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EFFECT OF ENDOPLASMIC RETICULUM STRESS MEDIATED BY

HYPERGLYCEMIA ON BARRIER FUNCTION IN RETINAL CELLS

BY

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ABSTRACT

Background: Diabetic Retinopathy (DR) is an inflammatory disease mainly characterized by the dysfunction of the retinal blood vessels. Several studies reported a possible link between the hyperglycemia and the activation of unfolded protein response (UPR) and hence initiating endoplasmic reticulum (ER) stress in various retinal cells. However, the mechanism underlying the ERS in the human retinal endothelial cells (HRMEC) remains poorly investigated and understood.

Methodology: In the present study, utilizing the HRMEC, we evaluated the effect of high glucose (30mM) on several ERS signaling pathway (HSPA5, EIF2K3, and XBP1) at the mRNA expression level; the inflammatory cytokines (NFkB) at the protein level; the adhesion molecules (ICAM-1 and E-selectin) by flowcytometry . Also, we assessed the barrier function of HRMEC on real-time analysis using the electric-cell substrate impedance (ECIS). Also, this study uses the Tunicamycin (TM) as positive stress inducer and Phenyl-butyric acid (PBA) as an ERS inhibitor and study their effects on the treatment groups.

Results: We found that hyperglycemia increases the cellular production of reactive oxygen species (ROS) and PBA decreasing it. Also, the barrier quality of HRMEC was affected significantly by the treatment of high glucose and improved with PBA. The expression of Adhesion proteins (ICAM-1 and E-selectin) were increased in hyperglycemia and decreased expression with PBA.

Conclusion: Our results suggest that hyperglycemia may activate ERS that could play a role in the angiogenesis in the diabetic retinopathy.

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LIST OF ABBREVIATIONS

ABB   Annexin binding buffer

ASK1   Apoptosis Signal-regulating Kinase 1

ATF-6   Activating transcription factor-6

BAX   BCL2 – Associated X protein

BRB   Blood retinal barrier

BCL2   B-Cell lymphoma 2 protein

cDNA   Complementary deoxyribonucleic acid

CHOP   CCAAT/enhancer binding protein homologous protein

CO$_2$ Carbon Di-Oxide

CSC   Complete Serum Medium

DMEM   Dulbecco's Modified Eagle Medium

DNA   Deoxyribonucleic acid

DR   Diabetic Retinopathy

ECIS   Electric-cell substrate impedance

ER   Endoplasmic reticulum

ERAF   Endoplasmic reticulum-associated folding

GADD34   Growth arrest and DNA damage-inducible protein

GRP78   Glucose-regulated protein 78

GRP94   Glucose-regulated protein 94

GRPs   Glucose-regulator proteins

GSH   Glutathione

GSSG	Glutathione disulfide
HDAC	Histone deacetylase
HRMEC	Human retinal endothelial cells
HRMEC	Human Retinal Microvascular Endothelial Cells
ICAM-1	Intracellular adhesion molecules 1
IRE1	Inositol-requiring enzyme 1
IRE-1	Inositol-requiring protein-1
JNK	C-Jun Amino Kinase
NV	Neovascularization
P38-MAPK	Mitogen-activated Protein Kinase
PBA	Phenyl-butyric acid
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PERK	Protein kinase RNA-like ER kinase pathway
PI	Propidium Iodide
RER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase - Polymerase chain reaction
SD	Standard Deviation
SER	Smooth endoplasmic reticulum
TJ	Tight junctions

TM	Tunicamycin
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
XBP1	X-Box binding protein
ZO-1	Zonula occludin 1

Units

µg	Microgram
µL	Microlitre
^{0}C	Degrees Celsius
cm	Centimetre
g	Gram
hr	Hour
KD	90 Kilo Dalton
L	Litre
mL	Millilitre
mM	Millimolar
mm	Millimeter
nm	Nanometer
min	Minutes
Sec	Seconds

ACKNOWLEDGMENT

DEDICATION

To my lovely family

1. Chapter One

1.1 Introduction of the project:

Endoplasmic reticulum (ER) is an important component of the human cell. It consists of the cytoplasmic membrane- bound tubules. Under normal condition, it plays the main role in protein folding and synthesis. Under certain circumstances, acquisition of misfolded proteins occurs leading to ER stress (ERS) and activation of the unfolded protein response (UPR) (Sage et al., 2012). ERS is correlated with several reported diseases like neurodegenerative diseases and diabetes mellitus. Several previous studies described ERS as a relevant factor for hyperglycemia, nephropathy, and atherosclerosis (Sage et al., 2012 & Werstuck et.al, 2006). Many studies have focused on the molecular level of the UPR. Three main proteins have been identified following UPR stimulation in cellular stress. These proteins include; protein kinase RNA-like ER kinase pathway (PERK), the inositol-requiring protein-1 (IRE-1), and activating transcription factor-6 (ATF-6) (Sage et al., 2012). These proteins are attached to ER chaperone Glucose-regulated protein 78 (GRP78) (Werstuck et.al, 2006). In normal state, GRP78 keeps the compound inactive while undERS conditions the GRP78 will be dissociated from the three proteins and, as a result, several downstream genes been activated for cell maintenance or driving the cell to apoptosis (Harding et.al, 2005; Lee et. al, 2003& Haze et.al,1999). Another experimental study suggested that ERS also caused inflammation in animal models of diabetic retinopathy and found that the three UPR pathways and the inflammatory gene expressions are activated in the retinal endothelial cells (Ma, Wang &Zhang,2014;& Li et.al, 2009).

Also, chaperone-like activity, 4- phenylbutyrate acid (PBA) was reported to diminish the effect of ERS as well as the inflammation of the retina and vascular leakage in the diabetic animals. PBA is a terminal aromatic substituted fatty acid and is a histone deacetylase (HDAC) activity inhibitor (Memori et.al, 2012). In Diabetic retinopathy, the interruption of the blood-retinal barrier is contributed by hyperglycemia (Ehlrich et. al, 2010). Disruption of Tight junctions between endothelial cells leads inflammation and increase production of adhesion molecules (through increased expression of vascular endothelial growth factor (VEGF) such as intracellular adhesion molecules 1 (ICAM-1), occludin and zonula occludin 1(ZO-1).

1.2   Hypothesis:

The hypothesis of this study based on that hyperglycemia will induce ERS in retinal endothelial cells leading to vascular pathology changes such as barrier dysfunction in the microvascular retinal endothelial cells.

1.3   Significance of the problem:

The endothelium is considered an important structure for conserving vascular integrity and homeostasis. Earlier studies suggested that endothelial dysfunction was significantly associated with increase adhesion molecules production and weak intracellular junction. ERS exerts a fundamental role in the vascular retinal physiology through stimulation of multiple intracellular signaling cascades. Also, it leads to the main physiological impact in retinal endothelial cells such as apoptosis, inflammation, increased VEGF production, and ultimately blood retinal barrier (BRB) breakdown, retinal

neovascularization (NV), and neuronal degeneration. The vascular effects of chronic hyperglycemia have been reviewed in some different related diseases, including the diabetic retinopathy and macular edema. All these data could help in some understanding the ER pathway which could be involved in diabetic retinopathy pathogenesis and thus could help in DR management.

1.4   Research Aims and objectives:

Hyperglycemia role on retinal cells and induction of ERS is not well understood, in this study we aimed:

1. Assessment of the expression of ERS protein markers (HSPA5, EIF2AK3, and XBP1) and the impact on vascular endothelial cells of the retina (oxidative stress, and apoptosis).

2. Assessment of the impact on mediators involved in the angiogenesis such as such as the angiogenesis markers such as (VGEF), and the inflammatory mediators such as (NFkB).

3. Determination of the effect and role of ERS in the permeability of the retinal barrier function in HRECs exposed to hyperglycemia with and without ERS blocker and elucidate molecules involved in it by utilizing the recent impedance technology as previously published using the Electrical Cell–Substrate Impedance Sensing (ECIS).

4. Assessment of the adhesion molecules (ICAM and Selectin) by flow cytometry in reepsone to hyperglycemia with and without ERS blocker.

2.   Chapter Two

2.1   Biology of the endoplasmic reticulum:

Endoplasmic reticulum (ER) is a complex cytoplasmic organelle in the human cell. The first description of the ER was in 1945 by Porter, Claude, and Fullam who described it as "delicate lace-work extending throughout the cytoplasm" (Porter, Claude & Fullam,1945). It is composed of the cytoplasmic membrane-bound tubules. It is divided into two types; the rough ER (RER) and the smooth ER (SER). While ribosomes are embedded in the tubules of RER, SER tubules lacked it and only characterized by the smoothness of the tubules (Agostinis & Samali, 2012). The proportion of RER to SER in all cells vary depending on the function. Both RER and SER are enriched with specific different proteins needed for specific ER functions (Chen et al., 2010). Although RER is enriched with proteins required for protein synthesis and SER are plentiful enriched differently with specific proteins for each cell type.

ER have multiple vital roles. First; are the protein synthesis, processing, and folding. The ribosomes of the RER are controlling the synthesis of the soluble proteins, proteins for exports and membranous proteins. The oxidizing environment of RER is of favor and best for protein folding, maturation, and disulfide bond formation.

Also, Chaperones were reported in regulating the correct folding and protein assembly whereas enzymes assist in protein modifications which include N-linked glycosylation and disulfide bond formation. Furthermore, ER contains other regulators like the glucose-regulator proteins (GRPs). These include Glucose-regulated protein 78 (GRP78) and

Glucose-regulated protein 94 (GRP94) (Lee, 2001). Both GRP78 and GRP94 are also assisting in protein folding by identifying and binding to the hydrophobic part of the peptide. However, GRP78 is the most abundant protein with multiple roles. It is involved in endoplasmic reticulum-associated folding (ERAF), endoplasmic reticulum-associated degradation (ERAD), recognition of faulty proteins folding, and inducing the unfolded protein response (UPR). These functions as a quality control step in ER. ERAD is a process where the misfolded proteins will be transferred to the cytoplasm for ubiquitination by the proteasome (Stolz and Wolf, 2010).

2.2 Endoplasmic reticulum stress:

2.2.1 Endoplasmic reticulum stress and unfolded protein response:

ERS is a process caused mainly by a disruption in the protein folding and maturation process. Some of the main factors causing ERS are chemical disruption of ER function (e.g. glycosylation), metabolic conditions (e.g. increased glucose level), disturbance in the Ca^+ homeostasis and mutations causing abnormality in some proteins or enzymes synthesis (Hampton et al., 2000). Glycosylation has an enormous role in the quality control as well as in monitoring the protein folding process and determination the fate of the protein direction either for further processing at Golgi apparatus or following ERAD process (Mori et al., 1992). Tunicamycin is a drug used in several trails, as an inhibitor for ER glycosylation and hence inducing ERS (Galan et al., 2014; Nakamura et al., 2013). The cell responding to ERS through activation of ERS pathway and the induction of UPR to

maintain the cell homeostasis. This occurs through controlling protein translation, protein folding, ERAD, and autophagy (Ng, Spear, and Walter, 2000).

2.2.2 ERS signaling pathway:

During stress, UPR is arbitrated by three ER transmembrane sensors which eventually causing activation of series of transcription factors (Back & Kaufman, 2012). These sensors include; protein kinase RNA-like ER kinase (PERK), the inositol-requiring protein-1 (IRE-1), and activating transcription factor-6 (ATF-6) (Sage et.al, 2012). Those proteins are binding to GRP78 which is also known as heat shock 70kDa protein (HSPA5) (Werstuck et. al, 2006). In normal state, GRP78 keeps the compound inactive while undERS conditions the GRP78 will be dissociated from the three sensors and as a result, several downstream genes and transcription factors been activated for cell maintenance or driving the cell to apoptosis as described in figure 1 (Harding et.al,2000; Lee et. al, 2003; Haze et.al, 1999). The pro-apoptotic pathway is activated as soon as ERS prolonged and UPR fails to revert the cell to a normal state resulting in apoptosis, retinal inflammation and angiogenesis.
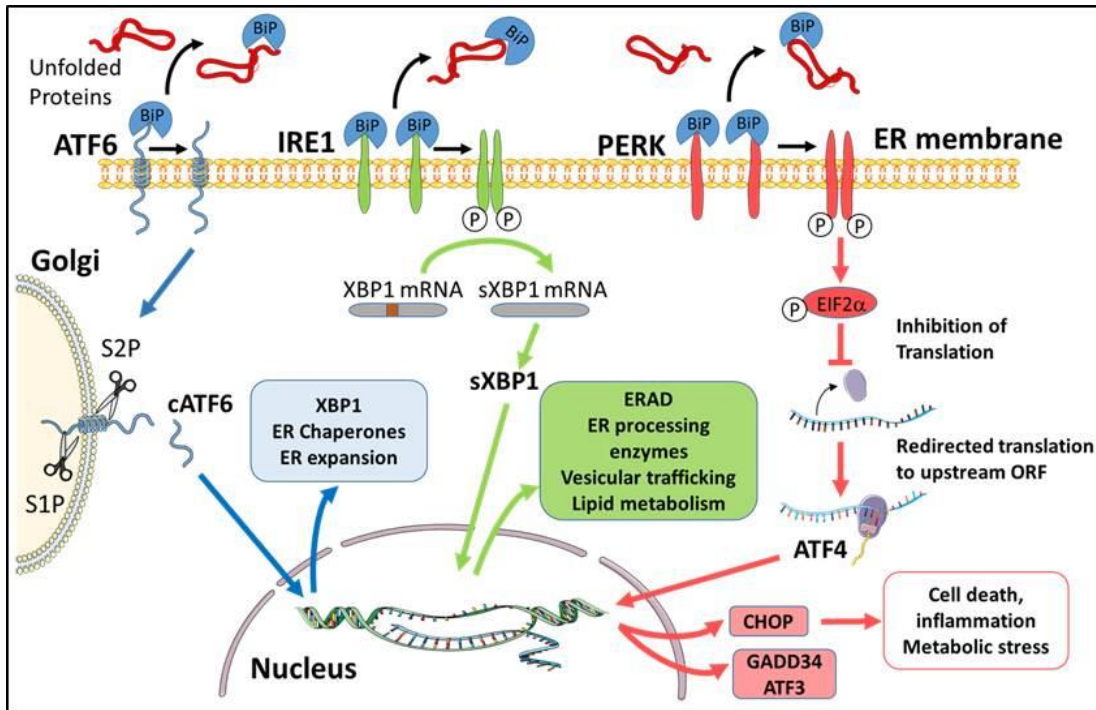
Figure 1: Endoplasmic Reticulum Stress signaling pathway. The three main sensors; ATF6, IRE1, and PERK, are activated due to exposure of endoplasmic reticulum to stress conditions. Adopted from Waldron, Pandol, Lugea &Groblewski, 2015.

2.2.2.1 ATF6 pathway:

ATF6 is a specific transcription factor involved in the activation of the ERS pathway. The regulatory role of ERS and the involvement of ATF6 as part of this pathway is not understood and was investigated for more than a decade ago. In ERS conditions, ATF6 is converted from 90 kD (p90ATF6) protein to 50 kD (p50ATF6) by a proteolytic process. P50ATF6 tends to accumulate in the nucleus and hence activating the transcription of GRP78 in the ER (Haze et.al, 1999).

