.

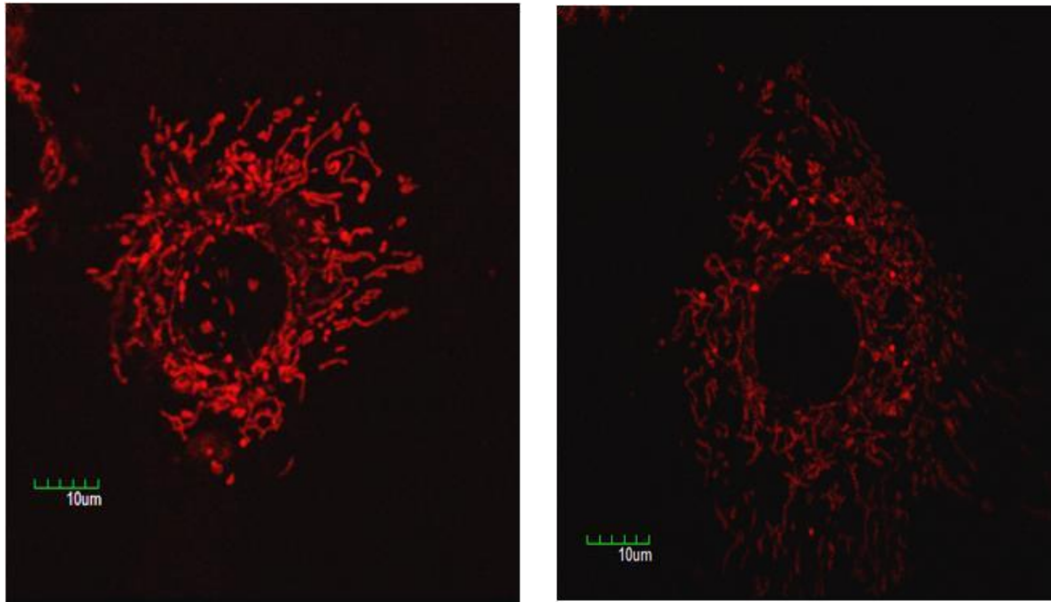

Figure 5.1: Cells with stained mitochondria in control under confocal microscope showing tubular mitochondria.

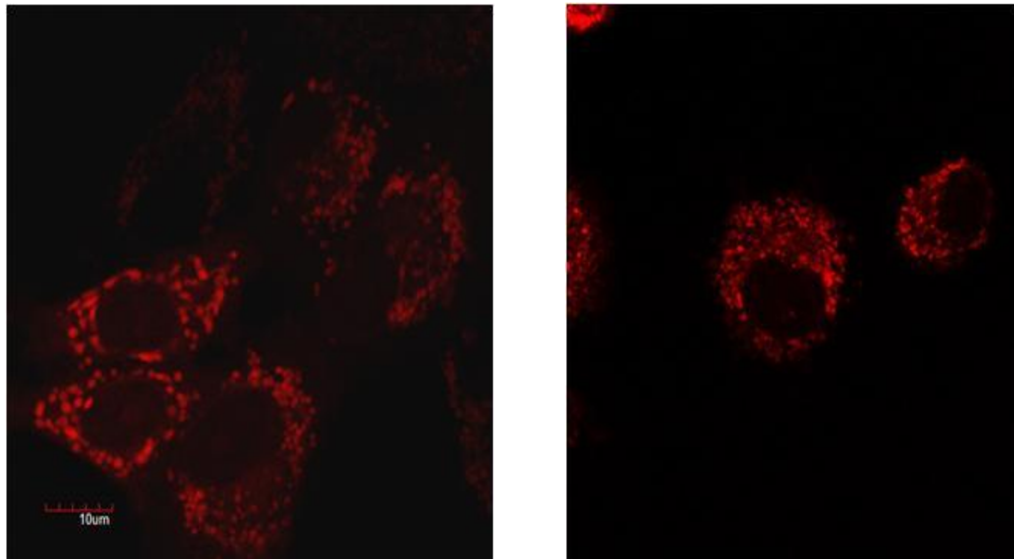

Figure 5.2: Cells with stained mitochondria in high glucose under confocal microscope showing condensed mitochondria.

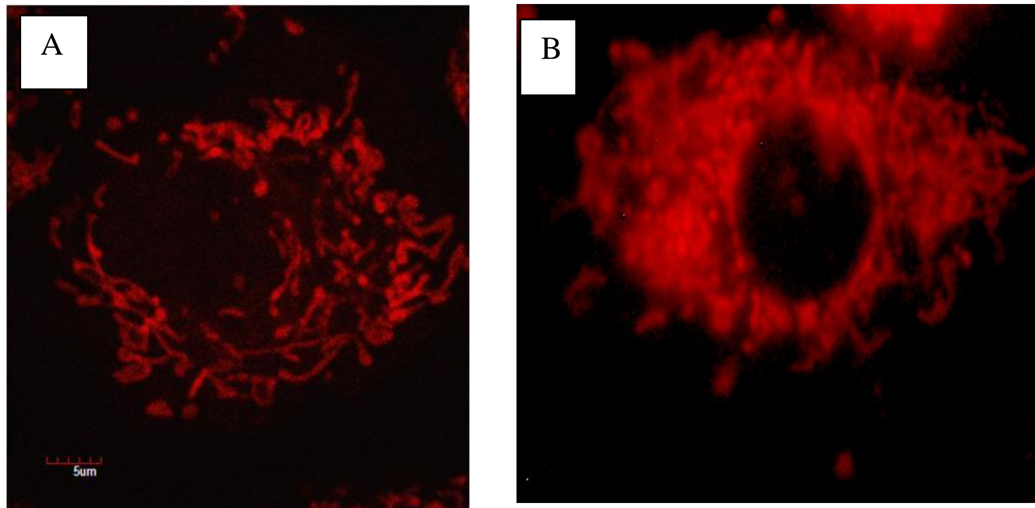

Figure 5.3: Cells with stained mitochondria in A) control glucose showing tubular mitochondria as compared to B) high glucose showing more of the condensed mitochondria but along with some tubular ones.

