QATAR UNIVERSITY

COLLEGE OF HEALTH SCIENCES

# THE CONTRIBUTION OF TOLL-LIKE RECEPTORS IN THE PATHOGENESIS OF DIABETIC RETINOPATHY IN HUMAN MICROVASCULAR RETINAL ENDOTHELIAL CELLS IN VITRO

BY

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# Abstract

Background: Diabetes Mellitus is a chronic systemic inflammatory disease including the eye causing macrovascular as well as microvascular complications known as diabetic retinopathy (DR), thus increasing the risk of vision impairment and blindness among working adults. The activation of the innate immune system during diabetes leads to an increase in certain biomarkers which in turn can antagonize the immune system leading to more complications. Toll like receptors (TLRs) are receptors of the innate immune system, known as pattern recognition receptors, among them TLR4 recently been linked to DR, but scanty data are available. Thus, this research focus on providing insight into the role of TLR4 in the pathogenesis of DR. Human microvascular retinal endothelial cells (HMVRECs) was used to evaluate the contribution of TLR4 in the pathogenesis of DR.

Methods: HMVRECs have been treated with high glucose (30 mM) and normal glucose (5.5mM) in addition to antioxidants, and the expression of TLR4, TLR2, NFkB and VEGFA mRNA are measured. The barrier function was assessed by trans electrical resistance impedance using ECIS (Applied Biophysics) in comparison to TLR4 siRNA-transfected cells treated in the same way. TNF-alpha was measured by the Elisa technique.

Results: High glucose treatment increases the mRNA expression of TLR4 while the TLR4 siRNA-transfected HG-treated cells attenuates the TLR4 mRNA expression. On the contrary, the inflammation (NFKB expression and TNF-alpha) was not attenuated by silencing TLR4. Additionally, antioxidant treatment did not help the cells to regain normal behavior when TLR4 was silenced. The barrier function disorder observed in normal cells exposed to HG did not improve significantly by silencing TLR4 in these cells.

Conclusion: Hyperglycemia induces TLR4 expression, and its downstream signaling induces inflammation, but silencing TLR4 does not restore normal barrier function, indicating that TLR4 alone does not contribute to the pathogenesis of DR.

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## Introduction

Diabetes Mellitus (DM) is classified as a worldwide epidemic and characterized by hyperglycemia leading to macrovascular and microvascular complications, thus increasing the risk of vision impairment and blindness due to diabetic retinopathy (DR). The progressive retinal microvascular changes result in retinal ischemia, permeability, neovascularization and macular edema. Several biochemical changes secondary to hyperglycemia affects the microvasculature of the retina. Such changes involve the augmented oxidative stress, increased inflammatory cytokines, chemokines and adhesion molecules.

Recently, it has been demonstrated that specific innate immune receptors are activated in diabetes, known as TLRs, they recognize the molecular patterns of different microbial infections. Among 10 human TLRs, TLR2 and TLR4 been studied to explore their role in diabetes. The role of TLR2 and TLR4 has been investigated in association with atherosclerosis, diabetic microvascular diseases, and diabetic nephropathy, whereas there are very sparse data available regarding the role of TLRs in DR.

Recent studies have been published aiming to explore the effect of hyperglycemia on the retinal pathology via TLRs activation. Therefore, in this study, we investigated the impact of TLR4 in the pathogenesis of retinal barrier dysfunction by exploring TLR4 in retinal cells treated with HG, and by application of siRNA technique for TLR4.

# Chapter 1: Literature Review

## 1.1 Diabetic Retinopathy:

Diabetes is classified as a worldwide epidemic, expected to affect up to 360 million people by 2030, therefore increasing the risk of diabetes complications including vision impairment and blindness due to diabetic retinopathy (DR), a common microvascular complication of diabetes among working adults (1-3). Diabetes is a pro-inflammatory state with hyperglycemia contributing to microvascular and macrovascular complications (1). Diabetic Retinopathy (DR) is a progressive retinal microvascular alteration leading to retinal ischemia, permeability, neovascularization and macular edema (3). As Diabetes mellitus (DM) becomes an epidemic, DR is rising to an alarming level. DR is classified into non-proliferative diabetic retinopathy (NPDR) including mild, moderate and severe stages, and proliferative diabetic retinopathy (PDR) which is differentiated by retinal neovascularization (growth of new blood vessels) (2). Both forms involve microvascular lesions and development of diabetic macular edema (DMO) that can complicate either forms (4). The root of vision impairment and blindness are PDR and DMO, almost affecting all type1 diabetic patients and more than 60% of type2 diabetic patients within the first decade of incidence of diabetes (2). Several biochemical changes due to hyperglycemia affect the microvasculature of the retina, including activation of several isoforms of protein kinase C (PKC), increased the formation of advanced glycation end-products (AGEs), enhanced the activity of polyol pathway and oxidative stress (5).

### 1.1.1 Features of DR:

Several abnormal features are characterizing the diabetic retinopathy, the most common are, microaneurysms, hemorrhages, hard exudates and cotton wool spots, which if left untreated, can lead to blindness (3, 6). DR progresses through several stages. First is due to an elevation of blood glucose level, the capillaries permeability increases, and endothelial elasticity decreases, this is classified as mild non-proliferative diabetic retinopathy. The first clinical sign of diabetic retinopathy is the appearance of non-specific microaneurysms (6). Continues change in the microaneurysms leads to the rupture of the capillaries and formation of intraretinal hemorrhages. As the disease progresses, the tight junction between the endothelial and the capillaries breaks leading to intraretinal accumulation of fluid (6). This is an indication of disease progression towards moderate and severe NPDR, which also is characterized by the formation of hard exudates. Leakage of microaneurysms is known as diabetic macular edema (DME) (3). The disease progresses to proliferative diabetic retinopathy when occlusion of the capillaries leads to hypoxia and therefore the release of vascular endothelial growth factor VEGF resulting in the formation of new retinal blood vessels (neovascularization), these blood vessels are leaky causing hemorrhage that is complicating the disease more (6). PDRP along with DME are the main reason of vision loss.

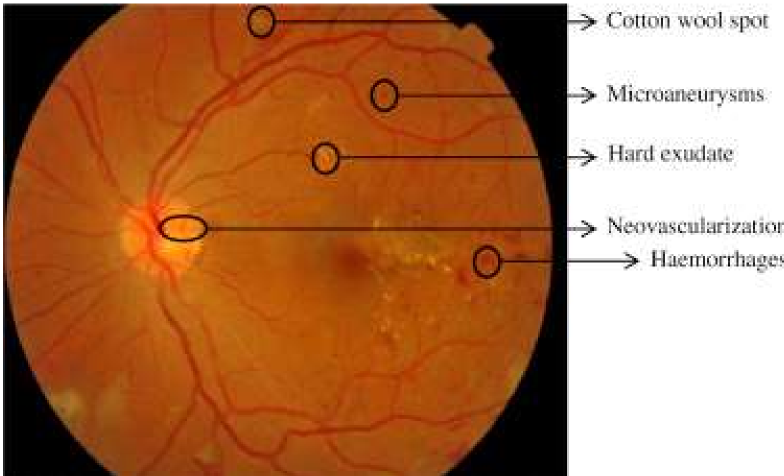

Fig. 1.1.1. Retinal fundus image. Clinical abnormal features of diabetic retinopathy.

## 1.2. Innate immunity and Toll-Like Receptors(TLRs):

As DM causes systemic inflammation, it affects the immune system by different aspects. The immune system is crucial in recognition self from non-self antigens and elimination of pathogens; it consists of two components; acquired immune system and innate immune system, both has been studied and characterized independently by an immunologist. The focus on the innate immune system started at the end of 20th century when toll receptor ability is shown to recognize fungal infections in Drosophila, which has only innate immunity and soon after that, it was proved to induce gene expression during inflammatory response in mammals (7). Thereafter, the researchers focused on the ability of toll-like receptors(TLRs) to activate the innate immune system and develop antigen-specific acquired immunity.

### 1.2.1. The structure and types of TLRs:

Toll like receptors are key receptors in the innate immune system which recognizes the molecular patterns of different microbial infections. As bacterial and fungal components are recognized by the cell surface TLRs, whereas endosomal TLRs recognize viral or microbial nucleic acids. There are different TLRs until now with 10 TLRs have been identified in humans, among them, TLR2 and TLR4 play a major role in diabetes, insulin resistance, and inflammation (8). These receptors are mainly expressed on the surface of macrophage and dendritic cells but also are expressed on cells of the central nervous system, kidney and liver cells (9).

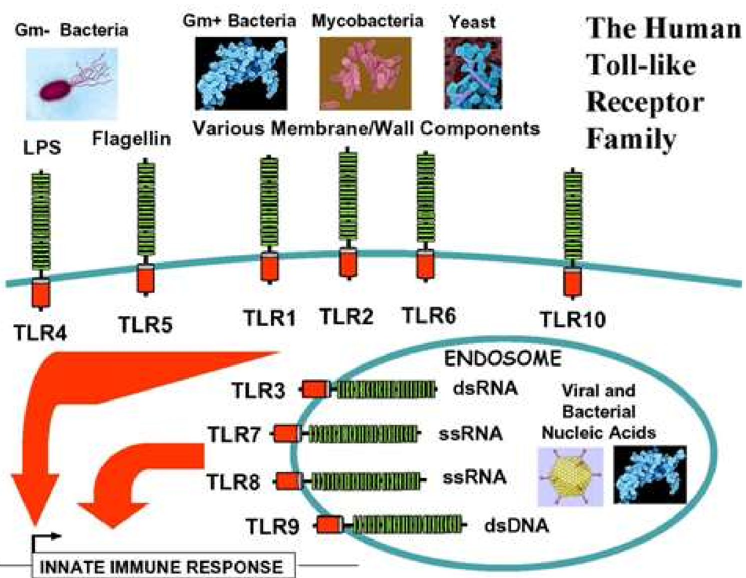


Fig.1.2.1a. Ligands recognized by TLR family. TLR2 heterodimerize with TLR1 or TLR6 and recognize triacyl lipopeptide or diacyl lipopeptide respectively. The ligand for TLR4 is LPS. The ligand for TLR3 is dsRNA. The ligand for TLR7 and 8 is ssRNA. The ligand for TLR9 is dsDNA, whereas TLR10 has no known ligands.

TLRs have two regions; cytoplasmic region which largely shares about 200 amino acid sequences as homology with IL-1 receptor family therefore known as the Toll/IL-1R (TIR) domain which is crucial for signaling, and extracellular diverse region of leucine-rich repeat (LRR) with varying number of repeats (22–29 residues in length) and is involved in the recognition of different pathogens(10).