2.2.2.2 PERK pathway:

UndERS conditions, the alpha subunit of eukaryotic initiation factor 2 (eIF2) is phosphorylated through certain protein kinases (Wek, Jiang & Anthony, 2006). The eIF2 kinases increased activation during ERS is Pancreatic eIF2 α kinase (PEK) (Harding et al., 2000; Kaufman, 2004). The eIF2 kinases involved in regulation of other pathways and resulted in blocking the protein translation and upregulating of the downstream gene that has an effect on the ERS pathway that leads to cell death. Activating transcription factor 4 (ATF4) is one of the main players that is regulated by eIF2 in the PERK and it is responsible for redirecting protein translation and activation of the pro-apoptotic factors such as CCAAT/enhancer binding protein homologous protein (CHOP) and ATF3 (Wek, Jiang & Anthony, 2006), also described in figure 1. The mechanism of this pathway is not yet understood.

2.2.2.3 X-box-binding protein 1 (XBP1 gene)

XBP1 is a transcription factor which also control UPR during ERS. XBP1 is required for the embryogenesis and the development of tissues such as exocrine pancreas and salivary gland. It modulates the cellular response to ERS in a PIK3R-dependent manner (PubMed:20348923). It is also involved in VEGF-induced endothelial cell (EC) proliferation and the retinal blood vessel formation during the embryonic development and the angiogenesis of adult tissues under ischemic conditions. One of the major functions also is working as a regulator of the UPR in obesity-induced insulin resistance and type 2 diabetes (T2D) for the management of obesity and diabetes prevention.

2.3 Role of Endoplasmic reticulum stress in Apoptosis:

Cellular apoptosis is a programmed cell death. This mechanism is activated due to several factors dependent or independent from the UPR pathway. MEKK (MAP3K) and reorganization of the ER membrane is one of the dependent factors and the PERK, and IRE1/XBP1 pathway was recognized as independent factors for apoptosis following prolonged ERS (Sano and Reed, 2015). In PERK pathway, a multifunctional transcription factor CHOP plays a vital role in both cell apoptosis and cell survival (Nishitoh, 2011). Although the CHOP activation leading to apoptosis is not clear, many studies suggested different models. According to, Fu et al., 2010, model; activation of CHOP causes down-regulation of the antiapoptotic BcL2 genes which lead to cell apoptosis. Under certain cellular stress, phosphorylation of CHOP by P-38 Mitogen-activated Protein Kinase (P38-MAPK) leads to apoptosis. This mechanism is activated as an effect of IRE1/XBP1 activation through TNF-Receptor Associated Factor (TRAF2) which mediate the activation of apoptosis signal-regulating Kinase 1(ASK1). ASK1, in turn, activate both C-Jun Amino Kinase (JNK) and P38-MAPK (Figure 1). Suggested models deficient of XBP1 induced oxidative stress, inflammation and cell death (Jing, Wang, Zhang;2012). Several genes which are controlled by CHOP such as GADD34 are causing induction of reactive oxygen species (ROS) (Kojima, 2003; Han et al., 2013). Also, genes activating CHOP such as ATF4, have been reported to induce ROS in the ER lumen and caused apoptosis (Tavender et al. 2010).

2.4   Role of Endoplasmic reticulum in oxidative stress:

Production of ROS is a crucial process for maintaining the hemostasis in normal cell conditions. The cellular production of ROS comes from different sources. It originates from the ER during the formation of the disulfide bonds and from the mitochondria during the mitochondrial respiration or detoxification. In the ER, the process of disulfide bond formation which occur as part of protein folding is mediated by enzymes like protein disulfide isomerase (PDI) and other chaperones like ERp72 and ERp57. Due to the nature of this process inside ER, the ER environment is considered highly oxidized, and the glutathione (GSH) will be catalyzed into glutathione disulfide (GSSH) (Hwang et al., 1992). The GSH is an essential antioxidant element.   On the other hand, protein misfolding in ER increase the formation of ROS over the normal limit causing oxidative stress. When ROS accumulate in ER, it affects the Ca^+ hemostasis causing Ca^+ to escape from ER lumen. UPR will be activated, and persistent ERS will activate the pre-apoptotic pathway (Malhotra & Kaufman, 2007). Another possible linking mechanism of ERS pathway and ROS production is activated by increased level of blood sugar or hyperglycemia. Hyperglycemia could cause activation of the ERS signaling as a result of diabetic retinopathy.

2.5   Endoplasmic Reticulum Stress and Diabetic Retinopathy:

Diabetes is a chronic illness mainly distinguished by hyperglycemia. Diabetes eventually leads to several complications such as macular edema, renal failure, cardiovascular diseases and Diabetic Retinopathy (DR). DR is a disease marked by loss of the blood

vessels. This leads to retinal ischemia and after that retinal neovascularization. Moreover, the breakdown of the blood-retinal barrier (BRB) is another feature of DR. During BRB breakdown, the tight junction molecules between the endothelial cells loosen and break down (Ma, Wang &Zhang; 2014).

Different molecular pathogenic mechanisms had been identified and caused ERS pathway activation. Molecular changes in both obesity and diabetes cause activation of the ERS pathway and leads to DR. Many studies reported that certain genes play a protective role against developing DR. This is causing the variety in the occurrence and the disease severity from one patient to another (Hu et.al, 2012). However, other genes are reported to be up-regulated in different cell types studies related to the retinal vascular damage and neovascularization; activating ATF4 and VEGF (Roybal et. al, 2004 and Abcouwer et.al, 2002).

2.6   Hyperglycemia and Blood Retinal Barrier Function:

BRB have a crucial role in the retina. It keeps the fluid control with the presence of another important protein which is the tight junctions (TJ) proteins. BRB also maintain the retinal endothelial cells integrity and polarity. BRB is divided into the inner part and the outer part. The inner part consists of retinal capillary endothelial cells whereas the outer part consists of retinal pigment epithelial (RPE) cells. Both the internal and external parts share a similarity in that these cells are linked together by TJ proteins. The most studied TJ are; occludin, claudin and zonula occluding (ZO-1) (Bauer et al., 2011).

Studies reported that BRB disruption is a leading cause of diabetic macular edema. RPE cell lines, when exposed to hyperglycemic conditions and increase IL1B, showed alteration in claudin-1 which suggests that there is a general alteration in the TJ organization (Trudea et al., 2011). However, RPE cell lines are previously known for demonstrating heterogeneity (reviewed by Goncalves, Ambrosio & Fernandes; 2013). Also, pro-inflammatory cytokines like VEGF was reported to arbitrate permeability in HRMEC during ERS (Li et al., 2009). Knowledge is still lacking how the exact role of ERS in BRB.

In this study, we utilized HRMEC exposed to hyperglycemia and ERS blocker to contribute some mechanisms understanding.

3.   Chapter Three

3.1   Materials:

Primary HRMEC; reference number ACBRI 18 were purchased from Cell System as passage 3. Complete CSC medium kit accompanied with serum and culture boost (4Z0-500), Complete Serum-Free Medium Kit accompanied with Rocket Fuel (SF-4Z0-500-R), CSC Attachment factor™ (4Z0-210), CSC Passage Reagents Group™ (4Z0-800), Bac-off® (4Z0-643), and CSC Cell Freezing Medium (4Z0-705) all were purchased from Cell System, 12815 NE 124th Street, Suite A Kirkland, WA 98034. These items are summarized in the following table.

Table 1. Cell culture reagents and cell lines required for this study. All items were purchased from Cell-System (USA).

Item	Cat #
Cryopreserved human retinal microvascular endothelial cells (HRMEC)	ACBRI 181
Passage Reagents Group	4Z0-800
Complete CSC Classic Medium: with 10% serum and culture boost	4Z0-500
Complete Serum-Free Medium Kit: with Rocket Fuel	SF-4Z0-500-R
Attachment Factor	4Z0-210
Bac-off (antibiotic)	4Z0-643

Chemical used generally in most of the experiments include; D-Glucose (G7021), D-Mannitol (M4125), Phosphate Buffered Saline (PBS) pH 7.4,10X (70011036) were purchased from Gibco (Life Technologies, UK), Chloroform 277105X purchased from BDH, and Ethanol 96% (20823.327) purchased from VWR (Radnor, PA 19087-8660, USA). The following table shows a list of these reagents.

Table 2: List of Chemicals and Reagents.

Item	Manufacturer	Cat #
D-Glucose	Sigma - Aldrich	G7021
D-Mannitol	Sigma-Aldrich	M4125
Phosphate Buffered Saline (PBS) pH 7.4 (10X)	Gibco (Life Technologies, UK)	70011036
Chloroform	BDH	277105X
Ethanol 96%	VWR	20823.327
Tunicamycin	Santa Cruz	Sc-3506A
4-Phenyl butyric acid	Santa Cruz	Sc-232961

Also, Tali® Image-Based Cytometer reagents include Tali® Apoptosis Kit - Annexin V Alexa Fluor® 488 &Propidium Iodide (A10788), and CellRox® Oxidative stress reagent (C10443) were obtained from Life Technologies USA. As shown in the following table.

Table 3 List of Tali-Image based Cytometer Reagents.

Item	Manufacturer	Cat #
Tali® Apoptosis Kit - Annexin V Alexa Fluor® 488 &Propidium Iodide	Life Technologies USA	A10788
CellRox® Oxidative stress reagent	Life Technologies USA	C10443
Tali® Cell Cycle Kit	Life Technologies USA	A10798

TRIzol Reagent Ambion RNA (15596026) was purchased from Life Technologies (5791 Van Allen Way, Carlsbad, California 92008, USA), High Capacity RNA-to-cDNA kit (P/N 4387406) and TaqMan Gene Expression Master Mix (P/N 4369016) were purchased from Applied Biosystems (5791 Van Allen Way, Carlsbad, California 92008, USA). Listed in table 4.

Table 4 List of Gene Expression Reagents.

Item	Manufacturer	Cat #
TRIzol Reagent Ambion RNA	Life Technologies (USA)	15596026
High Capacity RNA-to-cDNA kit	Applied Biosystems	P/N 4387406
TaqMan Gene Expression Master Mix	Applied Biosystems	P/N 4369016

3.2 Methods:

3.2.1 Human Retinal Endothelial Cell Culture:

Cryopreserved HRMEC were used for experiments between passage 5 and 6. Passage 3 cells were grown at 37 ^{0}C in 5% CO_2 in Tissue culture flasks (T75 cm^2). HRMEC were treated with 2 to 3 mL of the attachment factor, and a Complete Serum medium (CSC) was added to the flask. Then, HRMEC were grown in T75cm^2 and passaged using the Passage reagent Group. The attachment factor was used to enhance the cell growth and to support the polarity and cytoskeletal structure. CSC complete medium is composed of 10% serum, Dulbecco's Modified Eagle Medium (DMEM/F12), normal human blood glucose level (concentration of 0.9008 g/L) and was supplemented with the combination of animal-derived growth factors (Culture Boost) and an antibiotic treatment (Bac-Off® Tonic). The antibiotic is fluoroquinone ciprofloxacin.

For all experiments, HRMEC has seeded in either 100 mm or 6 well plates. The culture medium was changed after the first 24 hr and then every 48 hr as needed. At 70-80% cell confluency, cells were kept in starvation status for 6-8 h. Starvation was achieved by adding 6 mL of the serum-free medium. After the starvation, the drug treatment is added, and cells were kept in the treatment for the period designed in the plan.

3.2.2 Treatment Protocol:

During the pilot study, five groups of treatments at different time points were investigated. First, the untreated group (control) was representing the normal D-glucose concentration of 5 mM for 24 hr exposure. Second, Tunicamycin (0.1 µg/mL) was representing the

positive chemical ER inducer group for 24 hr exposure. Third, the treated group was represented with one high glucose concentration (30 mM) and observed at different exposure times; 24 hr, 48 hr, and 72 hr. Fourth, glucose oscillation group was observed for 72 hr starting from 30 mM glucose then changing to 5 mM after 24 hr and finally to 30 mM after 72 hr. Fifth, D-mannitol (30 mM) representing the osmotic control group with similar concentration and times as the third group of D-glucose. The following flowchart (figure 2) summarizes the treatment plan.
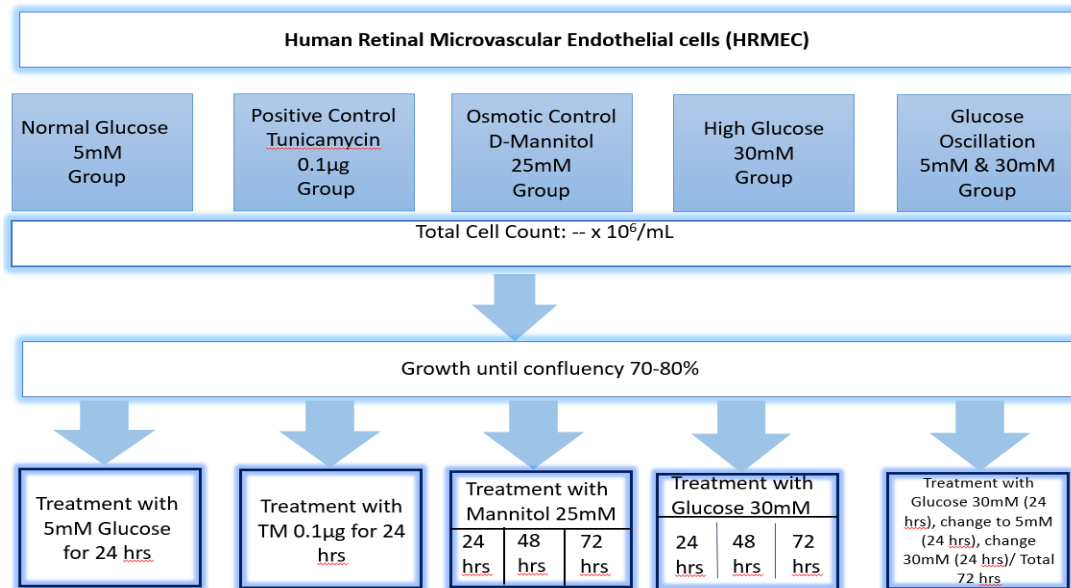
Figure 2. Work plan for the pilot study treatment groups. Different exposure times (24, 48 and 48hr) were performed for the glucose concentration of 30mM and the osmotic control group**.**

During the experimental study, four groups were used. These groups were further sub-grouped accordingly, as shown in the following workflow in figure 3. First, group (A) represented the untreated (control) group of D-glucose concentration of 5 mM for 24 hr. Second, the group (B) represented the positive control of 0.1µg/mL Tunicamycin (TM) for exposure of 24 hr. Third, group (C) represented the high glucose concentration treatment (30 mM) and were sub-grouped into (C1) and (C2). Subgroup C1 treated with high glucose for 24 hr whereas subgroup C2 was treated for 48 hr.   Fourth, group D represented the addition of ERS blocker which is 4-phenylbutyric acid (PBA) and was sub-grouped to into (D1), (D2) and (D3). In all subgroups, PBA was added half an hour before the treatment with either TM or Glucose(Humeres et al., 2013).
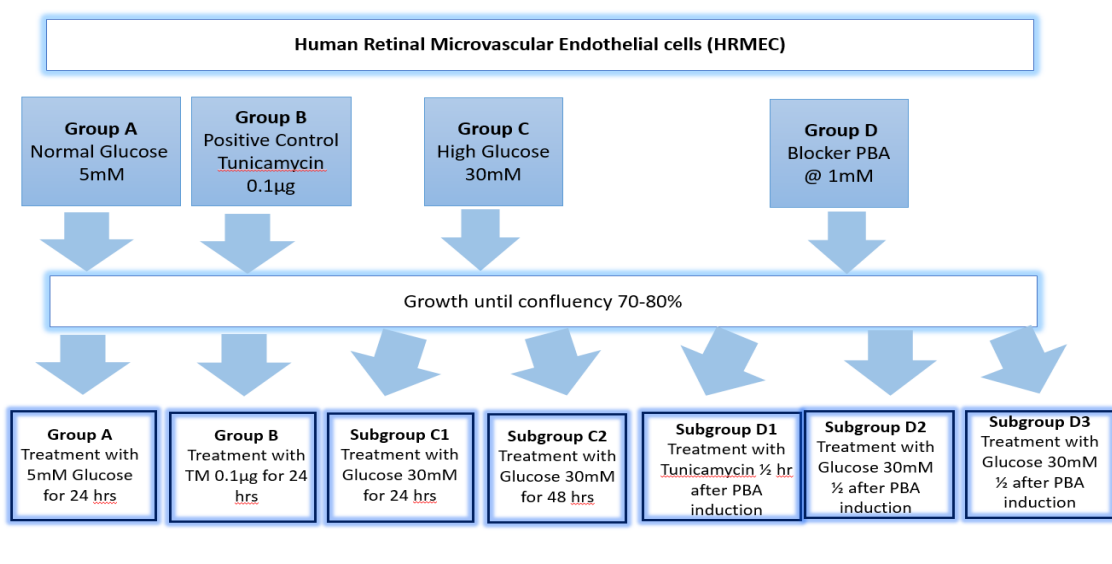
Figure 3. Work plan for the experiment study treatment groups. HREC were exposed to high glucose for 24 and 48 hrs. Additional groups of HREC exposed to high glucose 30mM and 4-phenylbutyric acid (PBA) were added.