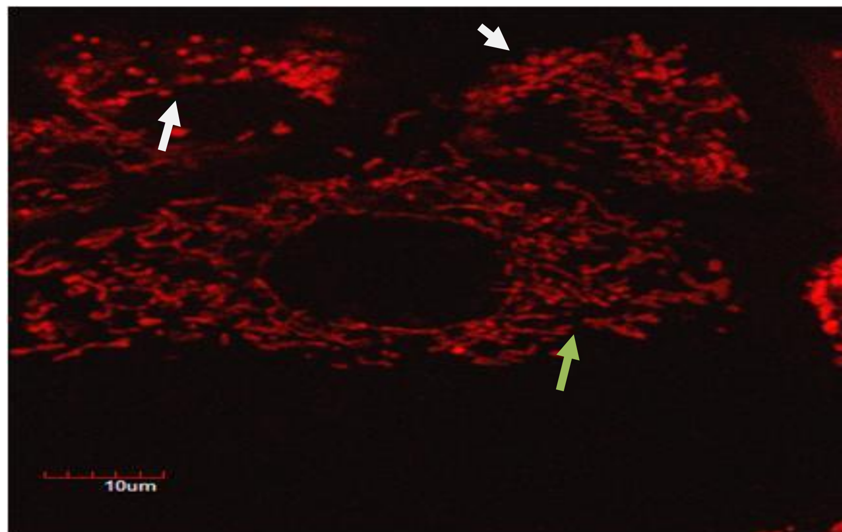

Figure 5.4: Cells with stained mitochondria in high glucose showing condensed (white arrows) as well as tubular mitochondria (green arrow).

As the cells were observed under confocal microscope both kinds of morphological states of mitochondria was observed in control as well as in high glucose conditions. So the mitochondrial shapes were analyzed for the quantification of mitochondrial morphology.

Cells were grown in fluorodishes in control and high glucose conditions, after three days treated with mitotracker red cmx ros and counted under confocal microscope by undertaking different fields and categorised under two different headings: 'cells with tubular mitochondria' and 'cells with condensed mitochondria'. These experiments were repeated five or six times. The cells counts were converted into a percentage and plotted as a graph.

The representative data showed the majority of the mitochondria from cells in high glucose conditions were small and condensed **(figure 5.6)** as compared to control **(figure 5.5).**

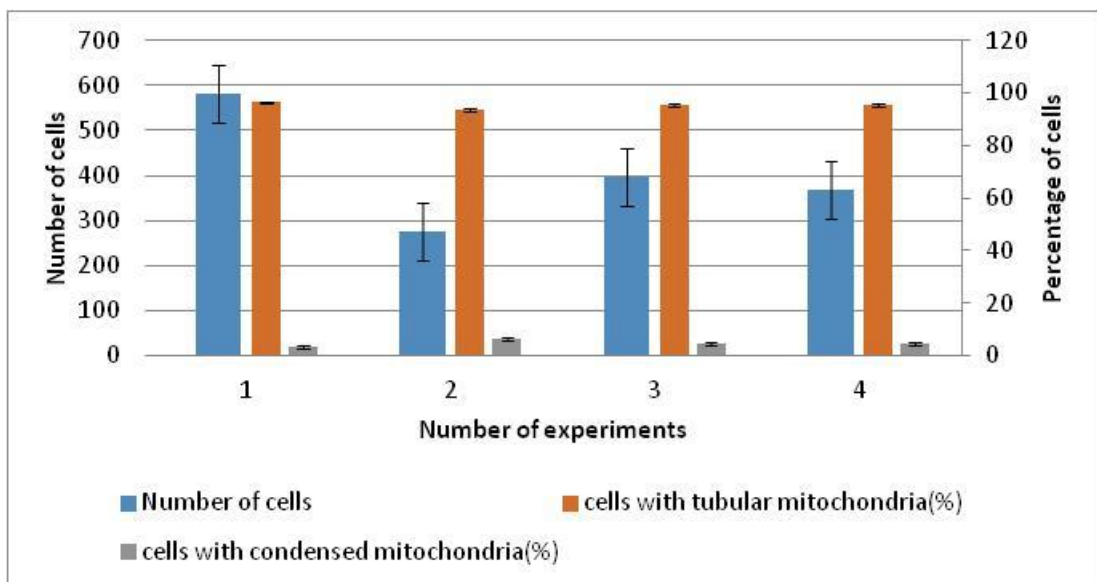

Figure 5.5: This figure showing the percentage of cells with tubular mitochondria and cells with condensed mitochondria in control glucose group (5mM). Error bars represents SEM.