Of the 10 TLR in humans TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed on the cell surface whereas TLR3, TLR7, TLR8, and TLR9 are expressed on the endosome; the intracellular compartment. Each TLR recognizes pathogens through pathogen-associated molecular patterns (PAMPs) that initiate an immune response. As the TLR family mediate the immune response through recognizing specific patterns of microbial components, TLR2 recognizes different lipopeptides, and TLR1/TLR6 function as co-receptors for TLR2, whereas TLR2 through its heterodimerization either with TLR1 or TLR6 it can differentiate between triacyl and diacyl lipopeptides, respectively (10-12). TLR3 can recognize viral dsRNA that is produced from many viruses during replication (7, 10, 13). TLR4 is vital for recognition of bacterial lipopolysaccharide (LPS) (14). TLR5 recognizes bacterial flagellin (15). TLR7 and TLR8 are receptors for ssRNA (16, 17). TLR9 recognize bacterial and viral CpG DNA (18, 19). Whereas, TLR10 remains an orphan without known ligand as it lacks a rodent homolog to be studied (20), but it has been implicated in viral and bacterial infections (21, 22).

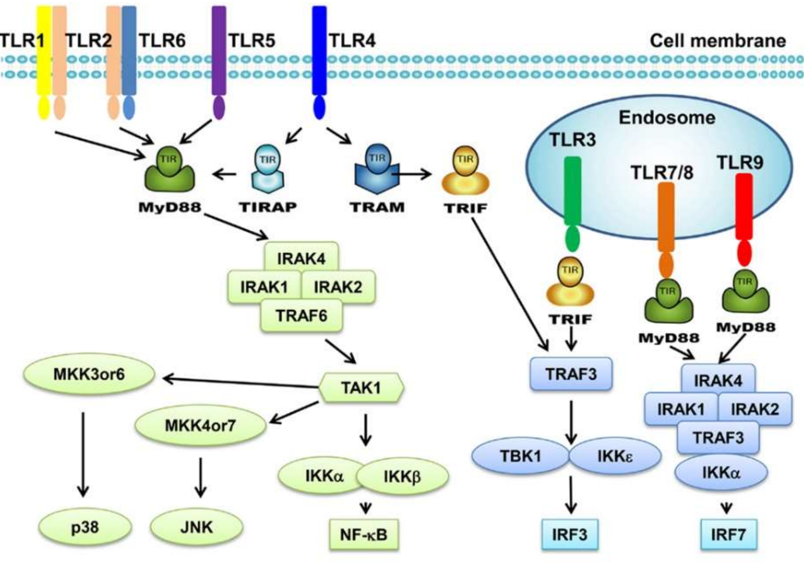


Fig. 1.2.1b. TLRs signaling pathway. All TLRs signals trough MYD88 dependent pathway, except TLR3, which signals trough TRIF pathway, or MYD88 independent pathway. A property of TLR4 is that is signals trough both pathways.

### 1.2.2: Signaling of TLRs:

Activation of TLRs leads to the expression of genes involved in inflammatory response. TLRs signals through myeloid differentiating factor (MYD88) either via dependent or independent pathways. Most TLRs gives signal through MYD88-dependant pathway activating its cytoplasmic TIR domain which is conserved among all TLRs, except TLR3 (23, 24). The importance of the cytoplasmic TIR domain in TLRs as the origin of signal transduction cascade, was first indicated by Poltorak et al. in 1998 using C3H/HeJ mice with mutation (change from proline to histidine) at 712 coding regions in the cytoplasmic TIR domain renders the mice irresponsive to LPS, also, it was the first study that recognized LPS as the ligand for TLR4 (14). TLR1, TLR2, TLR5, TLR6,

TLR7, TLR8, and TLR9 signals through activation of the MYD88-dependant pathway, whereas TLR3 signals through the MYD88-independent pathway, moreover TLR4 recruits both MYD88-dependant and MYD88-independant pathways (23).

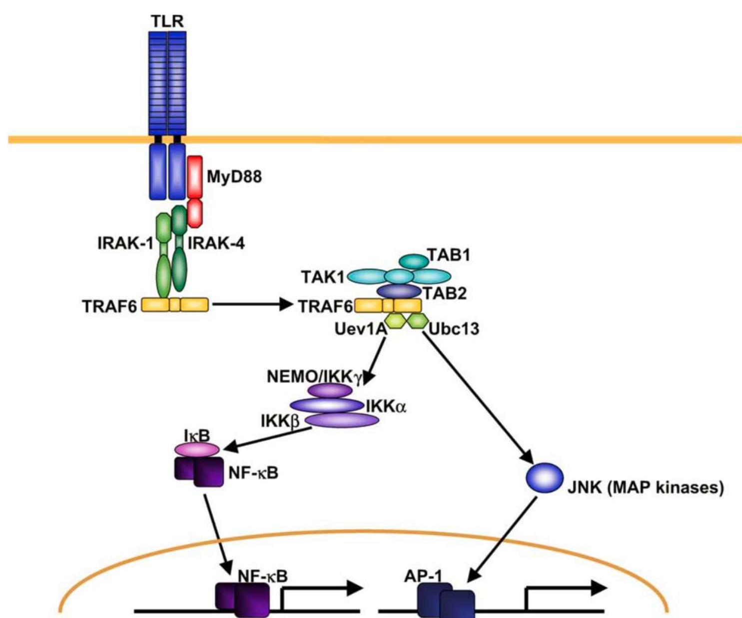


Fig1.2.2. MYD88 dependent signaling. Most TLRs activate MYD88 pathway that eventually activate NF-kB transcribing proinflammatory genes.

MYD88 consists of two domains; The C-terminal TIR domain and the N-terminal death domain, it binds the cytoplasmic TIR domain of TLRs and starts the signaling cascade (25, 26). The death domain of MYD88 interacts with the death domain of IRAK-4 (IL-1 receptor-associated kinase) stimulating its phosphorylation; activation of IRAK-4 will phosphorylate IRAK-1, which in turn will recruit binding of TRAF6 (tumor necrosis factor receptor–associated factor-6) to MYD88/IRAK-4/IRAK-1 complex (26-28). The

complex IRAK-1/TRAF6 will dissociate from the receptors and binds TGF-β-activated kinase1 (TAK1), TAK1-binding proteins, TAB1, and TAB2 at the membrane portion (26, 28). Further, IRAK1 stays and degrades in the membrane, while the TRAF6, TAK1, TAB1, and TAB2, migrates to the cytoplasm and form a larger complex. Consequently, this large complex activates c-Jun N-terminal kinase ( JNK) and inhibits κB kinase (IKK), which ends up activating NF-KB and AB-1(activator protein-1) in the nucleus. After that, NF-KB stimulates the transcription of proinflammatory cytokines and chemokines such as IL-8, IL-1β, IL-6 and TNF-α (24, 26, 28, 29).

Several studies demonstrated the importance of MYD88 mediated inflammatory response of TLR family members. In a study on TLR4 ligand (LPS), showed no response to the inflammatory mediators on MyD88 knockout mice (30, 31). Similarly, the cellular response to the TLR2 ligands was abolished through studying the MyD88 knockout mice (11, 30, 31). Furthermore, no response had been observed in cells from MyD88 knockout mice for both TLR9 and TLR7 ligand (32, 33). Nevertheless, the TLR5 ligand did not produce IL-6 in MyD88 knockout mice (15).

As mentioned above, in MYD88 knockout mice most of the TLRs ligand did not cause NF-KB activation and increase in the inflammatory markers. An exception was TLR4, which resulted in delayed phase activation of NF-KB indicating that it can signal through another pathway (30, 34). Whereas TLR3 ligand (dsRNA) exclusively results in NF-KB activation in MYD88 knockout mice (13). Altogether, the results reached to that, TLR4 signals through both MYD-88 pathway and non MYD-88 pathway, whereas TLR3

exclusively signals through the non-MYD88 pathway in which IRF3 plays a key role. IRF3 is a critical transcription factor for type I interferons and activation of NF-KB, and either of TLR4 and TLR3 activation phosphorylates it (10). The signaling from both IL-1R and TLRs are critical for activation NF-KB that is a vital transcription factor for proper immune function. TLR-mediated signaling pathways are regulated by TIR domain-containing adaptors, such two adaptors been identified during MyD88-independent pathway analysis, these adaptor protein are MyD88-adaptor-like (Mal) (TIRAP/Mal )and adaptor inducing IFN-B (TRIF) with adaptor molecule (TICAM-1) (TRIF/TICAM) (35-39). Further studies revealed that TIRAP/Mal is associated with MYD-88 dependent pathway but not with MYD-88 independent pathway (40, 41). However, other studies demonstrated that TRIF is essential for the TLR3 and TLR mediated MyD88-independent pathway (42, 43).

### 1.3. High Glucose and TLRs:

Recently is has been indicated that TLRs activation associates the activation of the innate immune system and inflammation during diabetes. This observation led to an increased interest to TLRs signaling pathways and subsequent inflammatory reactions (1, 8, 23, 44-46). Moreover, These receptors are precent on the cell surface and on endosome. The cell surface receptors can distinguish bwtween bacterial and fungal components, while the intracellular TLRs can distinguish between viral or microbial nucleic acids. In fact, they have the ability to recognize endogenous ligands that are

elevated in diabetes such as oxLDL (oxidized LDL), HSPs (heat-shock proteins) 60 and 70, fibrinogen, fibronectin and others (47-50), these are known as Damage associated molecular patterns (DAMPs). Among different TLRs, TLR2 and TLR4 have been indicated in insulin resistance (IR) and inflammation during diabetes, thus are the focus of this review.

One of such recent studies has been conducted in 2014 by Rajamani and Jialal (1) and published in the journal of diabetes research. They showed the effect of hyperglycemia on TLR2 and TLR4, in addition to NFKB activation. They applied different techniques to reach their goals including small inhibitory molecules and siRNA. Another study conducted by Wang et al. in 2015 (51), in which they used different samples to evaluate the expression of TLR4, including patients with proliferative diabetic retinopathy and diabetic mice.

In 2004, Michelsen et al.(52) provided a pathophysiological link between innate immunity, inflammation, and atherogenesis by studying mice deficient in apolipoprotein E prone to atherosclerosis that also lacked TLR4 or MyD88, demonstrating reduced aortic atherosclerosis and alters plaque phenotype. As diabetes leads to an increased risk for both microvascular and macrovascular complications, further studies followed this point seeking the role of TLRs in atherosclerosis (52-57). In 2006, Senn Joseph J. (58), studied the role of TLR2 in palmitate-induced insulin resistance in C2C12 myotubes, showing that treatment with palmitate caused TLR2 and MYD88 activation. His study provided strong evidence of TLR2 association with insulin resistance in muscle, when he

treated the cells with palmitate it inhibited insulin signal transduction, and when he applied a TLR2 antagonist in palmitate-treated cells, the insulin resistance was decreased (58). TLR4 also been linked to obesity and insulin resistance, increased the activity of pro-inflammatory kinases JNK, IKK, and P38, and increased transcription of the pro-inflammatory gene due to elevated expression of TLR4 in insulin targeted tissues, led to impaired insulin signal transduction and farther insulin desensitization (59). This association between the innate immune systems TLRs activation and inflammation during diabetes only recently been pinpointed, this led to an increased interest to TLRs signaling pathways and subsequent inflammatory reactions (8, 10, 23, 44, 46, 60).