3.2.3 Cell Growth Parameters Protocols:

3.2.3.1 Cell Count:

Cell count was performed routinely before each culture/subculture either manually or using the Tali® image-based Cytometer application for quick cell count. The manual method was conducted before the HRMEC seeding. A small quantity of the harvested cells (25 µL) were mixed with equal amount of Trypan blue (25 µL) to get 1:1 dilution. The suspension was then added to the hemocytometer chamber and let to stand for 5 min. The cells in the four corners and middle square were counted, and the average was calculated. The final count of cells have been computed as follow:

Cells count/ 1mL= Average square count x Dilution Factor x 10^4

Total cells count= Cells in 1mL x sample volume

For the automated cell count method, which was performed before the experimental tests, 25µL of the cell suspension was added to the Tali® analysis slide and loaded into the instrument for analysis.

3.2.3.2 Cell Viability and Apoptosis:

Apoptosis Kit was used to studying the apoptosis. The Apoptosis Kit contains both a green-fluorescent Annexin V–Alexa Fluor® 488 conjugate and red-fluorescent PI. The first is used to identify the apoptotic cells whereas used to determine the necrotic cells. The recommended concentration is 100000 to 10000000 cells/mL. HRMEC were harvested using 1 mL of trypsin-EDTA 0.25% followed by incubation for up to 7 min at 37^0C. First,

staining of the cells was performed with 1x Annexin binding buffer (ABB) and Annexin V Alexa Fluor® 488 in a microcentrifuge tube. Second, the stained mixture was kept in the dark for 20 min followed by suspending in 1x ABB and PI. After that, the cells were kept for short incubation for 5 min and sample cells were ready for analysis. Finally, 25 µL of cells were loaded into the analysis slide and loaded into the instrument. The wavelength reading for Annexin V is 488/499 nm and for PI is 535/617 nm.

3.2.3.3 Reactive Oxygen Species (ROS) measurements:

ROS were evaluated using the Cell-ROX® Oxidative Stress reagent kit. First, HRMEC were harvested using 1 mL of trypsin-EDTA 0.25 % followed by incubation for up to 7 min at 37^0C. Then, the cells were suspended in 1x PBS and stained with 5µM of Cell-ROX® orange reagent. This is followed by incubation of the mixture in the dark for 20 min. After that, cells were washed 4 to 5 times with 1X PBS. Finally, 25 µL of cells loaded into the analysis slide and loaded into the Tali® Image-Based Cytometer. The Readings were recorded at 545/565 nm wavelength.

3.2.4 Gene Expression Analysis:

3.2.4.1 Extraction of RNA

TRIzol® reagent was used to purify total RNA from each treatment group using. A slight modification was made to the kit instructions to enhance the concentration of RNA and improve the RNA purity. In brief, treated cells were homogenized with 1 mL TRIzol® reagent for 6 well plates and 3 mL for 100 mm plates. Cells were lysed and harvested using cell scrapers. Next, 0.2 mL of chloroform was added to the homogenized cells and

vigorously shaken by hand for 15 sec. The tube was kept for 5 min on ice and centrifuged at 12000 x g for 10 min at 4^0C. The upper aqueous phase was collected as it contains total RNA. After that, 0.5 mL of 100% isopropanol was added to the collected aqueous phase for RNA precipitation. The tube was inverted several times gently and incubated for 10 min on ice, followed by centrifuge of the mixture at 12000 x g for 15 minutes at 4^0C. Moreover, the supernatant was removed, and 1 mL of 75% ethanol was added per 1mL TRIzol® reagent for RNA washing. Gentle inversion of the tube is recommended followed by centrifugation for 5 min 4^0C. After removing the supernatant, an additional step of RNA washing was done. The pellet was kept at room temperature for maximum 10 min drying. Finally, the RNA pellet was resuspended in 20 µL RNase-free water and incubated at 56^0C for 10 min.

3.2.4.2   RNA concentration measurement:

RNA purity was estimated by calculating the 260/280 nm absorbance ratio using the Nanodrop 2000 spectrophotometer instrument. First, the instrument software was set to the type of nucleic acid to be measured which are RNA-40. The concentration unit for the nucleic acid measurements was set also set to ng/µL. After that, a default wavelength of 340 nm was used by the instrument for normalization using dH_2O. Then, a blank was established using the RNA suspension solution by pipetting 2 µL of the RNase-free water. Sample identification was entered in the appropriate field. Next, 2 µL of the RNA sample was loaded to the nanodrop, and concentration was recorded.

3.2.4.3 cDNA preparation:

Only up to 2 µg of total RNA was quantitatively reverse transcribed into complementary DNA (cDNA) using "High Capacity RNA to cDNA" kit. This resulted in synthesizing a single-stranded DNA. The procedure to synthesize cDNA was as follow. First, components of kits were thawed on ice before use. Next, the reaction mix was prepared by adding; 10 µL of 2X RT buffer, 1 µL of 20X RT Enzyme Mix into an Eppendorf tube. Then, up to 2 µg of total RNA was added and not to exceed the 9 µL (Table 5). Finally, the reaction as added to PCR thermocycler with a program as follow; the first step in 37 ^{0}C for 60 min and another second step in 95 ^{0}C for 5 min.

Table 5. RT-PCR reaction mix and cDNA synthesis.

Component	Volume/Reaction (µL)
2X RT buffer	10
20X RT Enzyme Mix	1
Nuclease-free water	Quantity sufficient to complete reaction volume
RNA sample	up to 2 µg
Total Reaction Volume	**20**

3.2.4.4 Real-time PCR analysis:

The expression of mRNA level of ERS genes performed by quantitative PCR (qPCR) with different oligonucleotides sense and anti-sense primers. The ERS genes selected for the study are HSPA5, EIF2AK3, and XBP1. Also, the samples were quantified for VEGFA and NFKB. The oligonucleotides used are listed in Table 6. cDNA prepared were ready to carry out the quantitative Real Time-PCR in Quanta Studio Flex6 thermal cycler instrument. The PCR reactions were prepared as in Table 7. PCR Reactions were run in duplicate and triplicate using GeneAmp PCR System 9700 (Applied Biosystems). Target genes were analyzed using delta-delta ($\Delta\Delta$Ct) application and results were normalized to the endogenous control (β-Actin).

Table 6 List of Oligonucleotides Probes.

Gene Symbol	Reference Sequence	Company
HSPA5	NM_005347	Life Technologies
EIF2AK3	NM_004836(1)	Life Technologies
XBP1	NM_005080(2)	Life Technologies
VEGFA	Hs.PT.56a.1149801.g	IDT
NFkB	Hs.PT.58.21008943	IDT

Table 7. qPCR Reaction Mix.

Component	Volume/Reaction (µL)
20x Primer/Probe	0.5
2x TaqMan Master Mix	5
cDNA	2
Nuclease-free water	2.5
Total Reaction Volume	**10**

3.2.5 Analysis of Barrier Function by Electric Cell-Substrate Impedance Sensing (ECIS):

HRMEC behavior quantification was detected by the electric cell-substrate impedance sensing (ECIS from Applied BioPhysics, USA) which is considered as a functional as well as an impedance based cellular assay. HRMEC were grown in a special chamber of eight wells. Each well coated with 10 gold electrodes. While HRMEC were growing and proliferated, an electrical current applied between the electrodes. The resulting potential is measured and calculated. HRMEC cell membrane will create resistance against the electrode current representing the study of the cell barrier function and attachment. The procedure for ECIS was carried out as in the manufacturer's operation manual. First, 200 µL of the attachment factor was added to the array wells. Second, after discarding the attachment factor 200µL of cold 10mM L-Cysteine was added and incubated for 15 min at room temperature. Cysteine works in providing reproducible capacitance to the experiment. Then, 200 µL of CSC complete serum medium was added to the 8 wells of the

array without adding any cells. After that, software was checked for electrode connectivity to each well. When all electrodes are connected, the instrument was started to collect data for free cells for 1 to 2 hr. Then, software was paused, and arrays were removed from the incubator to inoculate approximately 60.000 cells / well. Cells in the array were checked under the microscope for proper dispersion. Run was resumed until cells grow to 80% confluency (16 to 24 hr). Connectivity of the electrodes of the array was checked every time the array and the run were stopped. After cells had reached 80% confluency, the CSC medium was discarded, and 200 µL of serum-free medium was added to the wells. The array was inserted back into the incubator, and run was resumed for 8 hours. After 8 hr, the serum-free medium was discarded, and the specified treatments for each well were added to the time as in the work plan. Finally, when the run was completed, the data analysis was carried out through the ECIS ZӨ software (Applied Biophysics).

3.2.6   Adhesion molecules by Flowcytometer:

Expression of adhesion proteins CD56 (ICAM-1) and CD62E (E-selectin) on HRMEC was detected by BD® Accuri Flowcytometer. Generally, 500 µL of HRMEC groups resuspended in 1x PBS and then stained with 20 µL of PE Mouse anti-Human CD62E and PE Mouse anti-Human CD54. The suspensions were kept in the dark for 20 min and after that washed twice with 1x PBS. Analysis performed using BD® Accuri C6 software with minimum 20000 events. Unstained control was used to create the gating of cells based on cell granularity and cell size.

3.2.7 Statistical Analysis:

The analysis of continuous data that were normally distributed was presented as mean ±SD, for each experiment performed in triplicated for at least 2 times. Then tested by a one-way analysis of variance (ANOVA) then by post hoc for multiple comparison tests. A two-tailed P value was calculated, and results were considered significant for p value < 0.05. All statistical analysis were performed using Graph Pad Prism version 7

4.   Chapter Four

To reach our goal, we utilized HRMEC exposed to hyperglycemia and ERS blocker.

4.1   HMREC cells morphology:

The outcome of hyperglycemia concentration (30 mM) on HMREC in all treatment groups was monitored for growth and morphology. The untreated cells were normally grown and reached 70% confluency after 48hr. Tunicamycin (positive control) showed same cell morphology and growth confluency as the normal non-treated cells. Mannitol was used as an osmotic control with a concentration similar to the treatment groups used (5 mM and 30 mM), no differences were noticed in the cell morphology compared to the normal cells. The cell monitoring was obtained using the inverted microscope (10 X lens) and observations were recorded. Figure 4 shows the stages of HMREC growth and proliferation from 24 hr to the day of harvesting also it is indicating a comparison in cell count following each treatment. Although the morphology of the cells of the different treated groups was similar, the cell count at certain time points showed minimal differences, which requered further investigation.
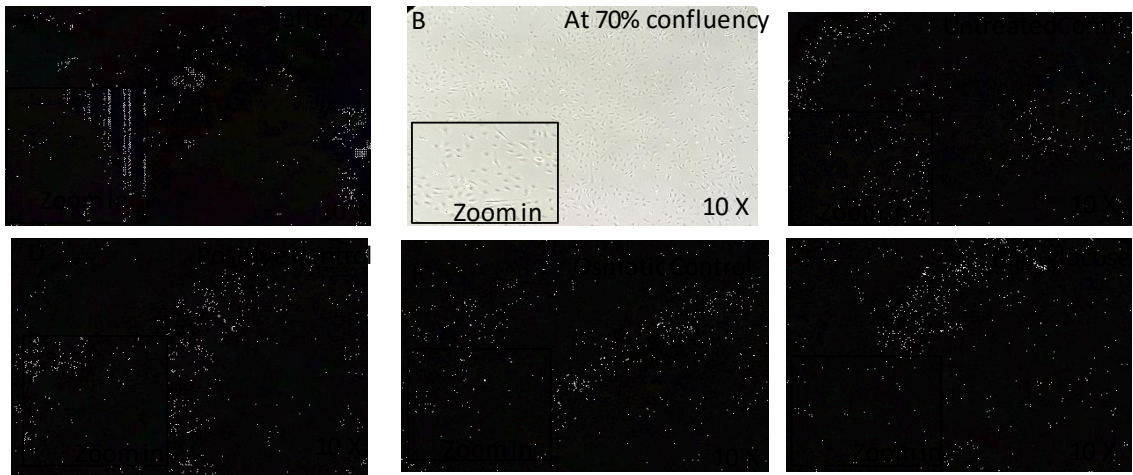
Figure 4.stages of HMREC growth and proliferation. **A**. HMREC after 24 hours of seeding. **B**. HMREC at confluency 70-80%. **C**. Untreated Control group (5.5mM glucose) at the time of cells harvesting. **D**. Positive Control group (Tunicamycin 0.1µg/mL) at the time of cells harvesting. **E**. Osmotic Control group (5.5mM Mannitol) at the time of cells harvesting. **F**. High glucose group (30 mM of D-glucose at 24 hours) at the time of cells harvesting.

4.2 Cell Growth Parameters:

4.2.1 Cell Count

The cell count for all treated cells was performed before each experiment using Tali® Image-based Cytometer Quick Count application to ensure equal seeding cell number. Cell number was recorded following each treatment at harvesting time. The cell count showed that the average cell concentration was 1.1×10^6 cells/mL to 2.5×10^6 cells/mL. The average cell size also measured by Tali® Image-based Cytometer was 9 µm. This results exhibited a higher number of cell counts of the non-treated cells and TM-treated cells (2X) which require investigation of cell death and viability in other treated cells including high glucose group.

4.2.2 Effect of Hyperglycemia on Cell Viability:

Assessment of the cellular viability is necessary to study the cytotoxicity of the chemical used on HRMEC. Tali® Image-based Cytometer affording a method to quantify both the live and dead cells population. Dead cells showed red staining as PI dye was used and the cells undergo apoptosis, or apoptotic cells showed green staining due to Annexin V binding.