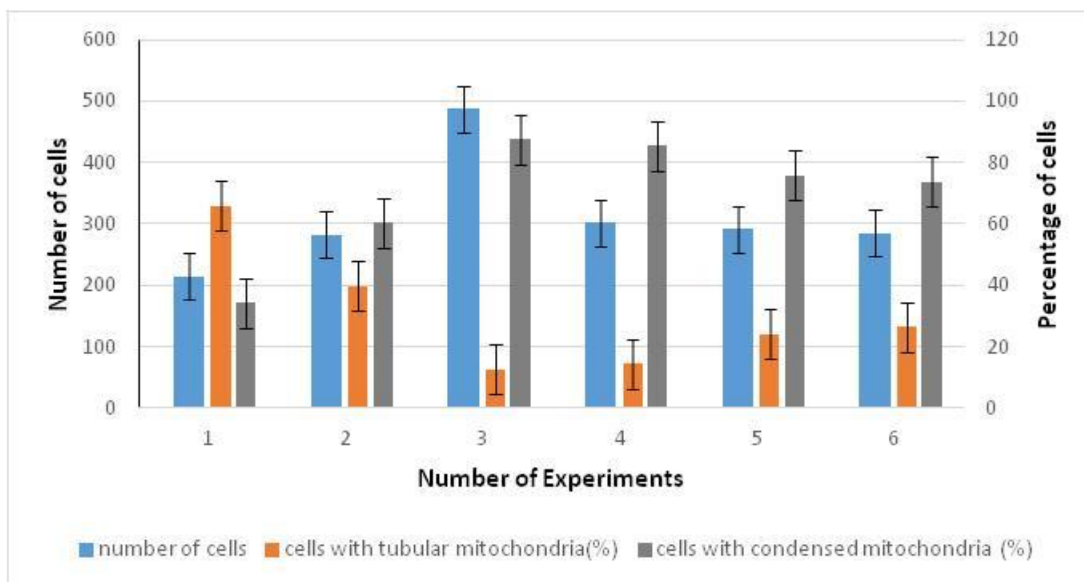

Figure 5.6: This figure showing the percentage of cells with tubular mitochondria and cells with condensed mitochondria in high glucose group(25mM). Error bar represents SEM.

5.4 Discussion

Mitochondria in most cells are in the form of interconnected networks that radiate from the nucleus and supply energy and metabolites to the cells. In this section, mitochondria were stained with mitotracker and mitochondrial morphology was observed using confocal microscopy. Change in mitochondrial morphology was observed and literature stated that this change was due to fission and fusion events.

Mitochondria are a dynamic organelle undergoes spontaneous fission and fusion events and play a very critical role when cell faces various stress . These stresses can be in the form of environmental stress, genetic mutations and metabolic stress. Fusion rescue the cells from stressful conditions by the fusion of dysfunctional mitochondria with healthy mitochondria and the mixing of contents between organelles. Fission plays its role by separation and removal of damaged mitochondria by mitophagy and contribute towards mitochondria quality control.

Mitochondrial morphology is mainly varied between two states: reticular network of fused mitochondria and fragmented mitochondria. As described in the literature review(section 1.3.1), mitochondrial morphology is maintained by the number of fusion and fission proteins. Any kind of disruptions in the fission, fusion or mitophagy results in mitochondrial damage and various diseases(Youle & van der Bliek, 2012).

In this chapter, Hela cells were grown in fluorodishes under normal and hyperglycaemic conditions (25mM) that act as mitochondrial stressors. When the cells were in their growth phase they were stained with mitochondria red cmx ros and mitochondrial morphology was studied under confocal microscope and images were taken. It was observed that cells in normal as well as in high glucose conditions showed both morphological states, the reticular network as well as fragmented state.

Further quantification of mitochondrial morphology was done by analyzing mitochondrial shapes. The results showed the majority of tubular mitochondria were in the control as compared to high glucose.

Evidences states that in high glucose conditions, mitochondria are more fragmented and there is an increased level of ROS. It was also demonstrated that mitochondrial fragmentation is mainly mediated by mitochondrial fission protein DLP1 which is necessary for fission process (Yu, *et al.*, 2008).It is also observed that dynamic change in the mitochondrial morphology in the form of fragmentation in high glucose conditions and founds to be associated with increased production of ROS(Yu, *et al.*, 2006). In vitro study in which 3T3-L1 adipocytes treated with high glucose conditions and free fatty acids induce mitochondrial dysfunction and more fragmented mitochondria observed as

compared to controls. This is due to a decreased level of mitofusion protein mfn1 and an increased level of mitofission protein Drp1(Gao*, et al.*, 2010).

Chapter 6

Effect of high glucose on HSP expression

6.1 Introduction

As the above results had shown that high glucose elicit mitochondrial stress and it was also well known that cell stress results in the induction of heat shock proteins. HSP60 expression is proven to be result of mitochondrial specific stress and HSP70 to be the result of general cellular stress(Martinus *et al.*, 1996). It has also been observed that hyperglycaemia and oxidative stress upregulate HSP60 expression in HeLa cells (Hall & Martinus, 2013). So the next step was to assess the expression level of HSP60 and HSP70 on exposure of high glucose (25mM) as well as in normal glucose conditions (5mM).

6.2 Methods

6.2.1 Quantification of HSP expression

Heat shock control cells: Some Hela cells were heat shocked so that can be used as a positive control. Hela cells were grown in the cells culture flask for 3 days at standard incubation conditions and with normal glucose level. When the cells were at exponential growth stage, media was replaced with media pre warmed at 45°C and then the flask was placed in the water bath at temperature of 45°C for 30 minutes. Again the media was replaced with pre warmed DMEM media at 37°C and placed in the incubator at the standard conditions for 12 hours. After that cells were harvested for protein extraction.

Protein extraction: Total protein was extracted using TENT buffer with freshly added 0.4mM phenylmethylsulfonyl (PMSF) as a protease inhibitor. Samples

were ruptured by vortexing in 1ml of TENT buffer and were placed in the fridge at 4°C for 30 minutes. Then the samples were centrifuged at 10,000rcf for 5 min at 4°C which separated the proteins from the cell debris and transferred to a new tube and stored at -20°C. Following the same procedure protein was extracted from culture flask with different glucose concentration and incubated at -20°C.

Protein estimation using BCA assay: Protein concentration was estimated using the Pierce BCA protein assay kit. The instructions were followed and albumin (BSA) standards and BCA (bicinchoninic acid) working reagents (WR) were prepared as explained in **chapter 2.4.3.**

Protein separation and transfer: To separate the total protein, 10% polyacrylamide discontinuous gel was prepared then samples were loaded onto the gel and run in a Bio-Rad mini protean 3 cell gel tank. Samples were prepared by taking 20μg of the protein and made up to 20μl by adding loading buffer. Samples were placed in the boiling water for 5 minutes which denatured the protein. Protein samples were loaded into the wells along with the molecular standards. As the gel was electrophoresed at 200v for 50min, the bands ran through the stacking cells and then the resolving gel.