**1.4. Inflammatory biomediators and DR:**

Retinopathy is the most distractive eye complication due to hyperglycemia, ending to blindness. Recently, attentions been drowned toward similarities between DR and chronic inflammatory diseases. As DR is a complication of diabetes, which is chronic inflammation, therefore inflammatory process involving structural and molecular alterations may play an important role in the pathogenesis of DR (61-64). Thus, many factors been suggested to contribute to this process such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-alpha (TNF-α), vascular endothelial growth factor (VEGF), and nuclear factor-kappa Beta (NF-Kβ).

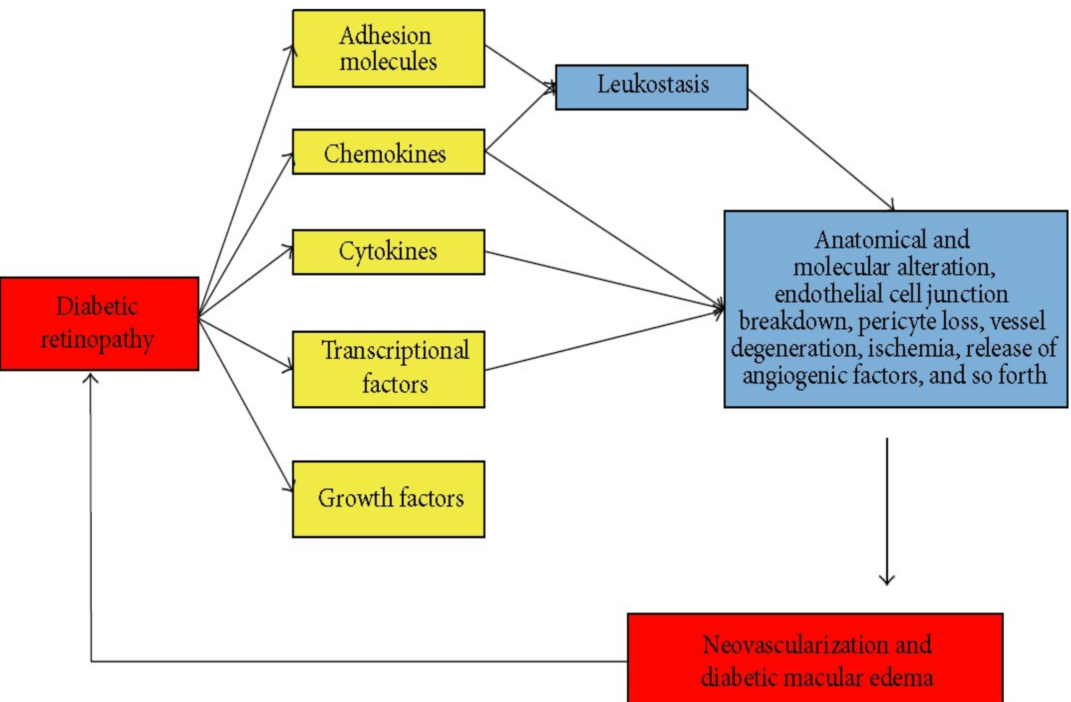


Fig.1.4. Contribution of different inflammatory mediators in DR development. Increase of different factors due to high glucose in diabetic lead to anatomical and molecular alterations which may play an important role in the pathogenesis of DR. Adapted from (66)

DR develops because of the many changes in the level of cytokines, chemokines and adhesion molecules that are dysregulated due to hyperglycemia, thus leads to such alterations including new vascularization of abnormal, leaky blood vessels in the eyes. One of such inflammatory markers is IL-1β, an inflammatory cytokine produced by the endothelium, which is considered being the initial source of IL-1β production in the retina; after that, it induces its own synthesis in an autocrine fashion. IL-1β is involved in angiogenesis and synthesizing collagen, most importantly it can activate the transcriptional factor NF-κB leading to transcription of different inflammatory cytokines (65, 66). The activation of NF-κB pathway, which is a pro-inflammatory signaling

pathway, leads to expression of several pro-inflammatory genes including cytokines, chemokines, and adhesion molecules (67), thus elevating inflammation in the eye. In the innate immunity; the nuclear translocation of NF-$\kappa$B results in induction of cytokines that regulates the immune response to TNF-α, IL-1, IL-6 and IL-8 and also adhesion molecules that recruits leukocytes to sites of inflammation, it also affect cell proliferation and apoptosis, in addition to vascularization through upregulation of VEGF and its receptors (68-72). Table1 shows the different inflammatory markers that found to be elevated in the vitreous of diabetic patients (65), these factors contributes in many different ways in the initiation and progression of DR.

Table 1.4: Vitreous mediators involved in the pathogenesis of DR (66).

| Vitreous mediators | Function |
|---|---|
| **IL-6** | (i) Regulating immune responses<br>(ii) Increasing vascular permeability<br>(iii) Angiogenesis<br>(iv) Regulating expression of metalloproteinases |
| **IL-8** | (i) Chemoattractant<br>(ii) Angiogenesis |
| **IL-1$\beta$** | (i) Angiogenesis<br>(ii) Synthesizing collagen |
| **TNF-$\alpha$** | (i) Antiangiogenic activity, but also proangiogenic effects under certain conditions<br>(ii) Increasing retinal endothelial permeability<br>(iii) Leukocyte adhesion<br>(iv) Oxidation |
| **NF-$\kappa$B** | (i) Regulating immune response, cell proliferation, and apoptosis<br>(ii) Synthesizing cytokines, chemokines, and proinflammatory molecules |
| **MCP-1** | (i) Recruiting and activating macrophages<br>(ii) Fibrosis and angiogenesis |
| **VEGF** | (i) Increasing vascular permeability<br>(ii) Angiogenesis<br>(iii) Endothelial cell migration and survival<br>(iv) Expression of ICAM and VCAM-1 |

Source (65)

### 1.5 The interaction between vascular cells and immune function:

Chronic blood glucose level eventually leads to the development of DR in diabetic patients; diabetes leads to increase in inflammatory factors affecting the endothelium. These inflammatory factors released after a nonspecific inflammatory response to damaged or stressed cells such factors include TNF-α and IL-1β, which in turn also induce expression of pro-inflammatory proteins (82). Indeed, there is an interaction between vascular endothelium and immune response cells thus affecting barrier function. During inflammation the vascular becomes activated, displaying increase leukocyte adhesion, permeability, the likeness of the vessels and increase in proangiogenic factors like VEGFA forming new blood vessels (82). The diabetic activated inflammation in DR believed to contribute to the retinal vascular damage and retinal neovascularization.

TLRs mainly expressed on the surface of macrophage and dendritic cells but also expressed on cells of the central nervous system, kidney and liver cells (9). Furthermore, recent studies demonstrated their expression in retinal vascular endothelial cells (1, 77). Of the 10 TLR in humans TLR1, TLR2, TLR4, TLR5 and TLR6 expressed on the cell surface whereas TLR3, TLR7, TLR8, and TLR9 expressed on the endosome. Each TLR recognizes pathogens through pathogen-associated molecular patterns (PAMPs) initiating an immune response. However, molecules released from stressed cells known as a damage-associated molecular pattern (DAMP) also activate TLRs (83, 84). As Diabetes is a pro-inflammatory state causing stress to cells, and several biochemical changes due to hyperglycemia affect the microvasculature of the retina, therefore the release of DAMPs activates TLRs, and among

different TLRs mainly TLR4 and TLR2 has been indicated in inflammation during diabetes and TLR4 is the interest of the current study.

**Hypothesis:**

Many mechanisms cause diabetic retinopathy, and several signaling pathways are involved. In this regard, we **hypothesize** that TLR4 may contribute to the pathogenesis of DR.

**Aims and objectives**

The main objective of this study is to investigate the role of TLR4 in the pathogenesis of DR by studying TLR4 in retinal cells treated with HG, and by application of siRNA technique for TLR4.

To achieve the general aim, the study has the following endpoints which are:

1) Assess the mRNA expression of TLR4 in glucose challenged HMVRECs
2) Assess the mRNA expression of NFKB, VEGFA, and TLR2 in glucose challenged HMVRECs
3) Assess the level of TNF- $\alpha$ in the cell supernatant of glucose challenged HMVRECs
4) Assess the mRNA expression of TLR4, TLR2, VEGFA and NFKB in siRNA transfected cells for TLR4
5) Provide dynamic real-time data using transelectrical resistance by applied biophysics system (ECIS) for the endothelial cell barrier function

# Chapter 2: MATERIAL AND METHODS

## 2.1: Materials:

Materials for cell culture were purchased from Cell System Corporation (Cell Systems, Kirkland, WA 98034, USA), including frozen vial of Primary Human Retinal Microvascular Endothelial Cells (HMVRECs) (Cat#ACBRI 181) passage3 [< 12 cumulative population doublings]; Complete Classic Medium Kit With Serum and CultureBoost (Certificate No: 4Z0-500); Complete Serum-Free Medium Kit (Certificate No: SF-4Z0-500) with RocketFuel (Certificate No: SF-4ZR-500); CSC Attachment Factor™ (Certificate No: 4Z0-210); CSC Passage Reagent Group™ (Certificate No: 4Z0-800), CSC Cell Freezing Medium (Certificate No: 4Z0-705) and Bac-Off® Tonic for antibiotic treatment of media (Certificate No: 4Z0-643). Gibco® 0.2% Trypsin-EDTA (1x) phenol red (REF#25200-072), Phosphate Buffered Saline (PBS) pH 7.4 (10X), TRIZOL® reagent [REF #15596026] were purchased from (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Chloroform HPLC grade [REF: 650498-1L], was purchased from Sigma-Aldrich, UAS. High Capacity RNA-to-cDNA kit was purchased from Applied Biosystems (P/N 4387406). TaqMan Gene Expression Master Mix (P/N 4369016).

The Invitrogen™ Tali™ Image-based Cytometer by Life Technologies (Catalog # T10796) has been used to detect red and green lights for apoptosis measurements by the following kits respectively: Annexin V Alexa Fluor® 488 & Propidium Iodide (Catalog

no: A10788), also used for automated cell counting. All purchased from Life Technologies Corporation, USA.

Pierce® RIPA Buffer (Cat# PF 201994) and Pierce® BCA Protein Assay Kit (Cat# 23227) were purchased from Thermo Scientific, Massachusetts, United States.

siRNA transfection reagents were purchased from Santa Cruz Biotechnology, USA; siRNA Transfection Medium (sc-36868) is a reduced serum medium added to siRNA suspension and siRNA transfection reagent; siRNA transfection reagent (sc-29528); TLR4 siRNA (h) (sc-40260) consist of pools of three to five target specific 19-25 at siRNAs designed to knockdown gene expression; Control siRNA-A (sc-37007) used as a negative control, consists of a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA and Control siRNA (FITC Conjugate)-A (sc-36869) used as a control for transfection efficiency monitored by fluorescence microscopy, also consists of a scrambled sequence but it is conjugated to a fluorescein that will not lead to the specific degradation of any known cellular message.