The evaluation of the percentage of live cell populations revealed that HRMEC treated with high glucose following 24 hr exhibited a non-significant increase in comparison to normoglycemia group (p>0.999). Also, the addition of the ERS blocker PBA exhibited a non-significant change in the percentage of living cells (p>0.999). The remaining of HRMEC treatment groups shows an insignificant reduction in the percentage of live cells in compared to the normoglycemic group. Figure 5 illustrates the % of living cells in each treated group.
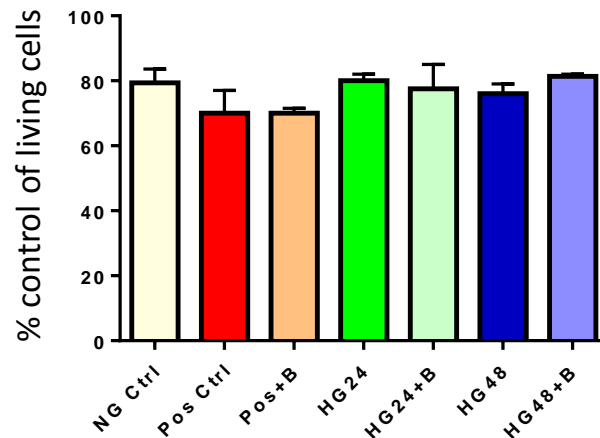
Figure 5. Effect of high glucose treatment for 24 and 48 hours measured by the percentage of Live Cells. Data are presented as means ±SD of 2-3 independent experiments analyzed by ANOVA with a post-hoc test for multiple experiments. The two-tailed p-value is significant at $p < 0.05$. Abbreviations, normoglycemic cells (NG Ctrl); Tunicamycin-treated cells (Pos Ctrl); Tunicamycin-treated cells and ER blocker (Pos+B); High Glucose for 24 hours (HG24); High Glucose for 24 hours+ER blocker (HG24+B); High Glucose for 48 hours (HG48); and High Glucose for 48 hours+ER blocker (HG48+B)

4.2.2 Effect of Hyperglycemia on HMREC on Apoptosis:

Apoptosis assay was used to detect the apoptotic percentage induced by the effect of high glucose treatment in the HMREC and ERS. The assay was performed by Tali Apoptosis Kit- Annexin V Alexa Flour® 488 and PI. HRMEC treated with TM revealed a significant increase ($p < 0.0001$) in apoptosis rate by 7.12 fold compared to the HRMEC with normoglycemia (2.667± 1.528 VS 19±8.458) respectively. HRMEC treated with high glucose treatment for 24 and 48 hr revealed a non-significant increase in the apoptosis rate in comparison to normoglycemia group (14.25± 3.182 and 11.33± 3.512) respectively.

Following addition of the ERS blocker PBA to HRMEC treated with TM, it showed a significant reduction in the apoptosis rate by 11.8 fold compared to HRMEC treated with TM ($p < 0.0001$). The addition of the same blocker to HRMEC treated with high glucose for 24 and 48 hr showed a significant decrease by 9.3 and 2.5 folds for both group with ($p=0.0001$), respectively as illustrated in figure 6.
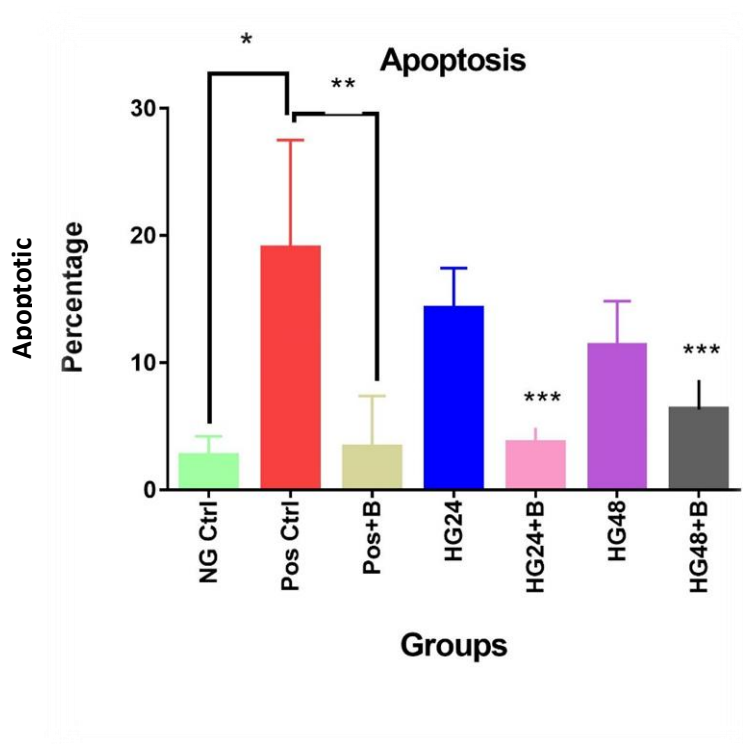
Figure 6. Effect of high glucose (30mM) and Tunicamycin (0.1µg/mL) on Apoptosis. Bars shows the data presented as Mean ±SD of 2-3 independent experiments analyzed by one-way ANOVA with a post-hoc test for multiple comparison tests. The two-tailed P- value is significant at p ≤0.05. * p≤0.05 versus the untreated control group, **p≤0.0001 compared with blocked Tunicamycin group, *** p≤0.0001 compared HG24+B and HG48+B groups with their respective HG24 and HG48 groups. Abbreviations, normoglycemic cells (NG Ctrl); Tunicamycin-treated cells (Pos Ctrl); Tunicamycin-treated cells and ER blocker (Pos+B); High Glucose for 24 hours (HG24); High Glucose for 24 hours+ER blocker (HG24+B); High Glucose for 48 hours (HG48); and High Glucose for 48 hours+ER blocker (HG48+B).

4.2.3 Effect of Hyperglycemia on oxidative stress:

To study the oxidative stress quantitatively in HMREC exposed to high glucose (24 and 48 hr), the percentage of the production of ROS was assessed by Tali® CellRox® Oxidative Stress reagent Kit using the Tali Image-based Cytometer. The total ROS production exhibited a significant increase in HMREC exposed to 0.1μg/mL TM by 12.6 fold compared to HRMEC normoglycemia cells ($p = 0.0001$). On the other hand, the addition of the blocker PBA to the HMREC treated with 0.1 µg/mL TM displayed a significant reduction in ROS by 2.9 folds (p=0.0476). Similarly, ROS produced by HRMEC treated with 30mM high glucose at 24 hr and 48 hr showed a significant increase of by 29.9 and 7.5 folds in comparison to HRMEC with normoglycemia (both p<0.0001). The addition of blocker PBA to the HRMEC treated with high glucose groups (30 mM for 24 and 48 hr) displayed a significant reduction by 14.3 and 13.8 folds compared to the hyperglycemic HMREC cells at 24hr and 48hr respectively (p=0.0001) (figure 7).
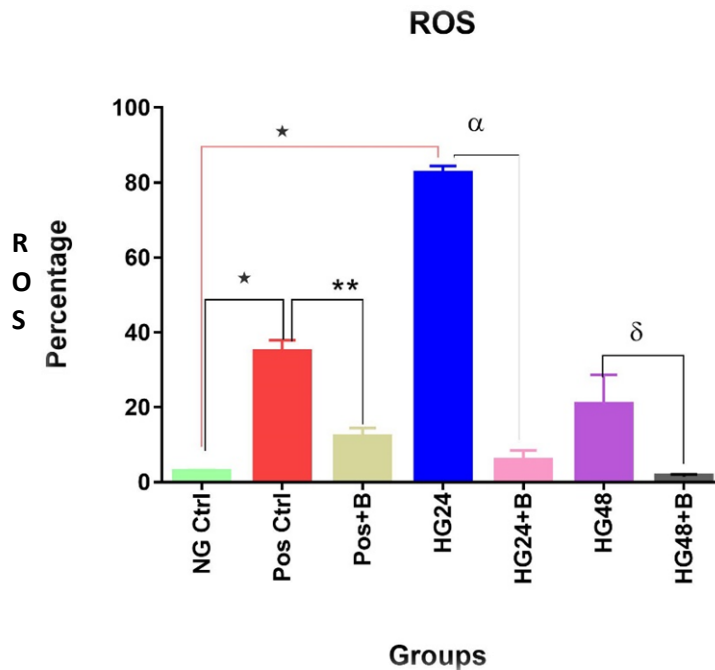
ROS

Figure 7. Effect of high glucose (30mM) treated for 24 and 48 hours and Tunicamycin (0.1µg/mL) on the production of reactive oxygen species in HMREC. Bars shows the data presented as Means ±SEM of 2-3 independent experiments analyzed by one-way ANOVA and post-hoc multiple comparison tests. P value is significant at p≤0.05. * p≤0.05 versus the untreated control group, ** p≤0.05 compared with Tunicamycin blocked by PBA group, $^{\alpha}$ p≤0.05 compared with high glucose group blocked with PBA (30mM for 24 hours) and δ p≤0.05 compared with high glucose group blocked with PBA (30mM for 48 hours). Abbreviations, normoglycemic cells (NG Ctrl); Tunicamycin-treated cells (Pos Ctrl); Tunicamycin-treated cells and ER blocker (Pos+B); High Glucose for 24 hours (HG24); High Glucose for 24 hours+ER blocker (HG24+B); High Glucose for 48 hours (HG48); and High Glucose for 48 hours+ER blocker (HG48+B).

4.2.4   Gene Expression Analysis of ERS pathway, NFkB, and VEFA:

To study the effect of high glucose and TM treatment on HRMEC, gene expression of

HSPA5, EIF2AK3, and XBP1 genes were examined and normalized to the β-actin

housekeeping gene. First, Total RNA was extracted from HRMEC study groups, and concentrations and purity were recorded as shown in the following table.

Table 8. RNA Concentration and Purity.

Treatment Group	RNA Concentration (ng/µL)	Purity (290/280)
Untreated- Normoglycemia	2001	2.1
Positive Control- TM	1299	2.13
TM+ PBA	1259	2.13
HG24 hours	951	2.08
HG24 +PBA	759	2.08
HG48 hours	1073	2.1
HG48+ PBA	1056	2.08

Expression analysis of HSPA5 mRNA in HRMEC exposed high glucose 30 mM (24), and TM revealed significance change in the expression levels normalized to the normoglycemia group. High glucose 24 hours showed a significant increase (p=0.0219) by approximately 2 folds compared to normoglycemia group whereas high glucose 48 hours showed an insignificant increase (p=0.452) compared to the normoglycemic group. Notably, the addition of ERS blocker PBA decreased the level of the HSPA5 expression significantly in each treatment group. In HRMEC with TM and PBA, the reduction was significant (p= 0.0014) by 1.8 folds compared with HRMEC with TM only. In HRMEC of high glucose

24 and 48 hr with PBA, the reduction was significant ($p= 0.0039$ and $p= 0.045$) by 3.6 and

3.5 folds respectively (figure 8).

Figure 8.Effect of high glucose (30mM) and Tunicamycin (0.1µg/mL) on HSPA5 gene expression. Bars shows the data presented as Mean ±SEM of 2-3 independent experiments analyzed by one-way ANOVA with a post-hoc test for multiple comparison tests. The two-tailed P- value is significant at p ≤0.05. * p≤0.05 versus the untreated control group, δp≤0.05 compared with blocked Tunicamycin group, α p≤0.05 compared with high glucose 24 hours. Abbreviations, normoglycemic cells (NG Ctrl); Tunicamycin-treated cells (Pos Ctrl); Tunicamycin-treated cells with ERS blocker PBA (Pos+B); High glucose treated cells for 24 hours (HG24); High glucose treated cells for 24 hrs with PBA (HG24+B); High glucose treated cells for 48 hrs (HG48); High glucose treated cells for 48 hrs with PBA (HG48+B).

Similar to HSPA5, the expression analysis EIF2K3 in HRMEC exposed to high glucose (24 hr) and TM revealed significance change in the expression level normalized to the normoglycemia group. HRMEC treated with TM showed a significant increase (P=0.0001) in EIF2AK2 expression by 4 folds compared to the normoglycemia group. Also, HRMEC of high glucose 24 hr showed a significant increase (p=0.005) by 3.1 folds compared to the normoglycemic group. Furthermore, HRMEC with TM and ERS blocker PBA decreased significantly the level of the EIF2AK2 expression (p=0.0027) by 2.7 folds compared HRMEC with TM only. In HRMEC of high glucose 24 with PBA, the reduction was significant (p= 0.0012) by 2.6 folds compared to HRMEC with high glucose only (figure 9).
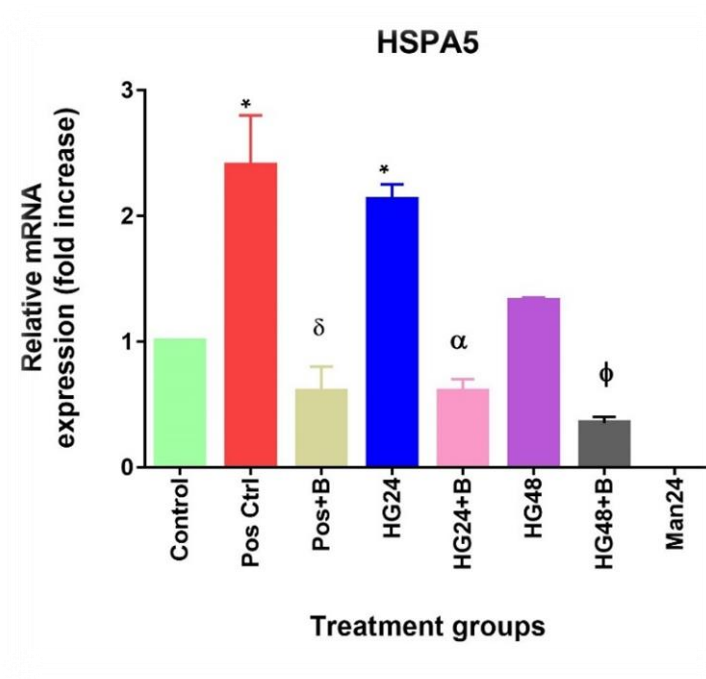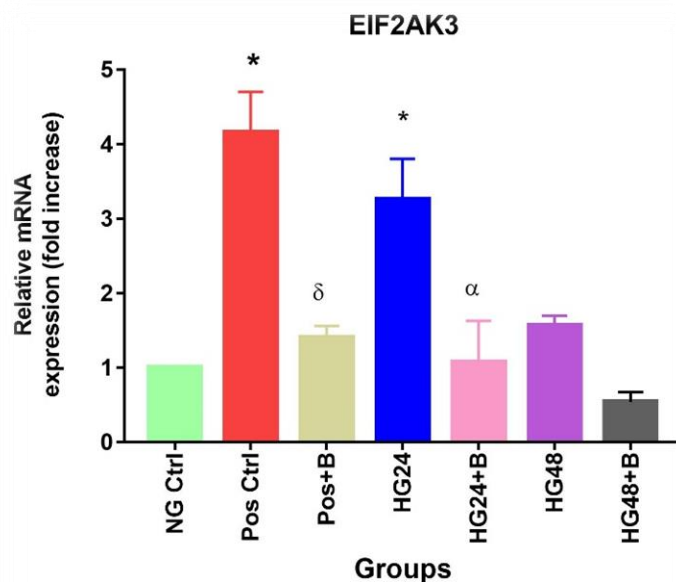
Figure 9. Effect of high glucose (30mM) and Tunicamycin (0.1µg/mL) on EIF2AK3 gene expression. Bars shows the data presented as Mean ±SEM of 2-3 independent experiments analyzed by one-way ANOVA with a post-hoc test for multiple comparison tests. The two-tailed P- value is significant at p ≤0.05. * p≤0.05 versus the untreated control group, δp≤0.05 compared with blocked Tunicamycin group, α p≤0.05 compared with high glucose 24 hours. Abbreviations, normoglycemic cells (NG Ctrl); Tunicamycin-treated cells (Pos Ctrl); Tunicamycin-treated cells with ERS blocker PBA (Pos+B); High glucose treated cells for 24 hours (HG24); High glucose treated cells for 24 hrs with PBA (HG24+B); High glucose treated cells for 48 hrs (HG48); High glucose treated cells for 48 hrs with PBA (HG48+B).