After the protein separation was completed, the proteins were transferred from the gel to the nitrocellulose membrane using the eBlot protein transfer system described in **chapter 2.4.4.** After the transfer of protein, the membrane was rinsed in distilled water and stained with ponceau stain for 15 sec to check the proper transfer of proteins on the membrane and then washed the membrane with distilled water to remove the stain off.

Western blotting: The membrane was blocked in 10% skim milk in TBST at 4°C overnight. The next day the membrane was washed with TBST 6 times and for 5 min each time on a gyro rocker followed by incubation with a primary polyclonal

rabbit hsp60(1:250) in 5% skim milk in TBST at 4°C in a humidified chamber for overnight. The following day the membrane was again washed with TBST six times for 5 min each time on gyro rocker. Then the membrane was incubated with secondary antibody Peroxidase conjugated affinipure goat anti-rabbit IgG 1:1000 diluted in 5% skim milk in TBST and placed in a humidified chamber on gyro rocker for 5 hours at room temperature. After 5 hours the membrane was washed with TBST 6 times and 5 min each time on a gyro rocker. After that the membrane was incubated with Thermo scientific super signal west pico chemilumiscent (1:1) dilution for 1 minute on a glass plate. After 5 min the excess was removed and the membrane was placed in the plastic sheet and visualized using the LAS-100 plus gel documentation.

6.3 Results

First the western blot was prepared using different amounts of proteins extracted from the control (5mM) : Lane 1 contained 10µg of protein sample, lane 2 contained 20µg, lane 3 contained 30µg, lane 4 contained 40µg and lane 5 contained 50µg. The membrane was first probed with primary and then secondary antibody for detection of HSP60 protein. The bands were visualised using chemilumisence imager. In a similar way after stripping same membrane, it was probed again with housekeeping protein actin. The blots showed bands of dissimilar sizes as the band with a low concentration of 10µg showed small band depicting a low level of HSP60 as compared to band with 50µg of protein with high level of HSP60 protein. The actin band also showed a high amount of loading result in high expression of HSP60 proteins. This western blot was performed just to find out the accurate amount of protein loadings **(figure 6.1).**

So for performing rest of the blot, 20µg of protein loading was selected.

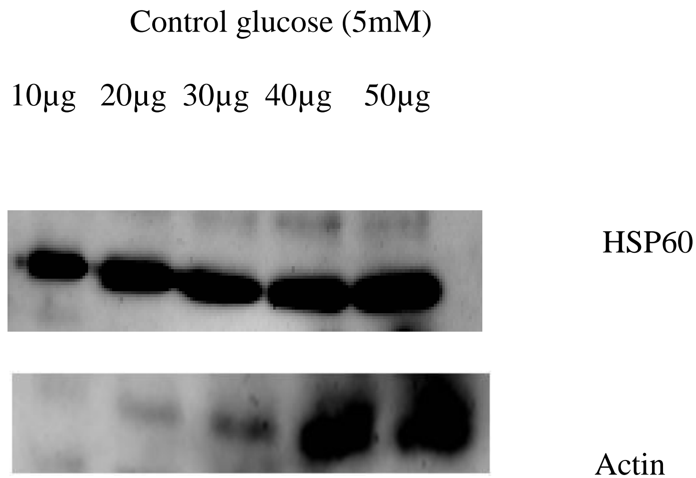

Figure 6.1: Western blots of HSP60 and actin in cells treated with control glucose (5mM) and different amount of protein samples loaded in each lane. 10μg in lane1; 20μg in lane2; 30μg in lane3; 40μg in lane4 and 50 μg in lane 5.

The second western blot was prepared with control (5mM) versus heat shocked sample. As the **figure 6.2 B** with gel stained with coomassie blue showed even loading was there. Clearly HSP60 expression was found to be upregulated in the heat shocked sample as compared to control sample :10μg protein in lane 1 and 20μg of proteins sample in lane2 **(figure 6.1A).** The bands were quantitated and HSP60 expression was plotted **(figure 6.3).**

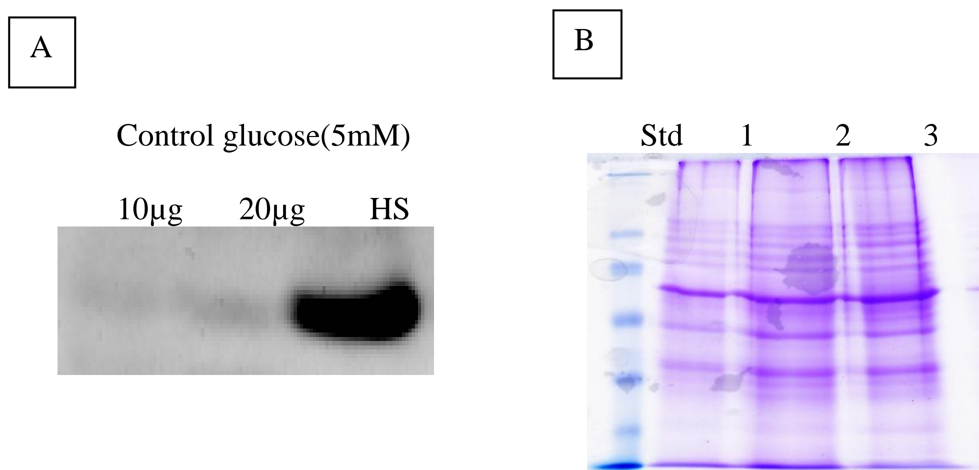

Figure 6.2: A) Western blots of Hsp 60 in cell treated with control glucose (5mM) in lane 1 (10μg) and lane 2(20μg) and heat shocked in lane 3. B) gel stained with coomassie blue showing equal loading of proteins samples.