**Ethical and Biosafety Approvals:**

Qatar University Institutional Biosafety Committee has approved this study, approval number: QU-IBC-14/14-15, see Appendix A

## 2.2 Methods

This is an experimental study in which different approaches were applied to reach the primary goal of the study.

### 2.2.1. Human Microvascular Retinal Endothelial Cell Culture.

Cryopreserved HRMECs passage 3 were rapidly thawed in a 37˚C water bath, than immediately added to complete medium (4Z0-500) and centrifuged for 7 min at 200rcf. The cells were grown in complete media with with 1ml Bac off (4Z0-643) in a $75cm^2$ flask plated with attachment factor (4Z0-210) and incubated at 37°C in 5% CO2 incubator until confluency. The media was changed at 24 hours after growth followed by every 48 hours. The cells were passaged till P5-P6 and used for experaments.

Before culturing or subculturing the cells, a manual cell count was performed routinely using Trypan Blue, 20µl cell suspension added in to 20µl of Trypan Blue and 15 µl of this mixture was loaded on to hemacytometer (Bright Line) and counted under a microscope. The number of cells counted in the 4 corner squares and middle 1 square was used in the below formula to retrieve the total number of cells:

[Total no.of cells counted / 5(no.of squares)] ×2(dilution factor)×10,000

The cells have also been counted using Tali™ Image-based Cytometer.

### 2.2.2 HRMECs Treatments:

Two groups of experiments have been conducted on these cells, groupA cell treatment without transfection and groupB cell treatment after TLR4 siRNA transfection; see 2.2.2(A) and 2.2.2(B).

### 2.2.2(A) GroupA Cells:

For conducting these experiments, the cells were grown on a 6 well plate in complete media without antibiotics, plated with attachment factor; the media was changed at 24 hours after growth followed by every 48 hours. Upon 75-85% of confluency, the cells were serum starved [changed the normal complete media containing 10% FBS to serum free media containing 0% FBS] for 6 hrs (SF-4Z0-500). Thereafter the cells were treated for 36-40hrs with different treatment groups: 1- Control (CN): normal complete media containing 5.5mM glucose. 2- High glucose(HG): 25mM of D-glucose added to normal complete media for a total of 30mM glucose. 3- Mannitol (M): 25mM D-Mannitol added to normal complete media containing 5.5mM glucose and used as an osmotic control. 4- Antioxidants N-acetyl cysteine (NAC) (Sigma-Aldrich, USA) (10mM NAC + 30mM HG) or Docosahexaenoic acid (DHA) (Sigma-Aldrich, USA), (30uM DHA + 30mM HG) to study the role of antioxidants in preventing TLR4 activation. Subsequently, the cell supernatants and cell lysates were collected for different assays. Experiments in this study were performed in three biological replicates with 3-4 technical replicates for each.

Figure2.2.2(A) GroupA Cells

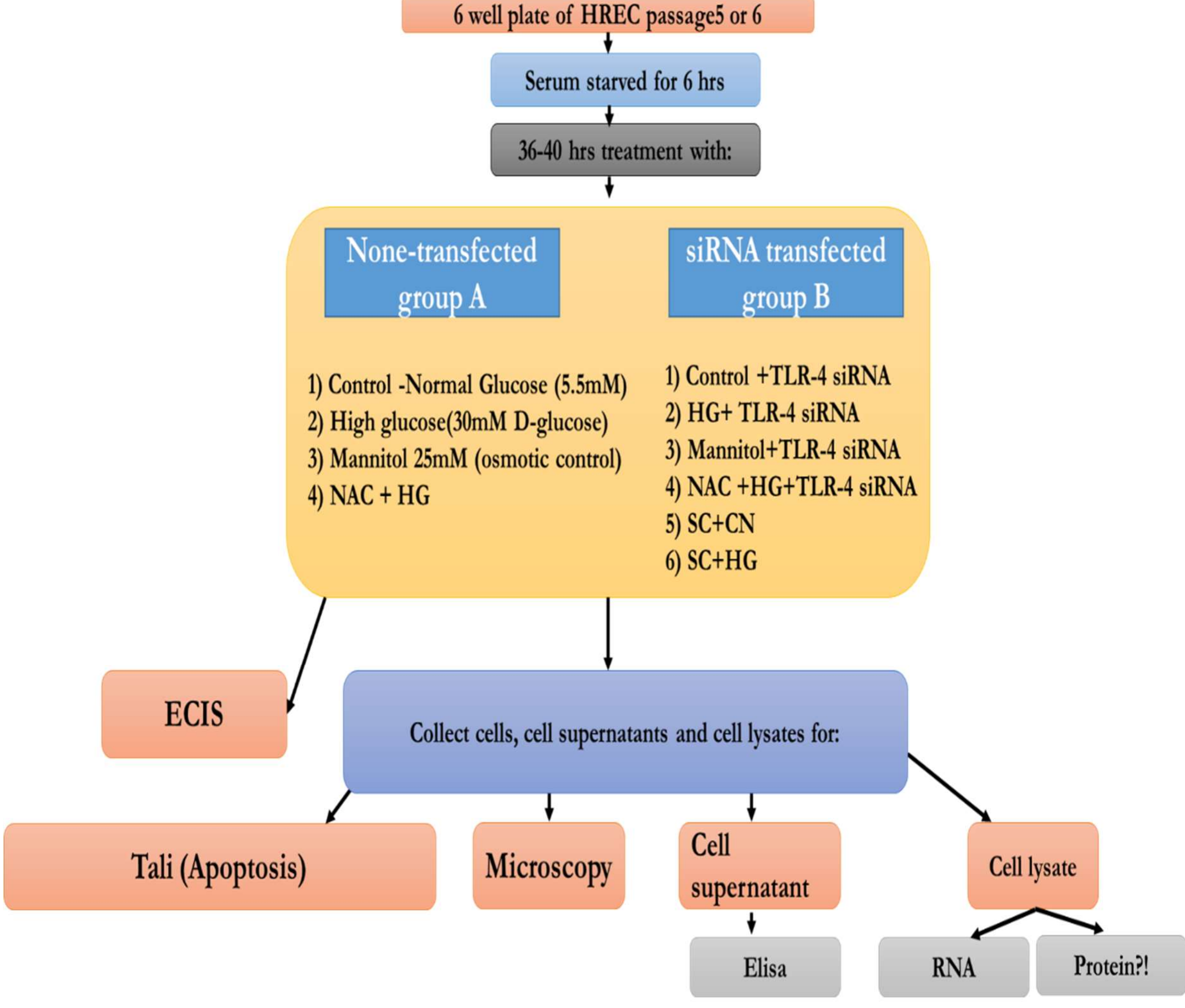


Fig 2.2.2(A): The different treatments plan of HMVRECs for both groupA and group B

### 2.2.2(B) GroupB Cells:

Small interfering RNA (siRNA) transfection technique is employed to silence the action of TLR4 specifically by cleaving and destroying the mRNA so that no protein can be synthesized. Thus, this study continued with siRNA consequence on retinal cell biology, functions, morphology, and behavior. This technique employee a cationic lipid-mediated delivery for siRNA into the cell, allowing for highly efficient transfection. With cationic lipid delivery, the negatively charged siRNA spontaneously binds to the positively charged liposomes, forming siRNA cationic lipid reagent complexes.

After some optimization on the manufacturer's protocol (Santa Cruz) for siRNA transfection, about $2 \times 10^5$ HMVRECs were seeded in a six-well tissue culture plate in 2ml antibiotic-free complete medium (4Z0-500), Incubated at 37°C in 5% CO2 incubator until the cells were 60-80% confluent. The transfection solutions were prepared as follow; Solution A: 5.7 µl (0.75 $\mu$g) of 10 µM TLR4 siRNA duplex (sc-40260) diluted into 100 µl siRNA Transfection Medium(sc-36868). Solution B: 5.7 µl of Transfection Reagent (sc-29528) diluted into 100 µl siRNA Transfection Medium (sc-36868). Whereas the controls were prepared as follow; Solution A: Negative control, the scrambled sequence siRNA(SC): 5.7 µl (0.75 $\mu$g) of 10 µM SC siRNA duplex (sc-37007) was diluted into 100 µl siRNA Transfection Medium (sc-36868); Solution B: 5.7 µl of Transfection Reagent (sc-29528) diluted into 100 µl siRNA Transfection Medium (sc-36868). The solution A for FITC control for monitoring transfection efficiency was prepared by adding 5.7 µl (0.75 $\mu$g) of 10 µM of Fluorescein Conjugate siRNa control

(sc-36869) and Solution B prepared by adding 5.7 µl of Transfection Reagent (sc-29528) diluted into 100 µl siRNA Transfection Medium (sc-36868).

Each solution A is directly added to solution B and mixed gently by pipetting the solution up and down than the mixture was incubated for 30-45 minutes at room temperature. The 60-80% confluent cells were washed once with 2 ml of siRNA Transfection Medium (sc-36868). Thereafter 0.8 ml siRNA Transfection Medium added to each tube containing the mixture (Solution A + Solution B), gently mixed and overlaid onto the washed cells. Following the incubation for 6 hours at 37° C in a CO2 incubator, the FITC control was assayed under the fluorescence microscope. Whereas 1 ml of normal growth medium containing 2 times the normal serum and antibiotics concentration was added to the other plates of cells without removing the transfection mixture; then the cells were incubated for an additional 18-24 hours. Subsequently, the cells were serum starved for 2 hours, glucose and antioxidant treatments were given as explained in section 2.2.2(A) while the SC cells either treated with CN or HG.

The transfection protocol was optimized by first applying the protocol on FITC control and monitoring the transfection efficiency with a fluorescence microscope, 0.75ug of siRNA duplex was used according to a 2014 study by Rajmani and Jailal(1), and according to transfection protocol by Santa Cruz in addition to trying different concentrations with FITC control. Transfection time was optimized, as the protocol states that after adding the transfection mixture the cells are incubated for 5-7 hours at 37° C in a CO2 incubator, whereas in this study the cells have been incubated for 6hrs after observing FITC control from 5-7 hrs. As it has been observed that at 5$^{th}$ hour there was

no or very faint green fluorescence, at 6$^{th}$ hrs the fluorescence was adequately observed whereas, at 7$^{th}$ hour and after, the majority of the cells were dead.