The gene expression of XBP1 for HRMEC treated with TM showed a significant increase (*P*=0.005) in the expression by 2.2 folds compared to the normoglycemia group. HRMEC of high glucose 24 hr showed a significant increase (*p*=0.019) by 0.95 folds compared to the normoglycemic group. Also, HRMEC with TM and ERS blocker PBA decreased significantly the level of the expression (*p*=0.001) by 1.45 folds compared HRMEC with

TM only. In HRMEC of high glucose 24 with PBA, the reduction was significant ($p=$ 0.001) by 1.4 folds compared to HRMEC with high glucose only (figure 10).
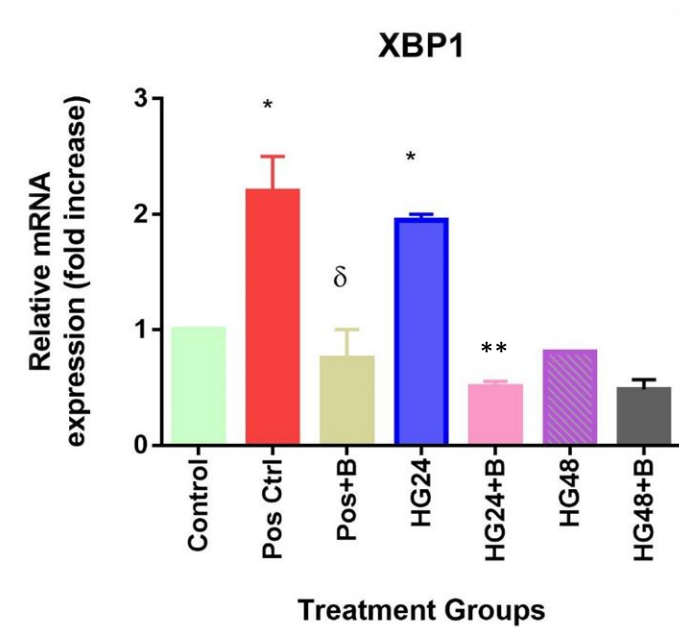
Figure 10. Quantitative Real time - PCR (q-PCR) analysis of XBP1 in HRMEC treated with Tunicamycin (0.1µg/mL) and high glucose concentration (30mM) for 24 and 48 hours. Bars shows the data presented as Mean ±SEM of 2-3 independent experiments analyzed by one-way ANOVA with a post-hoc test for multiple comparison tests. The two-tailed P- value is significant at p ≤0.05. * p≤0.05 versus the untreated control group, δp≤0.05 compared with blocked Tunicamycin group. Abbreviations, normoglycemic cells (NG Ctrl); Tunicamycin-treated cells (Pos Ctrl); Tunicamycin-treated cells with ERS blocker PBA (Pos+B); High glucose treated cells for 24 hours (HG24); High glucose treated cells for 24 hrs with PBA (HG24+B); High glucose treated cells for 48 hrs (HG48); High glucose treated cells for 48 hrs with PBA (HG48+B).

The mRNA expressions of the pro-apoptotic gene NFkB showed a significant difference between HRMEC exposed to high glucose for 24 and HRMEC of normoglycemia (p=0.011) by 1.5 fold while HRMEC exposed to high glucose for 48 hr showed an insignificant increase in NFkB expression compared to normoglycemia. HRMEC treated with high glucose for 24 hr and with ERS inhibitor PBA showed significance decrease compared to HRMEC with a high glucose only group (p≤0.05) (figure 11).

Figure 11. quantitative Real Time - PCR analysis of NFkB in RNA extract of HRMEC exposed high glucose level for 24 and 48 hours and addition inhibition of ERS PBA. Bars shows the data presented as Mean ±SEM of 2-3 independent experiments analyzed by one-way ANOVA with a post-hoc test for multiple comparison tests. The two-tailed P- value is significant at p ≤0.05. * p≤0.05 versus the untreated control group. Abbreviations, normoglycemic cells (NG Ctrl); Tunicamycin-treated cells (Pos Ctrl); Tunicamycin-treated cells with ERS blocker PBA (Pos+B); High glucose treated cells for 24 hours (HG24); High glucose treated cells for 24 hrs with PBA (HG24+B); High glucose treated cells for 48 hrs (HG48); High glucose treated cells for 48 hrs with PBA (HG48+B).

The mRNA relative quantitative of VEGFA genes, which is involved in the angiogenesis, in HRMEC exposed to 0.1µg/mL TM revealed a significant upregulation ($P= 0. 004$) in the expression level by approximately 4 folds in comparison to normoglycemia. Also, HRMEC exposed to 24 and 48 hr high glucose showed significance increase ($p < 0.05$) compared to normoglycemia. Likewise, the addition of ERS inhibitor PBA exhibited a significant difference between the treatment groups ($p < 0.05$) (figure 12).
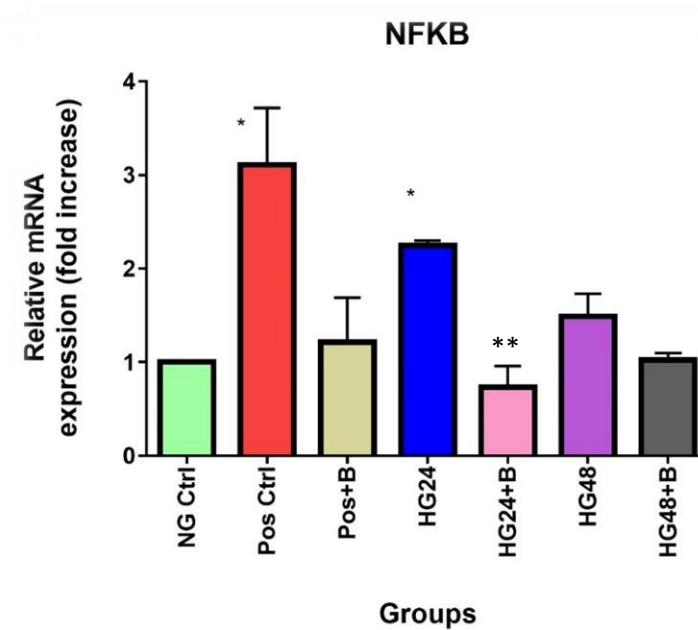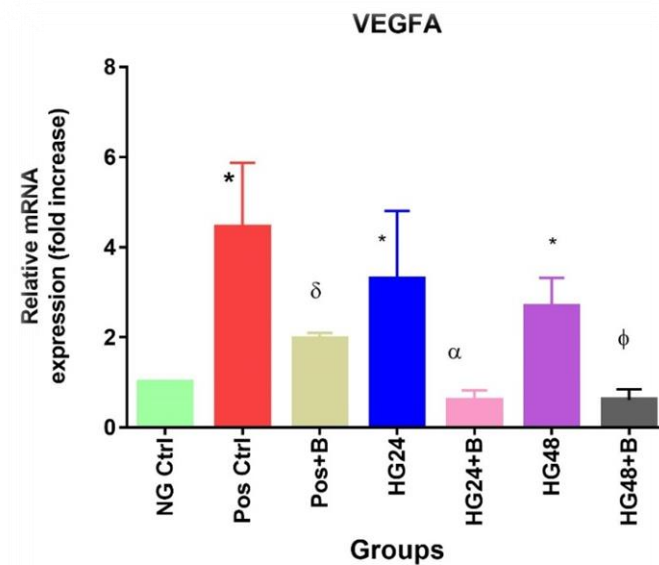

Figure 12. quantitative Real Time -PCR analysis of VEGF-A in RNA extract of HRMEC exposed to TM, high glucose level for 24 and 48 hours and addition inhibition of ERS PBA. Bars shows the data presented as means ±SEM of 2-3 independent experiments analyzed by ANOVA with multiple comparisons between individual groups. *p≤0.05 compared with the normoglycemic group. Abbreviations, normoglycemic cells (NG Ctrl); Tunicamycin-treated cells (Pos Ctrl); Tunicamycin-treated cells with ERS blocker PBA (Pos+B); High glucose treated cells for 24 hours (HG24); High glucose treated cells for 24 hrs with PBA (HG24+B); High glucose treated cells for 48 hrs (HG48); High glucose treated cells for 48 hrs with PBA (HG48+B).

4.2.5 Analysis of Cellular resistance at different time point by ECIS (Retinal Barrier Function):

To investigate and quantify the retinal barrier quality through assessment of the HRMEC proliferation using Electric Cell Impedance Sensing (ECIS) technology. The ECIS mainly measures the resistance between the cells which will indicate the changes in the cell to cell adherent or barrier function. Using HRMEC cultured cells and following glucose addition to the cells at 80 % confluency (30 mM) for 24 hrs as pre-treatment phase and total of 48 hrs for analytical phase. As shown in figure 13, Resistance monitored through HRMEC proliferation in the pre-treatment stage shows an initial increase and late decrease at 40 kHz in the pre-treatment phase. The results showed higher resistance in the high glucose + blocker treated cells in comparison to the high glucose only or TM control cells. Furthermore, the resistance in the high glucose + blocker treated cells significantly increased in the 48 h treatment phase compared to the pretreatment phase (figure 13). In addition, the resistance measured in the glucose treated cells was lower than the TM control treated (figure 13).
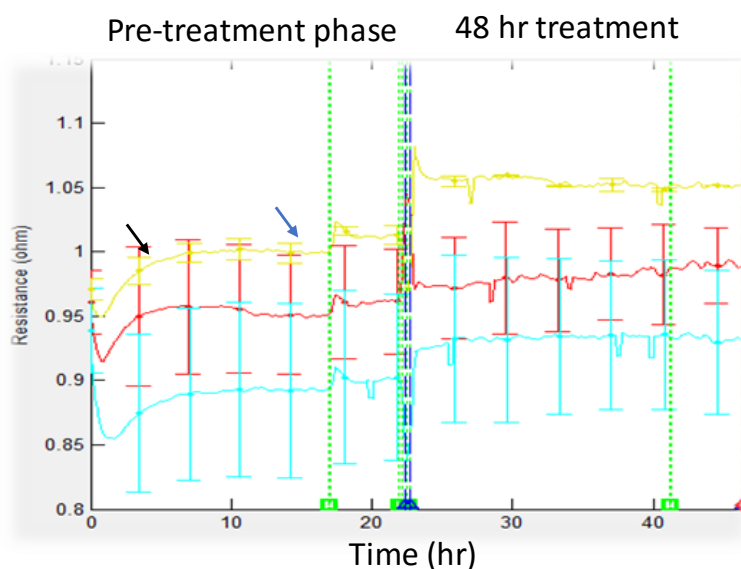
Figure 13. Resistance monitored through HRMEC proliferation in the pre-treatment stage shows initial increase pointed with a black arrow and later decrease pointed with blue arrow at 40 kHz. Yellow: 48h – high glucose + Blocker, red: TM, light blue: 48 h – high glucose.

This experiment was repeated, and the glucose, as well as the glucose +blocker, treated cells were compared to non-treated cells at 2 different time points (24 hr and 48 hr). the same results were obtained, as the Glucose + blockers showed higher resistance compared to the other groups. Moreover, its resistance was greater at 48 hr compared to 24 hr pre-treatment phase resistance. Data not shown.

The complex impedance of ECIS electrodes was monitored in real-time throughout the proliferation of HRMEC and presented as the resistance frequency of 40 kHz. The analysis of the pre-treatment stage showed an initial increase of the resistance accompanied by cell spreading and proliferation followed by a decrease in the resistance in the same stage of

proliferation (due to the changes in morphology and cell size). In the treatment phase, the resistance of HRMEC exposed to high glucose for 24 hrs and 48 hrs showed a significant decrease (both $p < 0.05$) compared to the normoglycemic group. Also, the HRMEC treated with TM showed a significant reduction ($p < 0.05$) in the resistance compared to the normoglycemic group. In contrast, the addition of PBA to HRMEC treated with TM and high glucose concentration of 24 hrs and 48 hrs revealed significant increase ($p < 0.05$) in the resistance in comparison to the same groups without the PBA (figure 14 A and B).
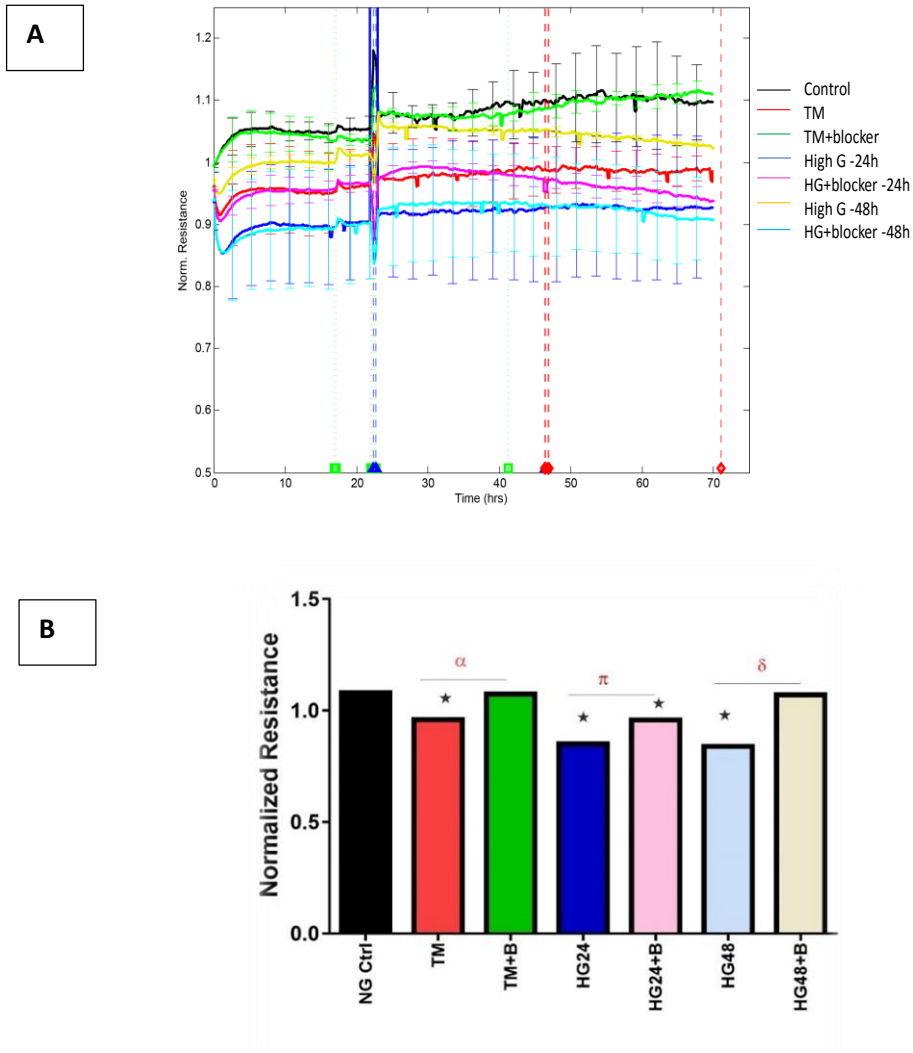
Figure 14. Characterization of trans-electrical resistance (barrier function) of HRMEC exposed to different treatment: high glucose concentration (30mM) for 24 and 48 hrs, TM, and the addition of ERS blocker PBA. A: Resistance measurement of different treatment groups presented in a plot from time zero for the seeding until hr 70. B: Bars shows the significance of resistance (barrier function) change between the different treatment groups presented as mean and SEM. *p <0.05 compared with the normoglycemic group. A p<0.05 compared with TM group. πp<0.05 compared to high glucose of 24 hrs. δp<0.05 compared with high glucose for 48 hours. Abbreviations, normoglycemic cells (NG Ctrl); Tunicamycin-treated cells (Pos Ctrl); Tunicamycin-treated cells with ERS blocker PBA (Pos+B); High glucose treated cells for 24 hours (HG24); High glucose treated cells for 24 hrs with PBA (HG24+B); High glucose treated cells for 48 hrs. (HG48); High glucose treated cells for 48 hrs. with PBA (HG48+B).