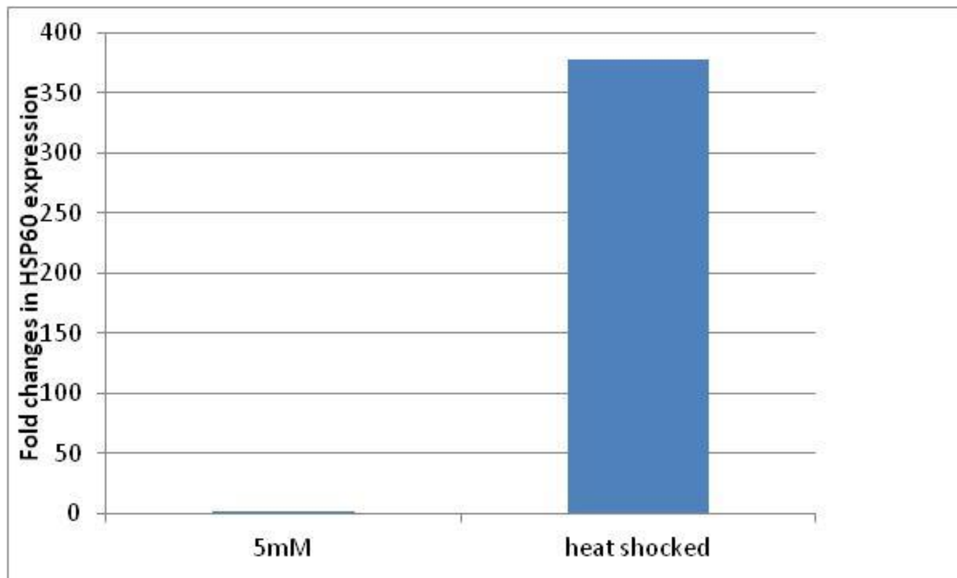

Figure 6.3: Relative HSP60 expression in cells treated with 5mM and heat shocked

The third western blot was prepared with different concentrations of glucose 5mM, 10mM, 15mM , 25mM and a heat shocked sample as well. The results showed a faint band with 5Mm (lane1), bands with 15mM(lane3) and band with heat shocked sample (lane 5)**(figure 6.3A).** Figure 6.3B shows the stained gel and loading. HSP60 expression was clearly upregulated with heat shocked sample as compared to control. The other concentration (10mM and 25 mM) did not show any bands which was very unusual result.

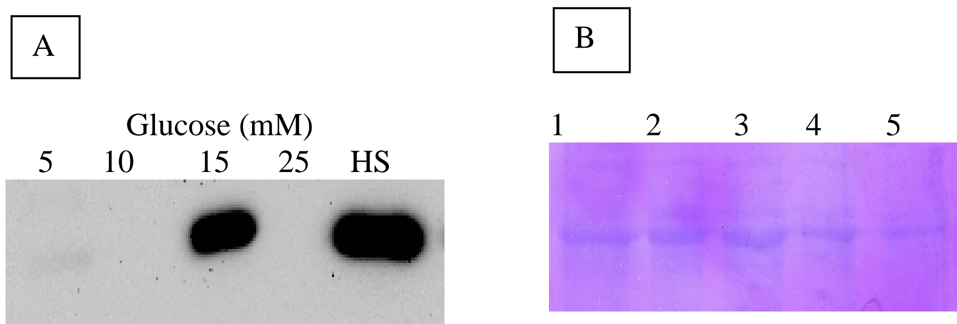

Figure 6.4: A) Western blots of HSP60 in cells treated with different concentrations of glucose and heat shocked sample. 1) 5mM 2) 10mM 3) 15mM 4) 25mM 5) heat shocked B) gel stained with coomassie blue showing loading in different lanes.

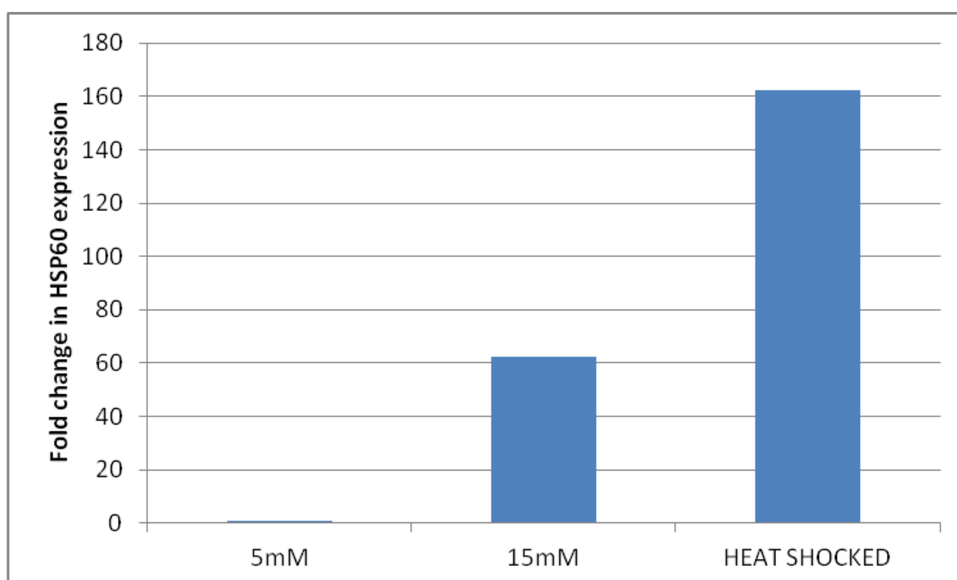

Figure 6.5: Relative HSP60 expression in cells treated with different concentrations of glucose but only 5mM, 15mM and heat shocked showing its expression.

Next the western blot was prepared again with 5mM, 25mM and a heat shocked sample. The nitrocellulose membrane was stained with ponceau red to check the even loading as well as the proper transfer of bands **(figure 6.6 B).** The results were very unusual this time also as only heat shocked sample showed the band illustrating an upregulated level of HSP60, but neither 5mM nor 25mM glucose concentration showed any band with HSP60 **(figure 6.6A).** But the ponceau red stained membrane clearly showed the presence of proteins in each lane indicating that absence of HSP60 signal was not due to lack of proteins in samples.