All siRNA reagents were obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

Figure 2.2.2(B) GroupB Cells

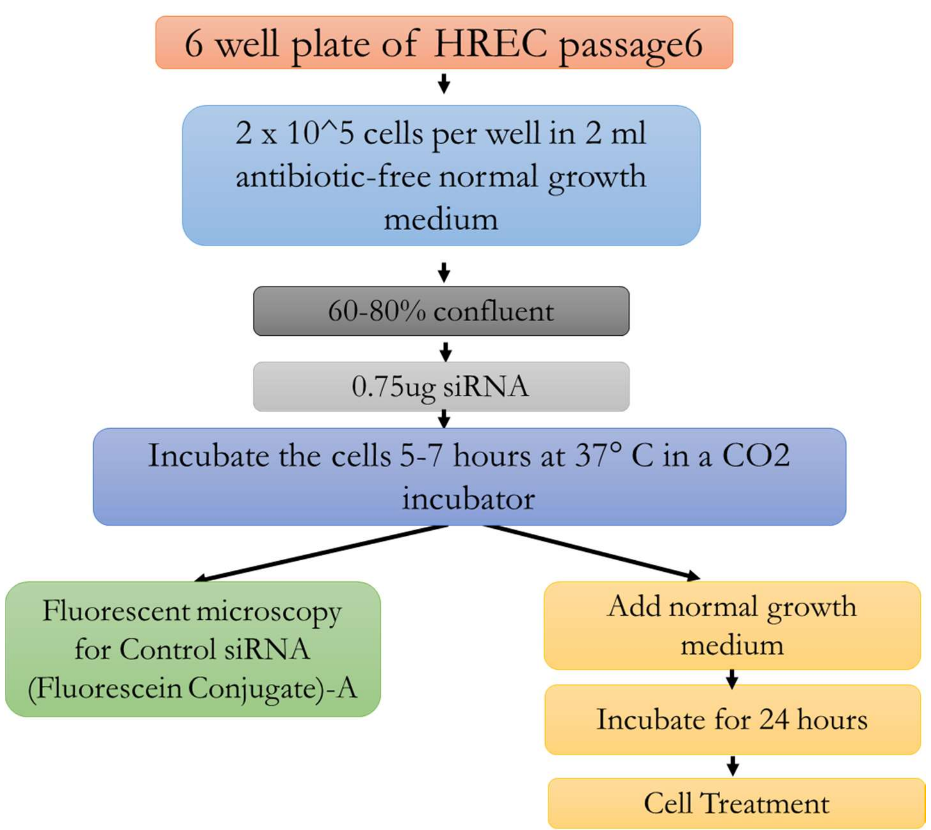


Figure 2.2.2(B): The work flow of transfection protocol.

### 2.2.3 Gene Expression Analysis:

#### 2.2.3(A): RNA Extraction

Total RNA from treated HMVRECs as described in 2.2.2(A) and (B) was extracted using TRIZOL® reagent (REF #15596026, Invitrogen, Carlsbad, CA). Briefly, 0.2ml of chloroform added to each 1ml of homogenized cells in Trizol reagent, after shaking vigorously, centrifuged at 12000xg for 15 min at 4$^{o}$ C. Thereafter the aqueous layer was transferred to a new 1.5 ml Eppendorf tube and 0.5ml of chilled 100% isopropanol was added, after incubation for 10min at room temperature, centrifuged at 12000xg for 10 min at 4$^{o}$C, followed by washing the pellet with 1ml of 75% ethanol, air dried the pellet and resuspended in 20µl of RNase-free water. The concentration of RNA was measured in the NanoQuant Plate on TECAN Infinite 200 PRO microplate reader (life sciences, Switzerland) at absorbance 260/280 nm.

#### 2.2.3(B) cDNA Preparation:

The extracted RNA was converted to cDNA using High Capacity RNA to cDNA kit (Applied Biosystems, Massachusetts, USA), 1000ng of total RNA was used with this kit to generate cDNA. A total of 20 µl of reaction mix is prepared with the following volume of each component per reaction: 10 µl of 2XRT buffer, 1 µl of the 20X enzyme mix, up to 9 µl of sample is used, and the remaining volume is completed with nuclease free water. Reverse transcription reaction was performed in Thermo cycler Gene Amp®, PCR System 9700 (Applied Biosystems, USA) according to the following heat cycles: incubation at 37$^{o}$C for 1hr and stopping the reaction by heating at 95$^{o}$C for 5 min followed by a hold at 4$^{o}$C.

### 2.2.3(C) Quantitative Real Time PCR:

Quantitative RT-PCR is done using Quantstudio 6 Flex Real Time PCR system (Applied Biosystems, USA) with comparative CT method for quantitative gene expression by TaqMan® Gene Expression Master Mix (Applied Biosystem, USA) and used primers specific for TLR2, TLR4 (Applied Biosystems, USA), NFKB, VGEFA (Integrated DNA Technologies, Coralville, USA) with β-actin used as endogenous control, the master mix was prepared according to the protocol in Table (2.2.3C), with total volume of 10 µl. All samples were run in triplicate; the relative expression values were normalized to β-actin value.

Table2.2.3C1. RT-PCR Reaction Mix:

RT-PCR Reaction Mix for TLR2, TLR4, NF-KB and β-actin

| **component** | volume/ reaction (µl) |
|---|---|
| **2x Master Mix** | 5 µl |
| **20X Primer** | 0.5 µl |
| **Nuclease-free Water** | 1.5 µl |
| RT-PCR Reaction Mix for VEGF-A | |
| **2x Master Mix** | 5 µl |
| **10X Primer** | 1 µl |
| **Nuclease-free Water** | 1 µl |

Table2.2.3C2. Oligonucleotide sequences RT-PCR:

| Genes | Primer Sequences (5'→ 3') |
|---|---|
| **Beta actin (Human)** | Taq man Hs99999903_m1 |
| **TLR4(Human)** | Taq man Hs00152939_m1 |
| **TLR2(Human)** | Taq man Hs01872448_s1 |
| **NFKB(Human)** | IDT Hs.PT.58.21008993 |
| **VEGF-A(Human)** | IDT Hs.PT.56a.1149801.g |

### 2.2.4 Protein processing:

Note: protein is extracted, measured and preserved in -80°C for future use.

### 2.2.4(A) Protein extraction:

Protein is extracted from the cells by RIPA buffer (Thermo Scientific, USA). After cell treatment as mentioned in section 2.2.2 A and B, cells were washed twice with 1xPBS to remove any traces of media. The cells were homogenized using RIPA lysis and extraction buffer. Kept for 5 minutes at room temperature, then scraped and collected in microcentrifuge tubes. Stored in -80˚C until used for further analysis.

### 2.2.4(B) Protein concentration:

The concentration of protein is determined using Pierce BCA Protein Assay Kit (Thermo Scientific, Massachusetts, United States), Nine different BSA protein standards from 0-2000μg/mL were prepared. In brief, 200ul of working reagent was added to each sample and standard in a microplate. Then 25ul of each standard (25-2000ug/ml) and

unknown sample added into microplate in duplicate. The plate was shaken briefly and incubated at 37$^{o}$C for 30 min. Finally brought to room temperature before reading it at 562nm on Epoch2 Microplate Spectrophotometer (BioTek, USA).

**2.2.5 Apoptosis:**

Apoptosis is physiological cell death, carefully regulated in cells and if regulated indicates to disease status. Apoptosis is distinguished from necrosis by the morphological and biochemical change; such changes include the translocation of phosphatidylserine (PS) to the inner leaflet of the plasma membrane. Normally PS is located on the cytoplasmic surface of the cell membrane. The Tali Apoptosis Kit- Annexin V Alexa Fluor® 488 and Propidium Iodide (PI) is used to assess percent of apoptotic cell death. As Annexin V has a high affinity for PS exposed on the outer leaflet, therefore, used identify apoptotic cells. Wherase PI only binds nucleic acid of dead cells.

The treated cells as described in Section 2.2.2(A) were washed with 1xPBS and 1ml of trypsin was added into each well, incubated at 37oC, 5% CO2 for 5 min, plate was taped from all sides to detach cells and cell suspension was collected into 15ml tube followed by centrifugation at 200rcf for 5-7 min and supernatant was discarded. The cells were resuspended in 100 µl of 1x Annexin binding buffer (ABB) and 5 µl of Annexin V Alexa Fluor® 488 in a microcentrifuge tube, mixed and incubated for 20min in dark at room temperature. Following incubation, the tube was centrifuged for 1-2 min and resuspended in 100 µl pf ABB with 1 µl PI, briefly mixed and incubated in dark at room temperature for 5 min. After incubation the apoptosis cell percentage of all samples were analyzed

using Tali® Image-Based Cytometer, the cells were mixed gently, and 25 μl was loaded on Tali® Cellular Analysis slide, the percentage was determined by reading Alexa Fluor® 488 annexin V at 488/499 nm and PI at 535/617 nm.

### 2.2.6. Multiplex ELISA by Luminex 200™.:

The supernatants of HMVRECs is collected to measure Interleukin-4(IL-4), tumor necrosis factor-$\alpha$ (TNF-$\alpha$), interferone gama (INF-γ) by Luminex 200™(Luminex Corporation, Austin, United States). Brifely, cell culture supernatant were centrifuged to remove debris and stored at -20ºC. The samples were thawed once ready for assaying. The Human Cytokine/Chemokine Magnetic Bead Panel 96 Well Plate Assay (Cat. # HCYTOMAG-60K) a Bead-Based Multiplex Assays using the Luminex technology to analyze multiple cytokine and chemokine biomarkers simultaneously; used to measure the level of TNF-$\alpha$, IL-4 and INF-γ. In each well 25 μL of culture supernatant added with 25 μL of assay buffer and 25 μL pre mixed Beads. Standard or control been used during this procedure to ensure reliable results. After overnight incubation at 4ºC the plate was washed twice and 5 μL of Detection Antibodies added into each well, the plate was covered and incubated with agitation on a plate shaker for 1 hour at room temperature. Followed by adding 25 μL Streptavidin-Phycoerythrin and incubated with agitation on a plate shaker for 30 minutes at room temperature (20- 25°C). followd by 2 times washes and after addition of 150 μL of Sheath Fluid the plate was run on Luminex 200™, and the results were calculated by the software Xpotent 3.1 and expressed in pg/ml.

### 2.2.7 ELISA for Human Toll Like Receptor 4

The supernatants of HMVRECs is collected to measure soluble TLR4, the kit uses enzyme-linked immune sorbent assay technology with anti-TLR4 antibody precoating the 96 well plate( abbexa, cat n. abex250274). Brifely, cell culture supernatant was centrifuged to remove debris and stored at -20ºC.  The samples were thawed once ready for assaying. The satndards were prepare at the time of experamnt ranging from 20ng/ml-0.32ng/ml in addition to blank having only dillution buffer and 100µl added in their assigned wells in triplicate. Followed by adding 100 µl of samples in duplicate, incubated at 37ºC for 90 min, after discarding the contents, 100 µl of bioten conjugated antibody is added in each well and incubated at 37ºC for additional 60 min. After washing the plate 3 times,  100 µl of SABC buffer added in to each well and incubated for 30 min at 37ºC, followed by 5 times wash, 90µl of TMB substrate added in to each well and incubated in dark at 37ºC for 25 min, the reaction was stoped by adding 50µl of stop solution and the plate was read in amicroplate reader at 450nm.