4.2.6 Analysis of Adhesion Molecules by Flowcytomery:

To study the effect of ERS-induced by hyperglycemia on the cellular adhesion proteins E-selectin and ICAM-1 in HRMEC, protein expression was measured by BD® Acuuri Flowcytometer. For ICAM-1, the results revealed a significant increase in ICAM-1 expression in TM (figure 15 B), high glucose of 24 hrs (figure 15 D) and high glucose 48 hrs groups (figure 15 F) ($p<0.001$) in comparison to normoglycemia group (figure 15 A) by 1.1, 1.19 and 1.77 folds respectively (figure 15 H) . The expression of ICAM-1 in HRMEC TM + inhibitor PBA or blocker treated cells (figure 15 C) and 24 hrs high glucose treated cells (figure 15 E) revealed non-significance effect in comparison to the normoglycemia.

The addition of PBA inhibitor or blocker resulted in significant reduction ($p<0.001$) in the ICAM-1 expression as combined with each treatment (e.g., the ICAM expression was lower in the TM+ blocker treated cells than in TM-treated HRMEC). Moreover, comparing high glucose group versus the high glucose with inhibitor PBA both treated for 48 hr showed a significant decrease ($p<0.001$) in ICAM-1 expression in the combined treatment group by 0.9 fold (figure 15 H).
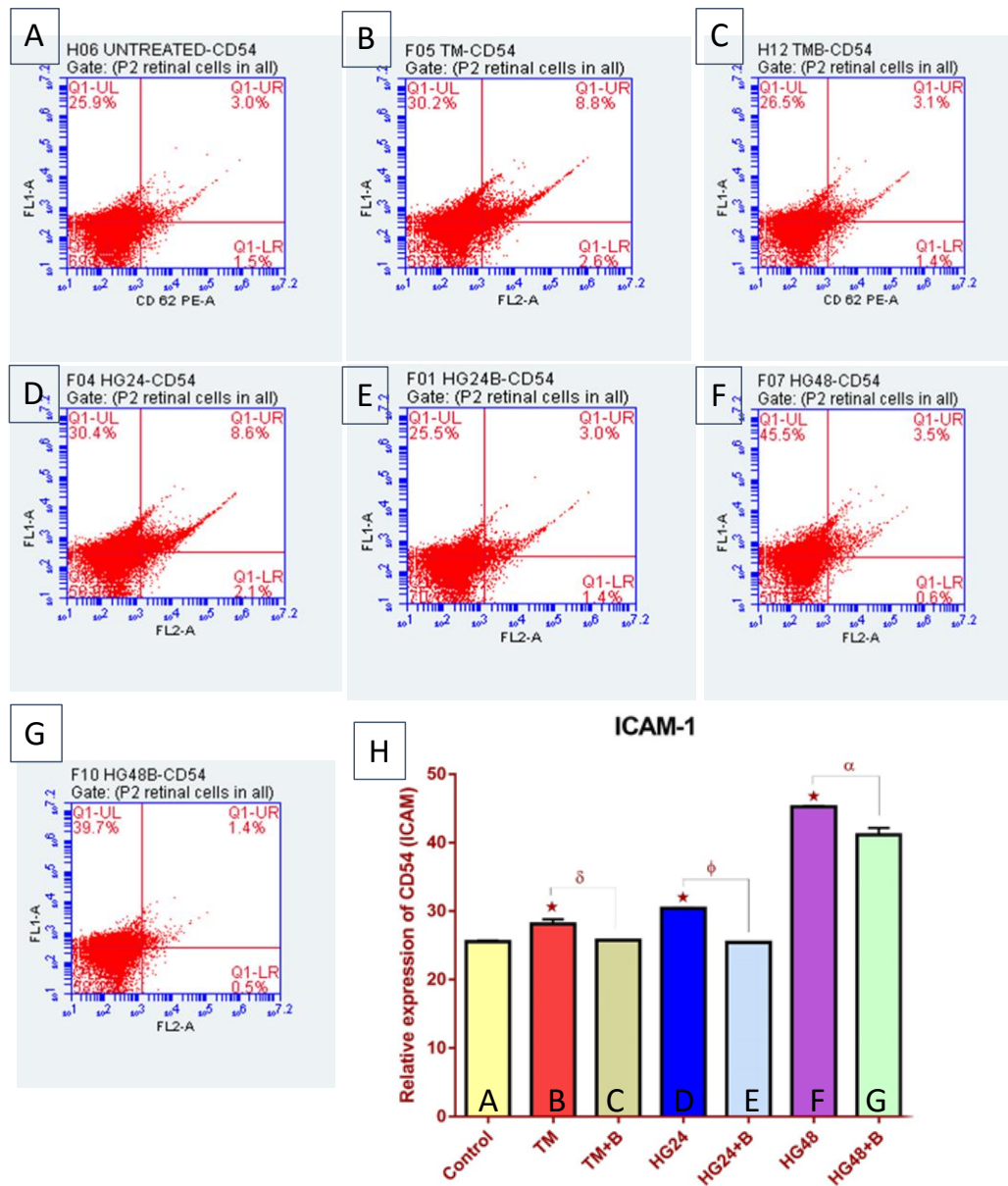
Figure 15. ICAM-1 (CD 54) Protein expression in HRMEC exposed to different treatments: 0.1µg/mL TM, 30 mM high glucose and inhibition of ERS PBA. Dot Plots and % of Protein expression of ICAM-1 (CD 54) in HRMEC exposed to different treatments: 0.1µg/mL TM, 30mM high glucose, and inhibition of ERS PBA. Data presented in bars shows the significance of expression change between the different treatment groups. *p <0.05 compared with the normoglycemic group. δ p<0.05 compared with TM group. Φp<0.05 compared to high glucose of 24 hours. αp<0.05 compared with high glucose for 48 hours. Abbreviations, normoglycemic cells (NG Ctrl); Tunicamycin-treated cells (Pos Ctrl); Tunicamycin-treated cells with ERS blocker PBA (Pos+B); High glucose treated cells for 24 hrs (HG24); High glucose treated cells for 24 hrs with PBA (HG24+B); High glucose treated cells for 48 hrs (HG48); High glucose treated cells for 48 hrs with PBA (HG48+B).

Similar to ICAM-1 expression results, E-selectin exhibited similar results. HRMEC treated with 24 hrs high glucose and treated with 48 hrs high glucose showed a significant increase (*p*=0.001) in comparison to normoglycemia. The expression of E-selectin for 24 hrs high glucose group versus HRMEC, 24 high glucose with PBA inhibitor group, revealed significant decrease (*p*=0.001) by 0.78 folds. In addition, HRMEC with 48 hrs high glucose group revealed significant decrease (*p*=0.001) compared to HRMEC with 48 hrs high glucose with PBA inhibitor by 0.88 fold (Figure 16).
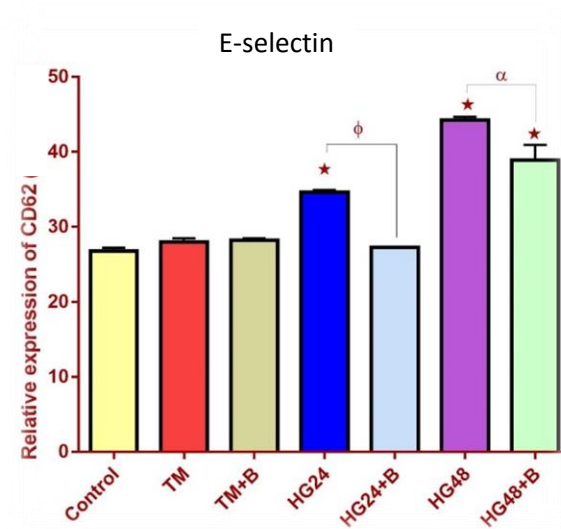
Figure 16. Protein expression of E-selectin (CD 62E) in HRMEC exposed to different treatments: 0.1µg/mL TM, 30mM high glucose, and inhibition of ERS PBA. Data presented in bars shows the expression change between the different treatment groups. *p <0.05 compared with the normoglycemic group. Φp<0.05 compared to high glucose of 24 hours. αp<0.05 compared with high glucose for 48 hours. Abbreviations, normoglycemic cells (NG Ctrl); Tunicamycin-treated cells (Pos Ctrl); Tunicamycin-treated cells with ERS blocker PBA (Pos+B); High glucose treated cells for 24 hours (HG24); High glucose treated cells for 24 hrs with PBA (HG24+B); High glucose treated cells for 48 hrs (HG48); High glucose treated cells for 48 hrs with PBA (HG48+B)

5. Chapter Five

5.1 Discussion:

Angiogenesis is a process of new blood vessels formation normally occurs in the embryonic development and other normal physiologic processes. It is also involved in pathologic processes like cancer and diabetes. In diabetic retinopathy mainly the new retinal blood vessels are formed (retinal neovascularization) due to increasing glucose level in the blood which leads to blindness at the end stage of the disease. Many proposed mechanisms underlie the retinal blood vessels dysfunction are widely investigated yet poorly understood. This is because of the effect of several common signaling pathways interfering in the pathogenesis of the disease. One of the novels known pathways is the ERS signaling pathway which is activated by the UPR. In an attempt to get a clear understanding of the underlying mechanism implicating in the retinal neovascularization and the angiogenesis pathophysiology, the current study assessed the consequences of hyperglycemia exposure in HRMEC on the activation of the endoplasmic reticulum stress pathways and its impact in regulating the inflammatory, apoptotic and the cellular adhesion interactions in vitro utilizing HREMC. The current study revealed major findings that may contribute to understanding the role of ERS in the early changes of the angiogenesis produced by short exposure of hyperglycemia (24 and 48 hrs) on HRMEC. We demonstrated that expression of the ERS genes; HSPA5, XBP1, and EIF2K3 are upregulated in response to hyperglycemia and decreased after ER blocker. Also, we noticed a significant elevation in VEGF-A, NFKB expression in response to the hyperglycemia with upregulation of the adhesion proteins ICAM-1and E-selectin, which

parallel the changes in the barrier function. Moreover, there is a significant increase in the oxidative stress and apoptosis in cultured HRMEC exposed to hyperglycemia. These findings are discussed in the next paragraphs to highlight the importance and relations to the observed barrier dysfunction in culture HREMC in response to high glucose and treatment with the ERS block (PBA).

5.1.1 The apoptosis rate and ROS production:

Apoptosis is initiated through intrinsic and extrinsic pathway as well as the reporter ERS associated apoptosis. Several studies reported that the UPR activation can regain the homeostasis of cells under mild ERS. Perpetuation ERS leads to retinal cell death which is a mark in the pathogenesis of DR (Zhang et al., 2014). In this study, we found a significant increase in the apoptotic rate and ROS production which is agreement with previous studies that reported the association of ERS with induction of apoptosis (Urra et al., 2013). In vivo study on the diabetic animal, retina suggested an elevation in the number of retinal neuronal cells death as well as increase ROS production when exposed to high glucose level (Gaspar et al., 2013). Another experimental study used different glucose concentrations on diabetic animal and cultured HRCEC showed a significant elevation in the apoptotic cells with the variable glucose used (Li et al., 2011). Furthermore, another study on suggested an involvement of ERS in retinal cell death upon administration of TM in cultured retinal ganglion cells (RCG2) (Shimazawa et al., 2007). Ikesugi et al., 2006 reported that apoptosis was induced in pericytes exposed to different glucose concentrations (Ikesugi et al., 2006). Furthermore, the significant

increase in ROS produced in HREMC in the present study in response to high glucose could be due to change in the redox status (Hwang et al., 1992). However, other studies suggested the participation of ERS pathway in overproduction of ROS in cells exposed to high glucose, which parallel the findings of the current study (Jing, Wang & Zhang, 2012).

5.1.2 ERS genes HSPA5, XBP1 and EIF2Ak3 in response to hyperglycemia for 24 and 48 hours:

The present study demonstrates a significant increase in the expression of ERS genes; HSPA5, XBP1, and EIF2Ak3 in the HRMEC exposed to 24 hours hyperglycemia. Previous studies showed the role of HSPA5 as a key regulator for three ERS sensors where it is dissociated during initial ERS. Nakamura et al. suggested HSPA5 role in the abnormal vascular changes by reporting upregulation of HSPA5 gene in HRMEC exposed to 10ng/mL of TM (Nakamura et al., 2013). Another study performed on retinal pigment epithelium (RPE) of diabetic subjects reported that HSPA5 expression is upregulated (Du et al., 2013), which parallel the current findings. Notably, other studies disagreed on this gene and indicated that HSPA5 expression on HRCEC and retinal pericytes (Li, 2011 & Ikesugi et al., 2009 respectively) did not increase. EIF2AK3 gene implicated in the part of the pathway through IRE1. This pathway involved in the inflammation and apoptosis. In Lakshmanan et al. study reported in diabetic rat model eIF2 increased in myocardial cells (Lakshmanan et al., 2013). The results of the present study parallel this study. This is also causing downstream genes to increase like CHOP the pro-apoptotic marker. Furthermore, knockdown of PERK in cardiomyocytes showed

a strong protective effect on apoptosis induced by hyperglycemia (Liu et al., 2013). XBP1 is reported in preceding studies as the gene from IRE1 pathway with an inflammatory, and a cell survival role, where the induction of XBP1 helps induction of ER other chaperones that maintaining the homeostasis (Jing, Wang & Zhang, 2012). Several studies reported that deficiency of XBP1 leads to increase oxidative stress and apoptosis (Jing, Wang & Zhang, 2012). In the same study, it was suggested that overexpression of XBP1 is linked to downregulation of the adhesion molecules ICAM-1 (Jing, Wang & Zhang, 2012).