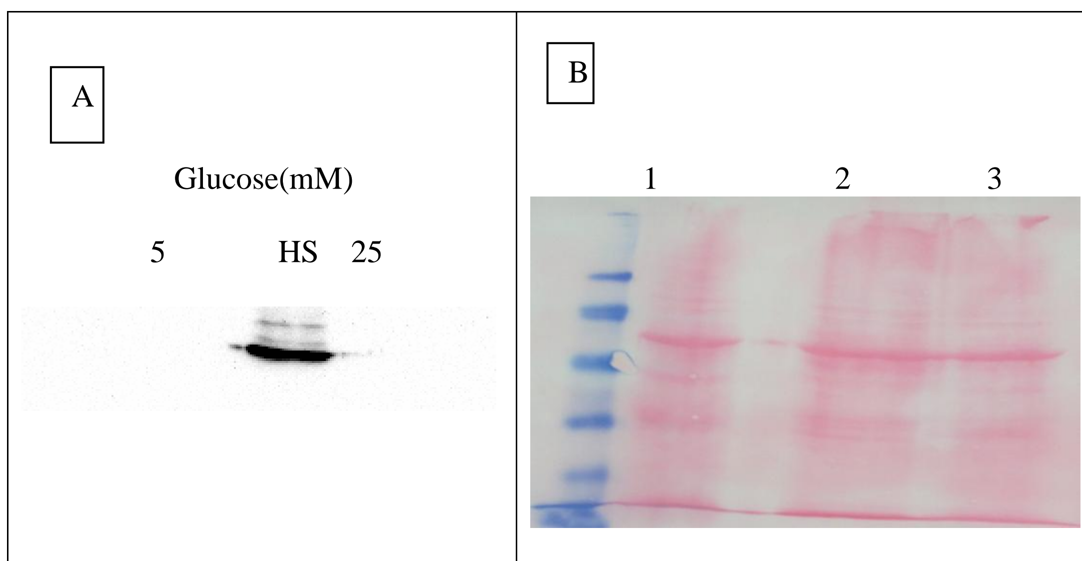

Figure 6.6: A) Western blots of HSP60 in cells treated with 5mM , 25mM and heat shocked but only showing HSP60 expression B) Membrane stained with Ponceau Red showing loading Lane 1) 5mM lane 2) heat shocked and lane 3) 25mM

It is well known that HSP60 does not always respond well with heat shock stress or other stress conditions((Barutta *et al.*, 2008; Shan *et al.*, 2010). On the other hand it is also well established that cytoplasmic HSP70 does get induced with heat shock. As one study demonstrated the induction of heat shock proteins mainly HSP60, HSP70 along with HSP32 and HSP27 in three different endothelial cell types by heavy metal and heat stress and also found that HSP70 induced at high rate as compared to HSP60 with heat shock stress(Wagner *et al.*, 1999). So thats why the next task was to look upon the expression of HSP70.

The membrane was prepared with similar samples as above which looked for the expression of HSP60 but this time membrane was probed with HSP70 antibodies and looked for HSP70 expression. So similarly the proteins were transferred to a membrane stained with Ponceau red (**figure 6.7B).** The bands were observed in all kinds of treatments: with 5mM (lane 1), heat shocked (lane2) and 25mM (lane 3).The bands were quantitated using gel quant software and expression was plotted **(figure 6.8).** HSP70 expression were clearly found to be upregulated more

in heat shocked sample as compared to 25mM which showed more upregulation of HSP70 as compared to control **(figure 6.8).**

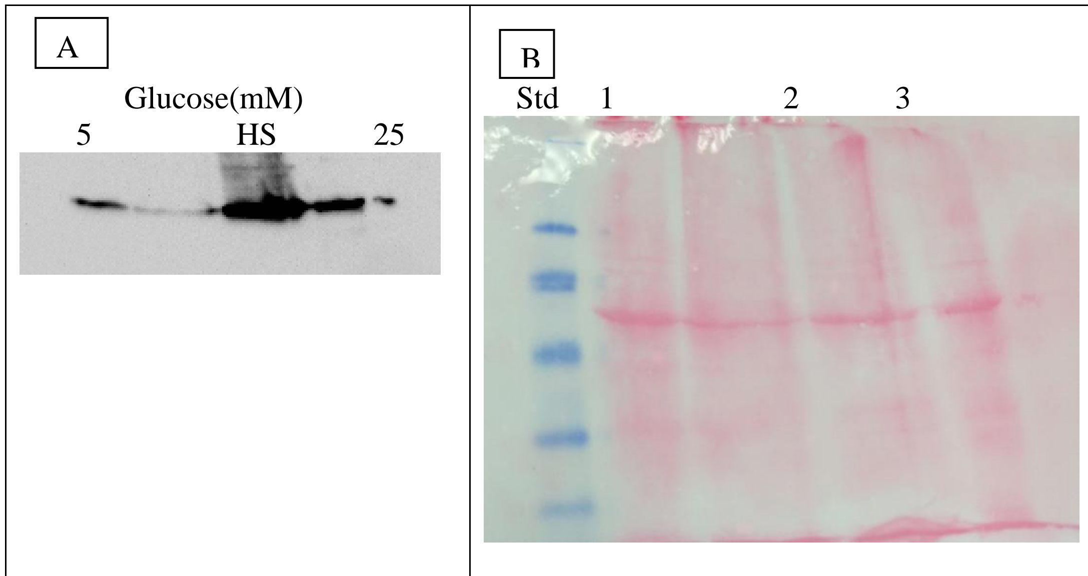

Figure 6.7: A Western blots of HSP70 in cells treated with glucose and heat shocked samples.1) 5mM 2) heat shocked 3) 25mM).The lane in between 1 and 2 was not taken into account because sample was not loaded correctly so discarded B) The membrane stained with Ponceau red showed transfer of proteins.