### 2.2.8 Electric Cell-substrate Impedance Sensing (ECIS)

ECIS (Applied Biophysics, USA) a quantified cell behavior system used for functional biological assays to provide real time data for cell behavior. ECIS plates were coated with L-system and incubated at room temperature for 15 min, followed by coating with attachment factor; as L-cysteine has a thiol group which will fill the gaps between the macromolecule of the attachment factor making the well surface uniform for cells to

grow. The incubator and the software connection was confirmed by checking the connection; then data collection was initiated with reading at multiple frequencies covering a range of 40000-27000 Hz and data been collected for 400 µl of cell-free media for 15-30 min. After pausing followed by connection checking, the microarray plate is removed and loaded with 400 µl of cell suspension (10000-60000 cells/$cm^2$) seeded onto ECIS 8W10E+ culture plates coated with gold electrodes (Applied Biophysics, UAS) and loaded into the ECIS incubator. The data has been collected for 18-24hrs, followed by treatment as mentioned in section 2.2.2(A) and 2.2.2(B). Every time the data collection was paused, the connection was checked before resuming data collection. For siRNA transfection in ECIS, same methods followed as section 2.2.2(B) with adjusting volumes to a total of 400 µl per well.

**2.2.9 Statistical analysis**

Statistical analyses were conducted using GraphPad 6 for Windows (Version 6 software; San Diego, California) and Microsoft Excel 2013 (Microsoft Corp., Redmond, WA). For in vitro studies, at least three biological replicates were prepared for each treatment group along with at least three technical replicates. The results are presented as the mean of three different experiments with a standard deviation and/or SE of the mean. Group differences will be evaluated using ANOVA followed by Tukey's post hoc test for multiple comparisons. For all analyses, two-tailed $P < 0.05$ will be considered sufficient to reject the null hypothesis.

# Chapter3. RESULTS

## 3.1 HRMECs morphology and cell count

The Passage3 HMRECs were grown on a complete media, the cell morphology and confluency was observed at different days, the media was changed after 24hr of growth than every 48 hrs. Once the proliferating cells became 75% to 80% confluent, the cells were introduced to serum free media according to the protocol used. Following Figure3.1 (A-C) shows morphology and different confluency of the cells observed on an inverted microscope.

Figure 3.1: Cell morphology

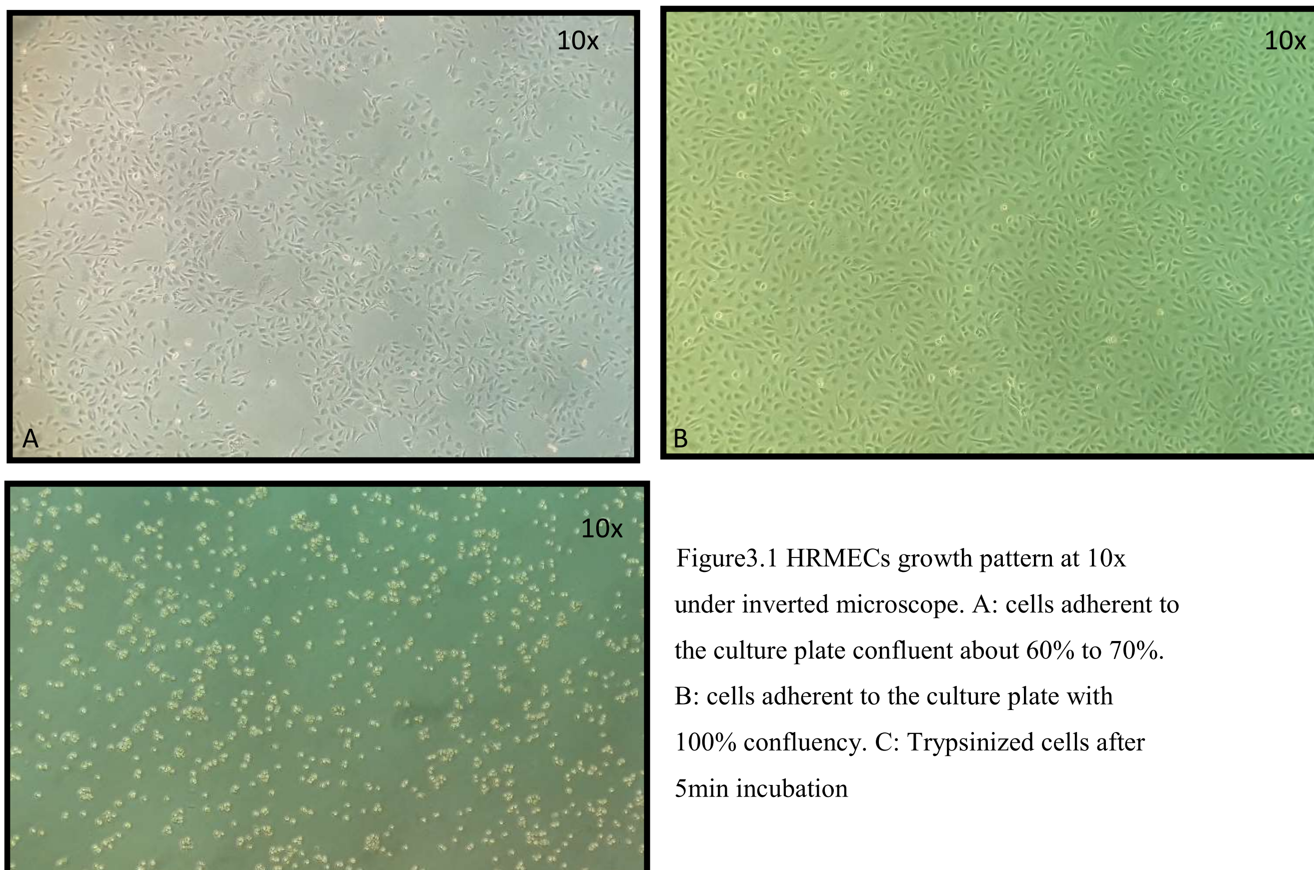


Figure3.1 HRMECs growth pattern at 10x under inverted microscope. A: cells adherent to the culture plate confluent about 60% to 70%. B: cells adherent to the culture plate with 100% confluency. C: Trypsinized cells after 5min incubation

Whereas the morphological assessment was carried out by Tali® Image-Based Flow Cytometer with different biological and technical replicates, revealing that on an average the cell size was 12-14µm.

In the next sections, data are presented first for normal HMVRECs (section 3.2-3.5) then in the second part data are presented for the transfected HMVRECs (section 3.6-3.10).

**3.2 High Glucose Induces Apoptosis:**

To investigate whether 30mM high glucose treatment to HMVRECs cell induces apoptosis and whether treatment of hyperglycemic cells with antioxidants (DHA, NAC) restores the normal cell condition. Tali Apoptosis Kit- Annexin V Alexa Fluor® 488 and Propidium Iodide kit was used to assess for apoptosis. Treating cells with 30mM High glucose significantly increased the percentage of apoptotic cells to17.46 (9.6) when compared to control cells treated with 5mM glucose 4.75(2.77) with $P<0.001$. On the other hand, treatment of HG cells with antioxidants whether by DHA or NAC both significantly reduced the percentage of apoptotic cells to 7.08 (6.04) with $P<0.001$ and 5.67(2.58) with $P<0.01$ respectively.

Figure 3.2: Apoptotic cell percentage

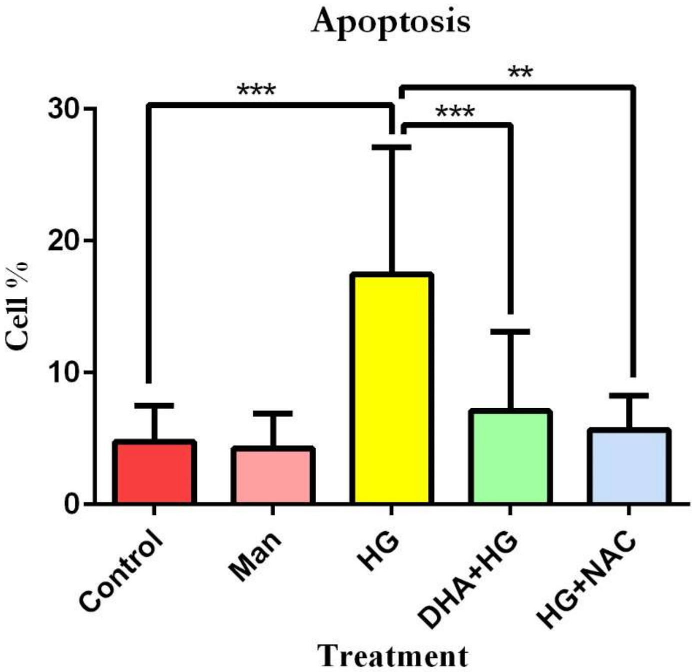


Figure 3.2 shows the percentage of apoptotic cells with different treatments compared with control and HG. Data presented as mean and (SD) of 3-4 independent experiments analyzed by One-Way ANOVA with Tukey-Kramer Multiple Comparisons Test between groups. Two-tailed *P*-value is significant <0.05. ***$P$<0.001, Control vs HG and HG vs DHA+HG, **$P$<0.01 for HG vs HG+NAC. Abbreviations: 5.5mM Glucose (control); 30mM Mannitol (Man); 30mM High glucose (HG); 30µM Docosahexaenoic acid (DHA)+ 30mM High glucose (DHA+HG); and 10mM N- Acetyl Cysteine(NAC) + 30mM High glucose (HG+NAC).

### 3.3 High Glucose Induces TLR4 mRNA expression:

HMVRECs cells were exposed to 5.5mM glucose (control) and 30mM high glucose (HG) for 36-40hrs, the mRNA expression was measured using real-time PCR. We observed that HG treatment significantly increased TLR4 and TLR2 mRNA expression when compared to control ($P$<0.001) (Figure3.3. a & b). On the contrary, no significant difference in the gene expression was detected forTLR4 and TLR 2 between the control group and the mannitol (osmotic control).

Figure3.3

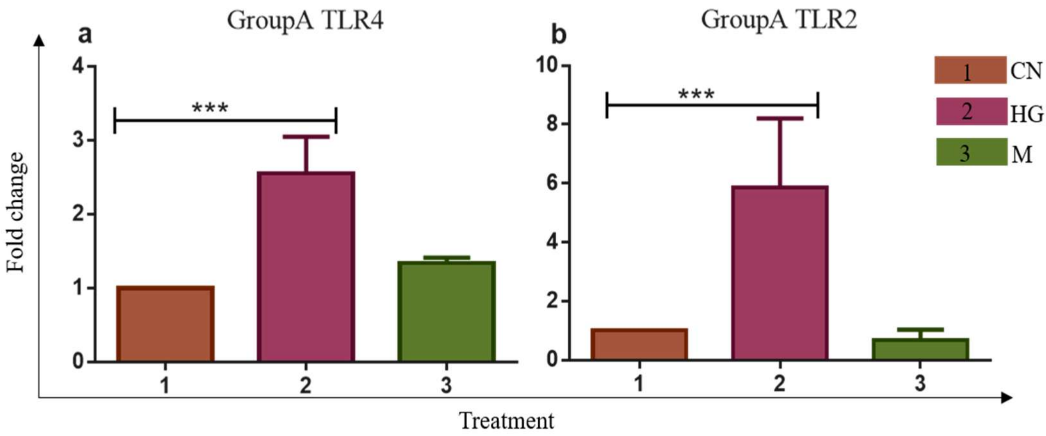


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Figure3.3 (a & b): Expression of TLR4 and TLR2 in HMVRECs. The bars show the relative quantity of TLR4 mRNA(a) and TLR2 mRNA(b) in HMVRECs normalized to β-actin under different treatment groups. The cells treated with 1 (5.5mM glucose -Control), 2 (30mM high glucose (HG)), and 3(25mM mannitol) as osmotic control. The data presented as means (SD) of 3-4 independent experiments, analyzed by One-way ANOVA with post-hoc Tukey-Kramer multiple comparisons test between individual groups. Two-tailed $p$ value is significant at $p$<0.05. *** $P$ <0.001 Control versus HG group.