5.1.3 Angiogenic gene VEGFA, and pro-apoptotic/pro-inflammatory NFκB, analysis:

The current study demonstrated that the angiogenic gene VEGFA expression is significantly increased in HRMEC exposed to 24h and 48 hours of high glucose and its decrease after application of the ER blocker; PBA. In addition, the treatment of HRMEC with 0.1µg/mL TM-induced significant increase in the expression of VEGFA. A study by Nakamura et al., 2013 that there was no change observed in VEGFA in response to TM treatment (Nakamura et al. 2013). Another study reported overexpression of VEGFA in HREC and Akita mice model suggesting its role in the retinal inflammation and angiogenesis (Li et al., 2009). Alternatively, another study aiming to study diabetic retinopathy at an early stage reported an increase in VEGFA expression in diabetic model versus control one (Li et al., 2011). The data of the current study is in agreement with these previous studies (Li et al., 2009), and (Li et al., 2011). The pro-inflammatory gene NFkB showed significance increase in the mRNA expression level for HRMEC exposed

with high glucose for 24 and 48 hours. The results of this study are in agreement with previous studies (Ornoy et al. 2010) and (Popov 2010). Many studies reported that NFkB could be regulated through the three ERS sensors which mean that three sensors participate in the inflammatory role (Hotamisligil, 2010).

5.1.4 Hyperglycemia increase adhesion proteins ICAM-1 and E-selectin:

Adhesion molecules are cell surface proteins that help cells to interact with each other. In the current study, exposure of HRMEC to high glucose upregulates ICAM-1 and E-selectin. Increased adhesion molecules reported causing vascular leakage which also reduce the retinal barrier leading to the main pathogenesis hallmark of DR. A study by Jingming, demonstrated that in HREC treated with 0.1µg/mL TM and followed by TNF-α treatment exhibited reduced ICAM-1 protein level. This study also suggested that the type of the chemical inducer used could affect the result of the adhesion molecules. Lie et al.; 2011 compared both TM and Thapsigargin as inducer where TM reduced ICAM-1 and the Thapsigargin increased the ICAM-1 level.(Li et al. 2011) Also, another study reported an increase in ICAM-1 protein level in rat retinal capillary EC line exposed to 25mmol of glucose (Zhang et al., 2015). ICAM-1 was reported in several studies as to be induced by several other markers like induction of VEGFA and TNF-α (Ehrich et al., 2010). However, our result suggests that the increase could be as a consequence of the collective effect of other pathways inducing the expression of ICAM-1 and E-selectin proteins.

5.1.5 Barrier quality of HRMEC were reduced by the effect of hyperglycemia:

Disturbance of blood retinal barrier induced by hyperglycemia lead to changes in the retinal vessels and exposure of the retina to the inner luminal vessels. In this study the assessment of barrier function was performed by ECIS which is permitting the real time and continuous monitoring of HRMEC during proliferation and treatment. In this study ECIS revealed that the HRMEC cell resistance (barrier quality) significantly decreased in the monolayer cells exposed to 24 and 48 hours high glucose compared to normal control. This could explain the effects of hyperglycemia on HRMEC and also supporting the result of increase expression of ICAM-1 and E-selectin proteins. The current data is supported by a previous study by Hussein et al 2014 that the barrier function was decreased in BREC of diabetic model animal after 2 hours treatment (Hussein, et al. 2014) and in culture HRMEC in response to high glucose (Rizk, et al. 2014).

5.1.6 Effect of 4-phenybutyric acid (PBA) as an inhibitor of ERS:

In this study, PBA was used as ER blocker as previously published (Li, et al. 2009). Results of apoptosis and ROS indicated a significant reduction in PBA treated HRMEC. Also, the significant reduction was noted in the adhesion proteins (ICAM and E-selectin), and in the improvement of the HRMEC barrier function. Overall, the reduction in ERS marker HSPA5, EIF2Ak3, XBP1, and pro-inflammatory NFκB and the angiogenic VEGFA biomarkers could refer to the role played by ERS in induction of the barrier dysfunction in retinal endothelial cells through its effects on ERS genes and its consequence on inflammatory, angiogenic, apoptotic and oxidative stress pathways. This

consistent with a previous study where PBA reported to be administered one hour before the treatment and decreased ICAM-1, TNF and VEGF retinal cell EC (Chen et al, 2012). In addition, PBA is an approved drug by FDA used in the treatment of urea-cycle disorder. We propose that cells exposed to hyperglycemia induces ERS as evident by upregulation of HSPA5, EIF2Ak3, XBP1with subsequent increase in ROS, apoptosis and VEGFA with marked effects of the barrier function associated with the upregulation of ICAM and p- selectin and subsequent decreased in these adhesion markers with corresponding improvement in the barrier function by ER blocker.

5.2   Conclusion:

In conclusion, our data suggest that glucose concentration of 30mM induced apoptosis and oxidative stress through the ERS which is the proposed mechanism pathway. ERS induced loss of barrier function in HRMEC by increasing the expression of ICAM-1 and E-selectin. Understanding the downstream of ERS pathway could support the current result that ERS is involved in the angiogenesis of DR. This study is limited by lack of in vivo experiments using diabetic animal models and also studying the protein expressions of ERS markers.

5.3   Future Direction:

The result of this study delivered valuable data related to ERS role in the retinal endothelial cells at an early stage of high glucose exposure. However, further studies are needed to clarify other ERS pathway expression genes. This study did not include the protein expression of ERS (e.g.CHOP and ATF4) as well as tight junction molecules

(e.g.Occludin). These proteins could provide a better understanding for the role of the downstream pathway genes in the retinal blood barrier breakdown and vascular permeability. Also, long term high glucose exposure could be studied in the same type of cells in the context of this study emphasizing on the complications of hyperglycemia on the retina.

In addition, the study can be expanded to include future investigation of in-vivo animal model to explore the ER role in details and . The success of such study can open the doors to investigate the ERS role in diabetic patients as clinical trials.

References

Back, S. H., Scheuner, D., Han, J., Song, B., Ribick, M., Wang, J., . . . Kaufman, R. J. (2009). Translation attenuation through eIF2alpha phosphorylation prevents oxidative stress and maintains the differentiated state in beta cells. Cell Metab, 10(1), 13-26. doi:10.1016/j.cmet.2009.06.002

Bauer, H. C., Traweger, A., Zweimueller-Mayer, J., Lehner, C., Tempfer, H., Krizbai, I., . . . Bauer, H. (2011). New aspects of the molecular constituents of tissue barriers. J Neural Transm (Vienna), 118(1), 7-21. doi:10.1007/s00702-010-0484-6

Bonizzi, G., & Karin, M. (2004). The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trends Immunol, 25(6), 280-288. doi:10.1016/j.it.2004.03.008

Cao, S. S., & Kaufman, R. J. (2014). Endoplasmic reticulum stress and oxidative stress in cell fate decision and human disease. Antioxid Redox Signal, 21(3), 396-413. doi:10.1089/ars.2014.5851

Du, M., Wu, M., Fu, D., Yang, S., Chen, J., Wilson, K., & Lyons, T. J. (2013). Effects of modified LDL and HDL on retinal pigment epithelial cells: a role in diabetic retinopathy? Diabetologia, 56(10), 2318-2328. doi:10.1007/s00125-013-2986-x

Ehrlich, R., Harris, A., Ciulla, T. A., Kheradiya, N., Winston, D. M., & Wirostko, B. (2010). Diabetic macular oedema: physical, physiological and molecular factors contribute to this pathological process. Acta Ophthalmol, 88(3), 279-291. doi:10.1111/j.1755-3768.2008.01501.x

Gargalovic, P. S., Gharavi, N. M., Clark, M. J., Pagnon, J., Yang, W. P., He, A., . . . Lusis, A. J. (2006). The unfolded protein response is an important regulator of inflammatory genes in endothelial cells. Arterioscler Thromb Vasc Biol, 26(11), 2490-2496. doi:10.1161/01.ATV.0000242903.41158.a1

Gaspar, J. M., Martins, A., Cruz, R., Rodrigues, C. M. P., Ambrósio, A. F., & Santiago, A. R. (2013). Tauroursodeoxycholic acid protects retinal neural cells from cell death induced by prolonged exposure to elevated glucose. Neuroscience, 253, 380-388. doi:http://dx.doi.org/10.1016/j.neuroscience.2013.08.053

Goncalves, A., Ambrosio, A. F., & Fernandes, R. (2013). Regulation of claudins in blood-tissue barriers under physiological and pathological states. Tissue Barriers, 1(3), e24782. doi:10.4161/tisb.24782

Goodall, J. C., Wu, C., Zhang, Y., McNeill, L., Ellis, L., Saudek, V., & Gaston, J. S. (2010). Endoplasmic reticulum stress-induced transcription factor, CHOP, is crucial for dendritic cell IL-23 expression. Proc Natl Acad Sci U S A, 107(41), 17698-17703. doi:10.1073/pnas.1011736107

Haze, K., Yoshida, H., Yanagi, H., Yura, T., & Mori, K. (1999). Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. Mol Biol Cell, 10(11), 3787-3799.

Hu, P., Han, Z., Couvillon, A. D., Kaufman, R. J., & Exton, J. H. (2006). Autocrine tumor necrosis factor alpha links endoplasmic reticulum stress to the membrane death receptor pathway through IRE1alpha-mediated NF-kappaB activation and down-

regulation of TRAF2 expression. Mol Cell Biol, 26(8), 3071-3084. doi:10.1128/mcb.26.8.3071-3084.2006

Humeres, C., Montenegro, J., Varela, M., Ayala, P., Vivar, R., Letelier, A., . . . Díaz-Araya, G. (2014). 4-Phenylbutyric acid prevent cytotoxicity induced by thapsigargin in rat cardiac fibroblast. Toxicology in Vitro, 28(8), 1443-1448. doi:http://dx.doi.org/10.1016/j.tiv.2014.07.013

Humeres, C., Montenegro, J., Varela, M., Ayala, P., Vivar, R., Letelier, A., . . . Díaz-Araya, G. (2014). 4-Phenylbutyric acid prevent cytotoxicity induced by thapsigargin in rat cardiac fibroblast. Toxicology in Vitro, 28(8), 1443-1448. doi:http://dx.doi.org/10.1016/j.tiv.2014.07.013

Hussein, K. A., et al. (2014) Bone morphogenetic protein 2: a potential new player in the pathogenesis of diabetic retinopathy. Exp Eye Res 125:79-88

Ikesugi, K., Mulhern, M. L., Madson, C. J., Hosoya, K., Terasaki, T., Kador, P. F., & Shinohara, T. (2006). Induction of endoplasmic reticulum stress in retinal pericytes by glucose deprivation. Curr Eye Res, 31(11), 947-953. doi:10.1080/02713680600966785

Ikesugi, K., Mulhern, M. L., Madson, C. J., Hosoya, K., Terasaki, T., Kador, P. F., & Shinohara, T. (2006). Induction of endoplasmic reticulum stress in retinal pericytes by glucose deprivation. Curr Eye Res, 31(11), 947-953. doi:10.1080/02713680600966785

Kojima, E., Takeuchi, A., Haneda, M., Yagi, A., Hasegawa, T., Yamaki, K., . . . Isobe, K. (2003). The function of GADD34 is a recovery from a shutoff of protein synthesis

induced by ERS: elucidation by GADD34-deficient mice. Faseb j, 17(11), 1573-1575. doi:10.1096/fj.02-1184fje

Lakshmanan, A. P., Harima, M., Suzuki, K., Soetikno, V., Nagata, M., Nakamura, T., . . . Watanabe, K. (2013). The hyperglycemia stimulated myocardial endoplasmic reticulum (ER) stress contributes to diabetic cardiomyopathy in the transgenic non-obese type 2 diabetic rats: a differential role of unfolded protein response (UPR) signaling proteins. Int J Biochem Cell Biol, 45(2), 438-447. doi:10.1016/j.biocel.2012.09.017

Li, B., Wang, H. S., Li, G. G., Zhao, M. J., & Zhao, M. H. (2011). The role of endoplasmic reticulum stress in the early stage of diabetic retinopathy. Acta Diabetol, 48(2), 103-111. doi:10.1007/s00592-009-0170-z

Li, J., Wang, J. J., Yu, Q., Wang, M., & Zhang, S. X. (2009). Endoplasmic reticulum stress is implicated in retinal inflammation and diabetic retinopathy. FEBS Lett, 583(9), 1521-1527. doi:10.1016/j.febslet.2009.04.007

Li, J., J. J. Wang, and S. X. Zhang. (2011) Preconditioning with endoplasmic reticulum stress mitigates retinal endothelial inflammation via activation of X-box binding protein 1. J Biol Chem 286(6):4912-21.

Liu, Z., Zhu, HT., Chen,KL., Wei,DJ. Qiu,C., and JH.Xue (2013). Protein kinase RNA like endoplasmic reticulum kinase (PERK) signaling pathway plays a major role in reactive oxygen species (ROS) -medicated endoplasmic reticulum stress- induced apoptosis in diabetic cardiomyopathy. Cardiovascular Diabetology, 12(158).

Ma, J. H., Wang, J. J., & Zhang, S. X. (2014). The unfolded protein response and diabetic retinopathy. J Diabetes Res, 2014, 160140. doi:10.1155/2014/160140

Mohammad, G., Siddiquei, M. M., Othman, A., Al-Shabrawey, M., & Abu El-Asrar, A. M. (2013). High-mobility group box-1 protein activates inflammatory signaling pathway components and disrupts retinal vascular-barrier in the diabetic retina. Experimental Eye Research, 107, 101-109. doi:http://dx.doi.org/10.1016/j.exer.2012.12.009

Nakamura, S., Takizawa, H., Shimazawa, M., Hashimoto, Y., Sugitani, S., Tsuruma, K., & Hara, H. (2013). Mild endoplasmic reticulum stress promotes retinal neovascularization via induction of BiP/GRP78. PLoS ONE, 8(3), e60517. doi:10.1371/journal.pone.0060517

Nishitoh, H. (2012). CHOP is a multifunctional transcription factor in the ERS response. J Biochem, 151(3), 217-219. doi:10.1093/jb/mvr143

Ozcan, U., Cao, Q., Yilmaz, E., Lee, A. H., Iwakoshi, N. N., Ozdelen, E., . . . Hotamisligil, G. S. (2004). Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science, 306(5695), 457-461. doi:10.1126/science.1103160

Ornoy, A., S. B. Rand, and N. Bischitz. 2010   Hyperglycemia and hypoxia are interrelated in their teratogenic mechanism: studies on cultured rat embryos. Birth Defects Res B Dev Reprod Toxicol 89(2):106-15.

Porter KR, C. A., Fullam EF. (1945). A study of tissue culture cells by electron microscopy: methods and preliminary observations. J Exp Med, 81(3), 233-246.

Popov, Doina (2010)   Endothelial cell dysfunction in hyperglycemia: Phenotypic change, intracellular signaling modification, ultrastructural alteration, and potential clinical outcomes. International Journal of Diabetes Mellitus 2(3):189-195.

Rizk, Nasser, et al.(2014) Docosahexaenoic Acid (dha) Remedies Hyperglycemia-induced Retinal Endothelial Dysfunction: A Potential Therapeutic Intervention For Diabetic Retinopathy. Qatar Foundation Annual Research Conference Proceedings:HBPP0251.