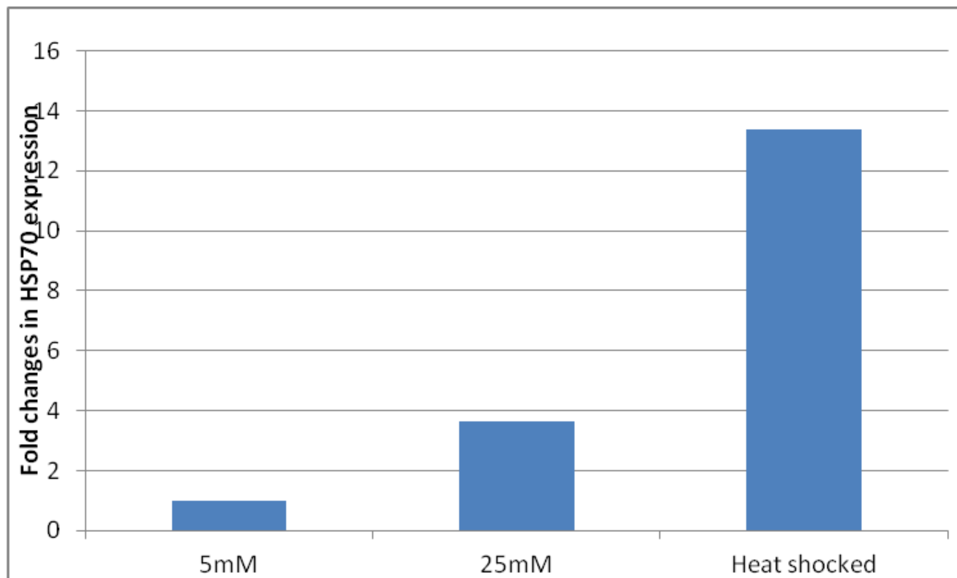

Figure 6.8: Relative HSP70 expression in cells treated with 5mM, 25mM and heat shocked.

6.4 Discussion

In this chapter, the expression of HSP60 and HSP70 was measured by western blotting using different glucose concentrations and heat shocked protein as positive control. The western blot results have shown that only heat shocked samples showed a high induction of HSP60 but all treatments showed expression with HSP70 from which it can be assumed that more general cellular stress was there as compared to mitochondrial specific stress for induction of HSP60 proteins.

As discussed previously in the literature review, heat shock proteins belong to a superfamily of stress proteins and are molecular chaperons who play an important role in correct protein folding, transportation to their localized cell organelles and the degradation of aged proteins. As previously mentioned in literature, HSPs were first discovered by Ferruccio Ritossa, accidently when Drosophila salivary gland cells were exposed to high temperature . This resulted in "puffing" of genes in the chromosome and elevation of proteins with molecular masses of 70 was observed(Ritossa, 1996). But its not only heat shock that results in the induction of these proteins. There are various stress conditions such as exposure of cells to various glucose analogs, viral bacterial or microbial infections, disease states, heavy metals, nitric oxide and hormones and various other cell insults that results in the induction of these stress proteins. Six well studied families of HSPs are HSP 40, 70, 60, 90 and 10. All these HSPs are localized in different cell organelles such as cytosol, endoplasmic reticulum, nucleus and mitochondria. HSP 60 is found to be localized in the matrix of mitochondria. Mitochondrial impairments founds to lead to the induction of HSP60 and general cell stress results in induction of Hsp70(Martinus, *et al.*, 1996).

It is also investigated that hyperglycaemia and oxidative stress upregulate HSP60 expression in HeLa cells (Hall & Martinus, 2013)

In this work, all together the results section showed upregulation of HSP60 expression with heat shocked sample (positive control) in all western blots experiments which put satisfactory remarks on the protocol followed. But the absence of the upregulation of HSP60 expression with a high glucose sample (25mM) and one western blots which showed nothing at all neither with 5mM and 25mM was very unusual to happens. The other interesting thing observed was that western blots with HSP70 protein showed its expression in all kinds of treatment and that a high level of HSP70 with heat shocked as compared to 25mM which was high as compared to control. So it clearly states the presence of general stress responsible for HSP70 expression and might be presence of inefficient level of mitochondrial stress for the HSP60 expression. The various questions and shortcomings that left behind with this work needs to be answered by some further works on this same project.

The consistency of the results in this work regarding increased expression of HSP60 and HSP70 with heat shocked implies that they are heat inducible and also play a role in thermo protection (Sharma *et al.*, 2007). When cells or organelles are exposed to heat stress , HSP60 has been observed to form complexes with polypeptides(Martin *et al.*, 1992). It is well established that various cellular stress results in the induction of heat shock proteins but it is also observed that during stress not all cells induce HSP60 expression. As one study found that when stress was applies to mesangial cells or podocytes no altered HSP expression was observed(Barutta*, et al.*, 2008). High expression of miR-1 and miR206 was also observed in high glucose conditions in rat myocardium which repress HSP60 expression(Shan*, et al.*, 2010). Some studies also suggest that HSP60 expression

is not affected by hyperglycaemia, in fact it is modulated by insulin. It was also found that insulin deficiency or inadequate insulin action as a novel mechanism that leads to downregulation of HSP60 expression in myocardium of STZ induced diabetic rats (Chen *et al.*, 2005a). So it states that in diabetic conditions it is not the hyperglycaemic conditions only that regulates that HSP60 expression.