### 3.4 Increased inflammatory cytokine production by HMVRECs cells in response to high glucose:

To investigate the inflammatory response of TLR4, we determined the expression of inflammatory biomarkers including TNF-α, INF-γ, IL-4 as well as angiogenesis marker VEGFA in HMVRECs cells under high glucose condition. The level of VEGFA mRNA after glucose challenge was significantly increased compared to control (Figure 3.4a) ($P<0.05$). We then measured TNF-α, IL-4 and IFN-γ protein in the cultural supernatant of HMVRECs cells by use of multiplexing assays. The level of TNF-α and IL-4 were significantly increased in HG-treated cells when compared to control cells (Figure3.4b) and (Figure 3.4C) ($P<0.01$) and ($P<0.05$) respectively. Whereas, HG treatment showed an insignificant change in the IFN-γ level. On the other hand, a significant change only been observed with TNF-α when the antioxidant treatment is given to HG- treated cells, carrying it back to the control level (Figur3.4 b) ($p<0.01$).

Figure 3.4(a-d)

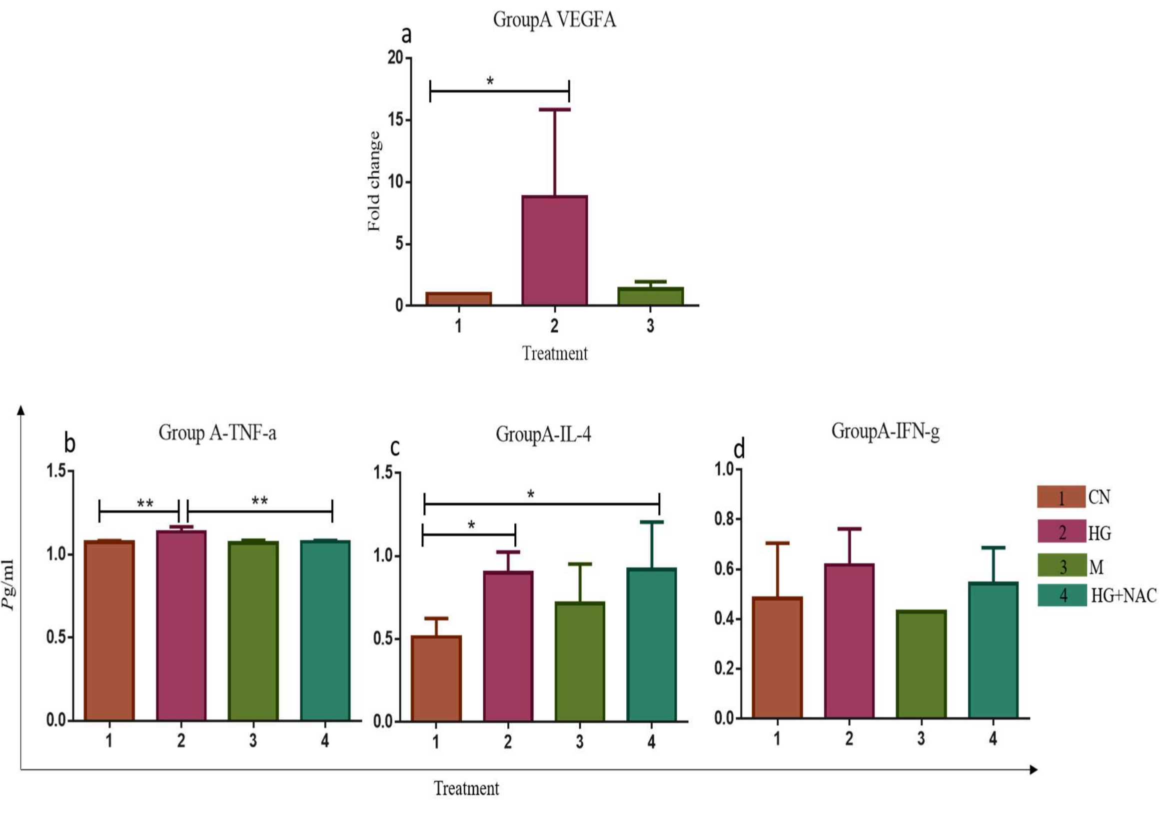



Figure3.4 (a-d): Inflammatory cytokine production by HMVRECs cells in response to high glucose. (a) Expression of VGEFA in HMVRECs. The bars show the relative quantity of VEGFA mRNA in HMVRECs treated with 1 (5.5 mM glucose (Control)), 2 (30mM high glucose (HG)) and 3 (25mM mannitol) normalized to β-actin as indicated in the methods section. (b) TNF-α protein in the supernatant of HMVRECs after different treatment as indicated using multiplex ELISA. (c) IL-4 protein in the supernatant of HMVRECs after different treatment as indicated using multiplex ELISA. (d)INF- γ protein in the supernatant of HMVRECs after different treatment as shown using multiplex ELISA. The data presented as means (SD) of 3-4 independent experiments, analyzed by One-way ANOVA with post-hoc Tukey multiple comparisons test between individual groups. Two-tailed p value is significant at $p<0.05$. **$P<0.01$, *** $P<0.001$ Control versus HG group. Abbreviations: 5.5 mM glucose control (CN); Mannitol (M); 30mM high glucose (HG); and N- Acetyl Cysteine(NAC) with 30mM high glucose (HG+NAC).

### 3.5 TLR4 activation results in the downstream signaling and activation of pro-inflammatory NFkB

TLR4 is known to signal through both MYD88 and non-MYD88 pathway that accumulate in NFKB activation and inflammatory cytokine release. Activation of NFkB leads to the expression of proinflammatory genes including cytokines, chemokines, and adhesion molecules. As a regulator of immune response, we thought to assess its expression in the high glucose treated HMVRECs. Therefore, we examined the effect of HG on the mRNA expression of NFkB. We observed that glucose challenge significantly increased NFkB mRNA expression compared to control with 2.245(0.9134) and $p<0.001$. (Figure3.5)

Figure 3.5

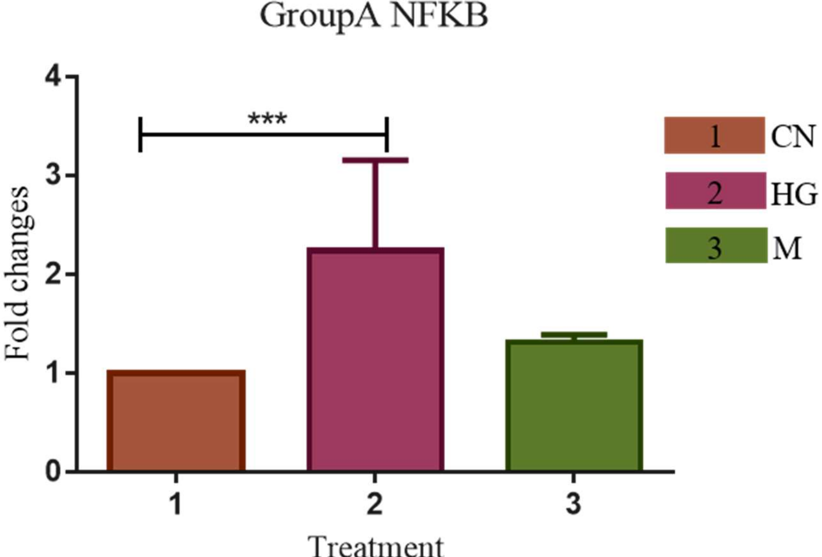



Figure 3.5: NFkB expression in HMVRECs under high glucose. The bars show the relative quantity of NFkB mRNA in HMVRECs treated with 1 (5.5mM glucose (Control), 2 (30mM glucose(HG)) and 3 (25mM mannitol) as osmotic control, normalized to β-actin. The data presented as means (SD) of 3-4 independent experiments analyzed by One-way ANOVA with post-hoc Tukey-Kramer multiple comparisons test between individual groups. Two-tailed *p* value is significant at $p<0.05$. *** $P <0.001$ Control versus HG group. Abbreviations: 5.5 mM glucose control (CN); 25mM Mannitol (M) and 30mM high glucose (HG).

### 3.6 siRNA efficiency with Fluorescein Conjugate siRNA control:

In this study, the cells have been incubated after adding transfection mixture- as mentioned in method section - for 6hrs. After observing FITC control from 5-7 hrs, we selected 6hrs incubation as transfection incubation time; it has been noted that at 5$^{th}$ hour there was no or very faint green fluorescence (picture not shown), at 6$^{th}$ hrs the fluorescence was adequately observed (Figure 3.6) wherase at 7$^{th}$ hour and after the majority of the cells were dead(picture not shown)

Figure 3.6(a&b)