Roybal, C. N., Yang, S., Sun, C. W., Hurtado, D., Vander Jagt, D. L., Townes, T. M., & Abcouwer, S. F. (2004). Homocysteine increases the expression of vascular endothelial growth factor by a mechanism involving endoplasmic reticulum stress and transcription factor ATF4. J Biol Chem, 279(15), 14844-14852. doi:10.1074/jbc.M312948200

Sano, R., & Reed, J. C. (2013). ERS-induced cell death mechanisms. Biochim Biophys Acta, 1833(12), 3460-3470. doi:10.1016/j.bbamcr.2013.06.028

Schuiki, I., Zhang, L., & Volchuk, A. (2012). Endoplasmic Reticulum Redox State Is Not Perturbed by Pharmacological or Pathological Endoplasmic Reticulum Stress in Live Pancreatic β-Cells. PLoS ONE, 7(11), e48626. doi:10.1371/journal.pone.0048626

Shin, E. S., Sorenson, C. M., & Sheibani, N. (2014). Diabetes and retinal vascular dysfunction. J Ophthalmic Vis Res, 9(3), 362-373. doi:10.4103/2008-322X.143378

Stolz, A., & Wolf, D. H. (2010). Endoplasmic reticulum associated protein degradation: A chaperone assisted journey to hell. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1803(6), 694-705. doi:http://dx.doi.org/10.1016/j.bbamcr.2010.02.005

Trudeau, K., Roy, S., Guo, W., Hernandez, C., Villarroel, M., Simo, R., & Roy, S. (2011). Fenofibric acid reduces fibronectin and collagen type IV overexpression in human retinal pigment epithelial cells grown in conditions mimicking the diabetic milieu: functional implications in retinal permeability. Invest Ophthalmol Vis Sci, 52(9), 6348-6354. doi:10.1167/iovs.11-7282

Yamazaki, H., Hiramatsu, N., Hayakawa, K., Tagawa, Y., Okamura, M., Ogata, R., . . . Kitamura, M. (2009). Activation of the Akt-NF-kappaB pathway by subtilase cytotoxin through the ATF6 branch of the unfolded protein response. J Immunol, 183(2), 1480-1487. doi:10.4049/jimmunol.0900017

Zhang, C., Wang, H., Nie, J., & Wang, F. (2014). Protective factors in diabetic retinopathy: focus on blood-retinal barrier. Discov Med, 18(98), 105-11

Appendix A. Institutional Bio-Safety Committee Approval:

Qatar University
Institutional Bio-safety Committee

QATAR UNIVERSITY
جامعة قطر

To: Dr. Nasser Rizk   4th June, 2015
Department of Health Sciences
College of Arts and Science

Dear Dr. Rizk,

Subject: Research grant QUST-CAS-SPR-14\15-40

Ref: Project titled "Effect of Endoplasmic Reticulum Stress mediated by Hyperglycemia on Barrier Function in Retinal Cells"

We would like to inform you that your application along with supporting documents provided for the above proposal have been reviewed by QU-IBC, and having met all the requirements, has been granted approval for a period of one year and renewable for each year thereafter, should be sought and approved by QU-IBC period to continue.

Please note that QU-IBC approval is contingent upon your adherence to the following QU-IBC Guidelines:

- Ensuring compliance with QU Safety Plans and applicable national and international regulations.
- Ensuring experiments that require prior IBC approval are not conducted until IBC approval is obtained and making initial determination of containment levels required for experiments.
- Notifying the IBC of any changes to other hazardous material experiments previously approved by the IBC.
- Reporting any significant problems, violations of QU Safety Plans and applicable regulations/guidelines, or any significant research-related accidents and illnesses to the QU-IBC. Also, ensuring personnel receive appropriate orientation and specific training for the safe performance of the work.

Your research approval No. is: **QU-IBC 13/14-15**. Please refer to this approval number in all your future correspondence pertaining to this research.

Best wishes.

Dr. Marawan Abu-Madi PhD, MLS(ASCP)cm

Appendix B. Research Arabic Summary

تـاثير اجهاد الشبكة الاندوبلازمية بواسطة ارتفاع السكر في الدم على وظيفة الحاجز في الخلايا البطانية الشبكية

يعتبر داء السكري من الامراض المزمنة والمنتشرة بكثرة في هذا العصر. تحتفظ الدول العربية بصدارة اكثر الدول المصابة بالسكري حسب احصائيات منظمة الصحة العالمية . لمرض السكري مضاعفات عديدة اهمها هو اعتلال شبكية العين المصاحب لارتفاع الجلوكوز بالدم. يعتقد ان من اهم اسباب اعتلال الشبكية هو خلل في تكوينات الحواجز الدموية للشبكية ومضاعفات التهابية.

هناك عدة آليات على مستوى الخليه لحدوث اعتلال الشبكية. احدث هذه الآليات هو اجهاد الشبكة الاندوبلامية. حيث تصاب الشبكة الاندوبلازمية بالخلل في وظائفها العادية نتيجة تراكم الغلوكوز في الدم. وتكمن اهمية الشبكة الاندوبلازمية في انها تعتبر المصنع لجميع البروتينات في الخلية وعند حدوث اي خلل يؤدي هذا الى تراكم البروتين الغير مكتمل التصنيع وبالتالي خلل الوظائف المترتبة على هذه البروتينات. وفي حالة استمرار هذا الاجهاد يؤدي هذا الى موت الخلايا المتعرضة للجلوكوز لفترات طويلة. هذه الآلية يعتقد ايضا انها مربطة بامراض اخرى بالاضافة الى السكري مثل السرطان و بعض الامراض العصبية.

في هذا البحث اعمل على فرضية ان اجهاد الشبكة الاندوبلازمية مرتبط تحديدا باعتلال الشبكية من خلال افراز الشبكة الاندوبلازمية لبعض لعوامل التي تؤدي الى خلل في ترابط وتكوين الخلايا المبطنة للشعيرات الدموية للعين (retinal endothelial cells). . وحيث ان هناك دراسات اثبتت وجود مستحضرات كيميائية ممكن ان تعاكس اجهاد الشبكة الاندوبلامية من خلال منعها او تقليلها، فان هذا المستحضر وهو phenylbutyric acid سوف يستخدم ايضا في هذا البحث.

المميز في هذا البحث ان الية للشبكة الادوبلازمية درست على نطاق واسع في معظم انواع خلايا العين ماعدا الخلايا المبطنة للشبكية. ونأمل بان تسهم نتائج هذا لبحث في فهم اعمق لهذه الآلية ومساعدة مرضى السكري واعتلال الشبكية بعلاج مناسب في المستقبل.

Appendix C. Research Poster:

An oral presentation for the study was performed during the Fifth research forum conducted at Hamad Medical Corporation 26th May 2016.

Postdoc and Graduate Students /Biomedical and Health Sciences

Effect of Endoplasmic Reticulum Stress Mediated by Hyperglycemia on Barrier Function in Retinal Cells

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ABSTRACT

Background: Diabetic Retinopathy (DR) is an inflammatory disease mainly characterized by the dysfunction of the retinal blood vessels. Several studies reported a possible link between the hyperglycemia and the activation of unfolded protein response (UPR) and hence initiating endoplasmic reticulum (ER) stress in various retinal cells. However, the mechanism underlying the ER stress in the human retinal endothelial cells (HRMEC) remains poorly investigated and understood.

Methodology: In the present study, utilizing the HRMEC, we evaluated the effect of high glucose (30mM) on several ER stress signaling pathway (HSPA5, EIF2K3, and XBP1) at the mRNA expression level; the inflammatory cytokines (NFkB) at the protein level; the adhesion molecules (ICAM-1 and P-selectin) by flowcytometry . In addition we assessed the barrier function of HRMEC on real-time analysis using the electric-cell substrate impedance (ECIS). Also, this study uses the Tunicamycin (TM) as positive stress inducer and Phenyl-butyric acid (PBA) as an ER stress inhibitor and study their effects on the treatment groups.

Results: We found that hyperglycemia increases the cellular production of reactive oxygen species (ROS) and PBA decreasing it. Also the barrier quality of HRMEC was affected significantly with treatment of high glucose and improved with PBA. The expression of Adhesion proteins (ICAM-1 and P-selectin) were increased in hyperglycemia and decreased expression with PBA.

Conclusion: Our results suggest that hyperglycemia may activate ER stress that could play a role in the angiogenesis in the diabetic retinopathy.

INTRODUCTION

- Under normal condition, Endoplasmic Reticulum plays the main role in protein folding and synthesis.
- Under certain circumstances, accumulation of misfolded proteins occurs leading to ER stress and the formation and activation of the unfolded protein response (UPR) (1).
- Three main proteins have been identified following UPR stimulation in cellular stress. These proteins include; protein kinase RNA-like ER kinase pathway (PERK), the inositol-requiring protein-1 (IRE-1), and activating transcription factor-6 (ATF-6) (1).
- These proteins are bound to ER chaperone Glucose-regulated protein 78 (GRP78) also known as Binding Immunoglobulin Protein (BiP).
- Another experimental study suggested that ER stress also caused inflammation in animal models of diabetic retinopathy and found that the three UPR pathways and the inflammatory gene expressions are activated in the retinal endothelial cells (6 &7).
- Chaperone-like activity, 4- phenylbutyric acid (PBA) was reported to diminish the effect of ER stress as well as the retinal inflammation and vascular leakage in the diabetic animals. PBA is a terminal aromatic substituted fatty acid and is a histone deacetylase (HDAC) activity inhibitor (8).
- In Diabetic retinopathy, the interruption of the blood-retinal barrier is contributed by hyperglycemia (9).
- Disruption of Tight junctions between endothelial cells leads inflammation and increase production of adhesion molecules (through increased expression of vascular endothelial growth factor VEGF) such as intracellular adhesion molecules 1 (ICAM-1).

Figure 1: ER stress signaling pathway.
Source: Waldron, Pandol, Lugea & Groblewski, 2015
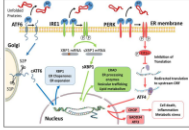
RESULTS

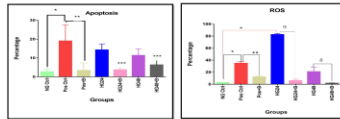**1. Effect of High Glucose on HMREC on Apoptosis and Oxidative stress**

Figure 8: **(Left)** Apoptosis increased in hyperglycemia 24 & 48 hrs treatment and reduced with addition of ER stress blocker PBA. **(Right)** Reactive oxygen species percentage in Tunicamycin group, and high glucose group (24 &48 hrs) showed significant ROS increase. Addition of PBA decreased ROS production in all groups.

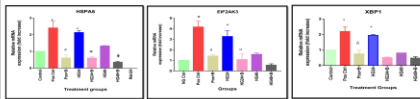**2. Effect of High Glucose on ER stress markers**

Figure 9: Gene expression of ER stress markers. Tunicamycin and High glucose 24 hrs. revealed significant increase in all three markers whereas High glucose 48 hrs. group was increased but insignificantly. Addition of ER stress blocker PBA decrease the mRNA expression in all groups significantly.

4. Effect of High Glucose on inflammatory marker NFKB & angiogenesis marker VEGFA
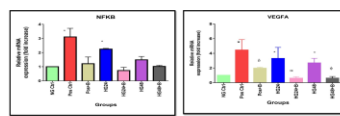
Figure 10: Gene expression of inflammatory marker NFkB **(Left)** revealed significant increase in Tunicamycin and high glucose 24 hrs. groups while high glucose 48 hrs. increase insignificantly. Addition of ER stress blocker PBA showed decrease in the gene expression but insignificant in all groups. **(Right)** Gene expression of VEGFA showed significant increase in all high glucose groups and addition of PBA decreased expression VEGFA significantly in all high glucose groups .

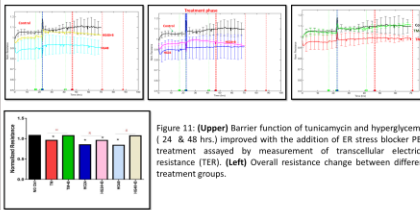**5. Barrier Function analysis under effect of hyperglycemia and ER stress blocker**

Figure 11: **(Upper)** Barrier function of tunicamycin and hyperglycemia (24 & 48 hrs.) improved with the addition of ER stress blocker PBA treatment assayed by measurement of transcellular electrical resistance (TER). **(Left)** Overall resistance change between different treatment groups.

RESULTS

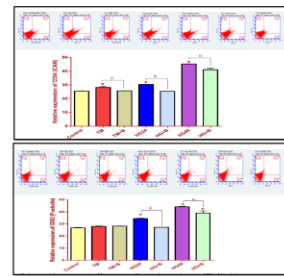**6. Effect of hyperglycemia and PBA on expression of adhesion protein ICAM and P-selectin in HMREC**

Figure 12: Treatment of HMREC with hyperglycemia (24 & 48 hrs) significantly increased ICAM-1 **(upper)** and P-selectin **(lower)** expression while treatment of PBA significantly decreased the expression in both proteins.

CONCLUSION

This study suggest that high glucose concentration induced apoptosis and oxidative stress through the ER stress which is the proposed mechanism pathway. ER stress induced loss of retinal vascular barrier function in HRMEC by increasing the expression of ICAM-1 and P-selectin. Also this study shows the potent therapeutic effect of PBA on ER stress. Understanding the downstream of ER stress pathway could support the current result that ER stress is involved in the angiogenesis of diabetic retinopathy. This study is limited by lack of in vivo studies using diabetic animal models.

REFERENCES

1- Sage AT et al. Metabolic Syndrome and Acute hyperglycemia are associated with endoplasmic reticulum stress in human mononuclear cells. Obesity 2012; 20: 748-755.
2- Werstuck GH et al. Glucosamine-induced endoplasmic reticulum dysfunction is associated with accelerated atherosclerosis in a hyperglycemic mouse model. Diabetes 2006; 55(1): 93-101.
3- Harding HP, et al. PERK is essential for translational regulation and cell survival during the unfolded protein response. Molecular Cell 2000; 5 (5): 897-904.
4- Lee AH, Iwakoshi NN, and Glimcher LH. XBP-1 regulates a subset of endoplasmic reticulum chaperone genes in the unfolded protein response. Molecular and cellular biology.2003; 23(21): 7448-7459.
5- Haze K,et al. Mammalian transcription factor ATF6 is synthesized as transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. Molecular biology of the cell. 1999; 10 (11): 3787-3799.
6- Ma JH, Wang JJ, and Zhang SX. The unfolded protein response and diabetic retinopathy. Journal of diabetes research. 2014; 160140- p.14.
7- Li J, et al. Endoplasmic reticulum stress is implicated in retinal inflammation and diabetic retinopathy. FEBS letters. 2009; 583(9): 1521-1527.
8- Mimori S. et al. Protective effects of 4-phenylbutyrate derivetives on the neuronal cells death and endoplasmic reticulum stress. Bio.Phar,.Bull. 2012; 35(1):84-90
9- Ehrlich R, et al. Diabetic macular oedema: physical, physiological and molecular factors contribute to this pathological process. Acta Ophtho. 2010;88(3): 279-291.
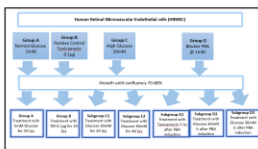
METHODOLOGY

Figure 2: Study design for the treatment groups. Study utilized with Human Retinal Endothelial Cell (HREC) Culture passage 5 and 6.

Figure 3: Apoptosis and oxidative stress were assessed using Tali® Image-based Cytometer (Life Technologies, USA).

Figure 4: Gene expression analysis for ER stress marker, NFkB and VEGF were carried out using QuantaStudio™6 Flex Real time PCR(Life Technologies, USA).

Figure 5: Adhesion molecules ICAM-1 and P-selectin were assessed using BD Accuri™ Flowcytometer (BD Biosciences).

Figure 6: Barrier function was analyzed by the Electric Cell Substrate Impedance Sensing (ECIS).

ACKNOWLEGEMENTS