Chapter 7

Final summary and future directions

In this study, the effects of different concentrations of glucose on HeLa cell growth, mitochondrial activity and mitochondrial morphology were investigated. To study the impact of different glucose concentrations on HeLa cells growth, physiological levels of glucose concentrations were used (5mM, 10mM ,15mM and 25mM). 5mM concentration was taken as the control and 25mM was taken as the high glucose concentration. The World Health Organisation describes diabetes as >11.1mM concentration (world health organisation 2006). HeLa cell growth was observed for 7 days in different glucose levels and then plotted as a graph which showed reduced cell growth with 25mM level as compared to control. The results confirm earlier studies which indicate that growing cells under hyperglycemic conditions lead to impaired cell growth. Literature has linked this growth inhibitory action by high glucose mainly with the secretion of TGF-β1(Rocco *et al.*, 1992; Kolm-Litty*, et al.*, 1998), production of ROS(Quagliaro*, et al.*, 2003) as well as accumulation of ammonium and lactate(Lao & Toth, 1997).

Furthermore, we demonstrated that impaired cell growth is most likely due to mitochondrial effects by using MTT assay and the results showed that 25mM concentration was found to significantly reduce the mitochondrial activity. Thus 25mM concentration was identified as the concentration for mitochondria targeted cell stress that impaired cell growth as well as have impact on mitochondrial activity .Literature readings have clearly pointed out that this mitochondrial

dysfunction is mainly associated with hyperglycaemia induced oxidative stress(Rolo & Palmeira, 2006; Akude*, et al.*, 2011) Dysregulation of intramyocellular fatty acid metabolism observed in insulin resistant off spring of patients with type 2 diabetes which is found to be caused by inherited defects of mitochondrial oxidative phosphorylation or impaired mitochondrial activity(Petersen *et al.*, 2004). So there are various evidences which observed altered mitochondrial activity in diabetic conditions and also found to be induced by oxidative stress. This study did not looked at ROS levels, these could be further investigated.

Furthermore, cells were grown in high glucose (25mM) and HSP60 expression was observed by western blotting. HSP60 is a mitochondrial specific protein and upregulation of HSP60 was observed in hyperglycaemic conditions. Some cells were heat shocked (42°C) which were set as a positive control for western blotting. Proteins were extracted using TENT buffer and western blotting was performed. HSP60 expression was found to be upregulated with heat shock treatment but the induction of HSP60 was not observed in high glucose conditions. In fact one western blot result unusually showed expression only with heat shock treatment but not even a faint band with 25mM and control . But the same samples and same treatment showed significant HSP70 expression with control, high glucose and heat shocked treatment. So it can be concluded that there was general cellular stress that elicited the HSP70 expression and not a sufficient level of mitochondrial stress that could elicit HSP60 expression. Further work is needed to be done on this project so that shortcomings and questions that left behind can be fulfilled and get answered.

Finally, the most significant results were the changes in mitochondrial morphology as shown by live cell confocal image analysis after mitotracker staining.

To study the impact of high glucose on mitochondrial morphology cells were grown in high glucose for three days and mitochondria stained with mitotracker red cmx ros and observed under confocal microscopy. Mitochondrial shapes were analyzed for the quantification of mitochondrial morphology and images of different shapes were also captured. It was observed that the majority of mitochondria in the control were tubular and branched as compared to high glucose (25mM) mitochondria which were found to be short and condensed. Various studies have shown that mitochondrial dynamics are altered in diabetic conditions along with an increased production of ROS and increased mitochondrial fission. Disruptions of mitochondrial networks and increased expression of fission proteins , Drp1 and Fis1 was observed when human aortic endothelial cells were exposed to 30mmol glucose(Shenouda*, et al.*, 2011). In high glucose conditions , mitochondrial fragmentation as well as metabolic dysfunction was observed in retinal pericytes that contributes further to diabetic retinopathy(Trudeau *et al.*, 2011). Studies also link insulin signalling with altered mitochondrial dynamics in diabetic conditions. It is observed that in diabetic cardiomyopathy defective insulin signalling is associated with the fragmentation of mitochondrial networks and Drp1 protein is found to be important as it regulates mitophagy and maintain cardiac health(Westermeier *et al.*, 2015)

Selective and constant fission and fusion events plays a very crucial role in maintaining the quality of the mitochondria. Asymmetric mitochondrial fission by Drp1 produces two distinct daughters. One of the daughters is healthy which further grows by undergoing fusion with the healthy mitochondria and other

daughter is damaged which undergoes an elimination process or the autophagic process known as mitophagy. Mitophagy is the process by which the cells eat their own mitochondria. To maintain cell homeostasis, defective organelles are trapped by autophagosomes and degraded by lysosomes(Shirihai *et al.*, 2015).

There are several fission and fusion proteins and various regulatory factors (as described in detail in literature review) that maintains the mitochondrial morphology and which can be further investigated and tracked to understand mitochondrial dynamics changes in more proper way. Because disruption to any of fission , fusion event and mitophagy can produce cell death and also linked to several diseases.

This study overall concluded that 25mM is sufficient to inhibit cell growth, reduce mitochondrial activity and also alter mitochondrial morphology. However according to this work 25mM does not elicit HSP60 expression. In future more projects and works are needed to repeat this experiment so that significant results can be produced regarding HSP60 expression. First, these HSPs are recognized as intracellular proteins only but there are increasing evidence that found that these HSPs are released in stress conditions and are associated with several diseases. In future revealing the source and pathways used by these HSPs in various diseases would prove to be helpful in designing the diagnostic means for clinical purposes and also open pathways for new research and discoveries. Further studies investigating the pathways used by HSP60 under mitochondrial stress in type2D will also enhance its value in clinical practice.

Regarding mitochondrial morphology this study agrees with literature , that high glucose conditions result in increased ROS production and increased

mitochondrial fragmentation. Researchers found that mitochondrial fission and fusion events are responsible for responsible for the maintainance and regulation of mitochondrial morphology. Disrupted mitochondrial fission and fusion events results in altered morphology which are further linked to various diseases(Galloway, *et al.*, 2012; Galloway & Yoon, 2013). In future, projects involving the fission and fusion proteins, regulatory factors and post translational modifications that regulates mitochondrial morphology proteins would be beneficial and also act as a desired therapeutic target in various diseases.

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