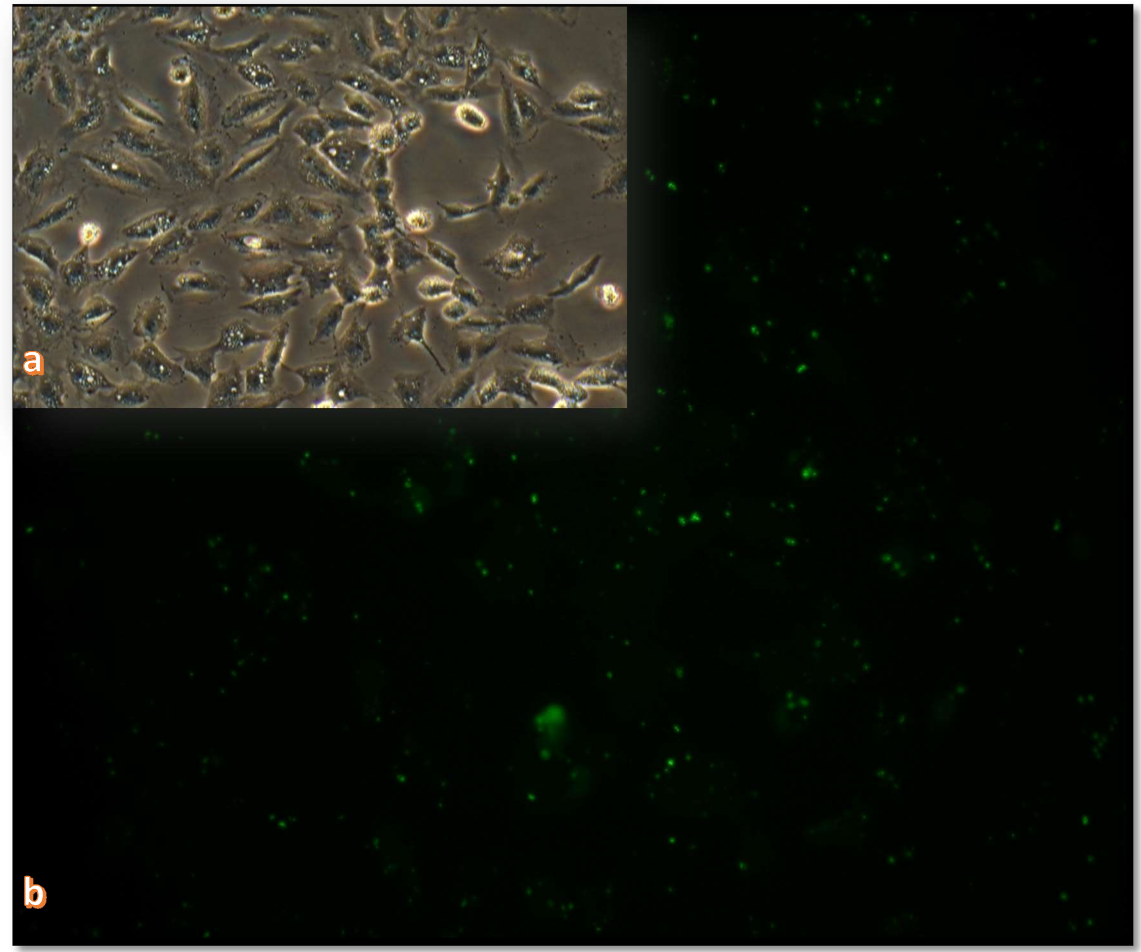
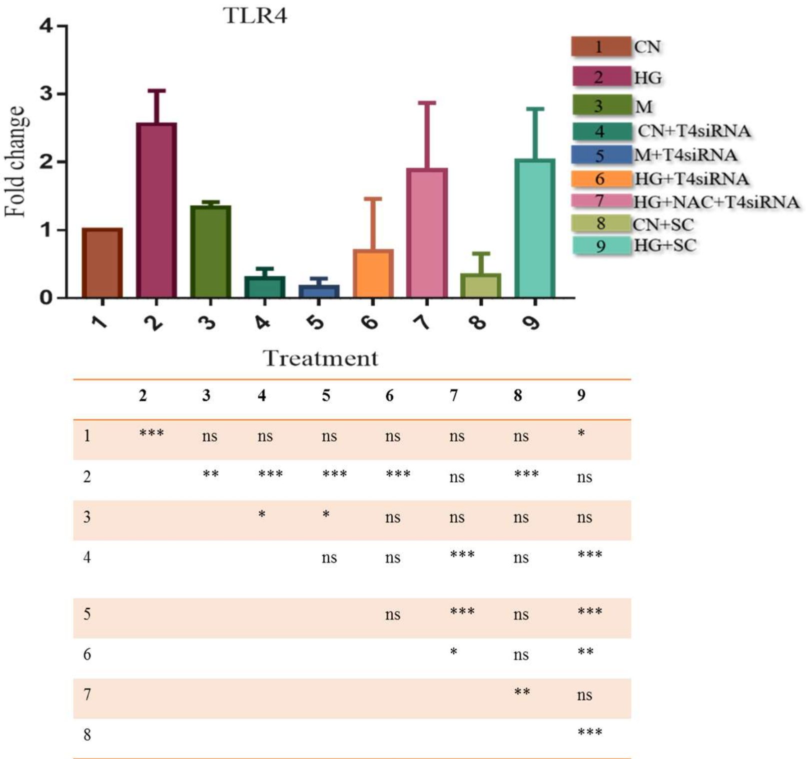



Figure3.6: FITC conjugated control-showing fluorescein in cells under fluorescent microscope 20X. The HMVRECs with FTIC conjugated siRNA control been incubated for 6hrs at 37 °C with 5% $CO^2$ and analyzed using fluorescent microscopy. (a) Viewing cells under Bright Field. (b) Cells with green fluorescence indicator of transfection.

### 3.7 Silencing TLR4 with TLR4 siRNA reduced the TLR4 mRNA expression in HG-treated HMVRECs cells.

To investigate the role of TLR4 in the pathogenesis of DR. siRNA specific for TLR4 was used to silence TLR4 action. Since we have shown an increase in the mRNA expression of both TLR4 and TLR2 with high glucose (Figure3.3 a-b), therefore after siRNA transfection we measured the mRNA expression and compared with the non-transfected group. We observed a significant difference in the TLR4 mRNA expression level between the two groups with $p$-value$< 0.001$. As shown in Figure3.7, TLR4 siRNA significantly attenuates the TLR4 mRNA level in the HG-treated group [2.556(0.4919) ($p<0.001$)]. As well as, there is a significant difference between HG and HG siRNA transfected cells ($p<0.001$) bringing the TLR4 mRNA expression back to normal level as evident by no significant difference between control and TLR4 siRNA transfected HG. The efficiency of transfection is evident the insignificant change in the TLR4 mRNA level in control and control with scrambled sequence siRNA (negative transfection control) ($p>0.05$) Figure 3.7.

Figure 3.7

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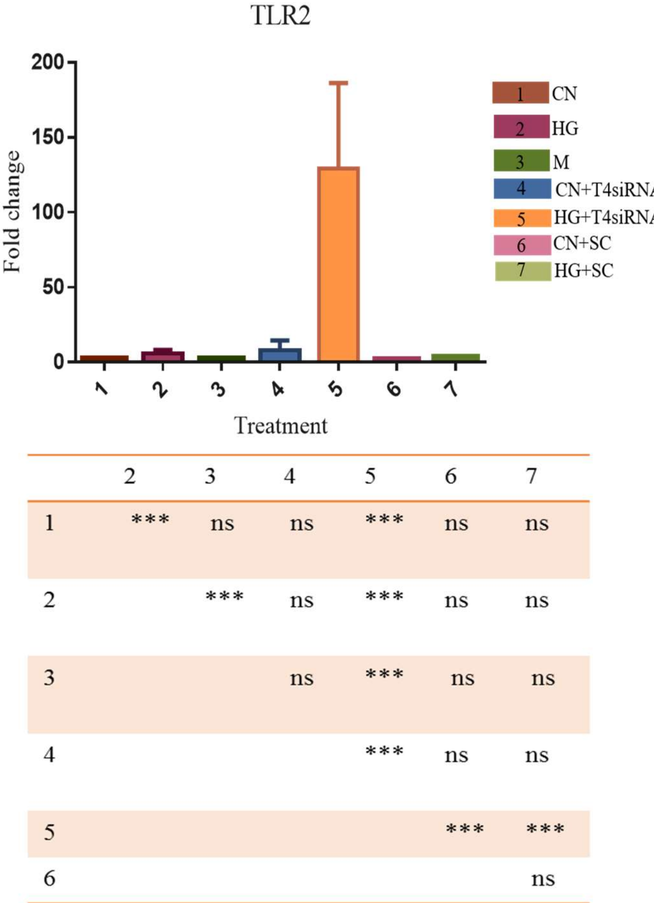
| 8 | | | | | | | | *** |

Figure3.7: Expression of TLR4 mRNA attenuated by TLR4 siRNA technique. The bars show the relative quantity of TLR4 mRNA in HMVRECs with TLR4 siRNA transfection. The mRNA expression normalized to β-actin under different treatment groups. After 6hrs transfection, the cells were treated with 4 (5.5mM glucose (control)), 5 (25mM mannitol) as an osmotic control and 6 (30mM high glucose (HG)). The data presented as means (SD) of 3-4 independent experiments, analyzed by One-way ANOVA with post-hoc Tukey-Kramer multiple comparisons test between individual groups. The two-tailed *p*-value is significant at $p<0.05$. *** $P<0.001$ CN versus HG group. **$P<0.01$ between Control and Control siRNA transfected (CN+siT4). *$P<0.05$ between HG+siT4 and SC+HG. Abbreviations: 5.5 mM glucose (control); Mannitol (M); 30mM high glucose (HG); 30µM Docosahexaenoic acid(DHA)+ 30mM high glucose (DHA+HG); and 10mM N- Acetyl Cysteine(NAC) + 30mM high glucose (NAC+HG). TLR4 silenced Control cells (CN+T4siRNA); TLR4 silenced HG-treated cells (HG+T4siRNA); TLR4 silenced HG and antioxidant-treated cells (HG+NAC+T4siRNA); TLR4 silenced Mannitol treated cells (M+T4siRNA); Scrambled sequence siRNA to control cells (CN+SC); and Scrambled sequence siRNA to HG-treated cells (HG+SC), not significant (ns).

### 3.8 Silencing TLR4 in high glucose treated HMVRECs cells increases TLR2 mRNA expression:

As shown before that, TLR2 mRNA expression increases under high glucose condition (Figure 3.3b), we examined the mRNA expression level of TLR2 in TLR4 silenced cells under high glucose condition. TLR2 mRNA expression significantly increased when exposed to 30mM high glucose in TLR4 silenced cells with 129.24(57.05) folds $p<0.001$, this increase in TLR2 mRNA was even higher than treating the cells with high glucose alone (see fig 3.3), in addition to a significant difference in the means of different treatment groups $P<0.0001$, as shown in Figure 3.8

Figure3.8

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| 6 | | | | | | ns |

Figure 3.8: Increased TLR2 mRNA expression in TLR4 silenced cells. The bars show the relative quantity of TLR2 mRNA in HMVRECs cell with TLR4 siRNA transfection. The mRNA expression normalized to β-actin under different treatment groups. After 6hrs of transfection, the cells were treated with 4 (5.5 mM glucose (control)), 5 (30mM High glucose (HG)), whereas SC added to 6 (control cells) and 7 (HG) treated cells as a negative transfection control. The data presented as means (SD) of 3-4 independent experiments, analyzed by One-way ANOVA with post-hoc Tukey-Kramer multiple comparisons test between individual groups. The two-tailed *p*-value is significant at $p<0.05$. *** $P<0.001$, **$P<0.01$, *$P<0.05$. Abbreviations: 5.5 normal glucose control (CN); Mannitol (M); 30mM high glucose (HG); TLR4 silenced Control cells (CN+T4siRNA); TLR4 silenced HG-treated cells (HG+T4siRNA); Scrambled sequence siRNA to control cells (CN+SC); and Scrambled sequence siRNA to HG-treated cells (HG+SC), and not significant (ns).

**3.9 TLR4 siRNA did not attenuate the increase in NFkB expression by HMVRECs cells under high glucose condition:**

As MyD88 and NFkB are common downstream signaling components of all TLRs except TLR3. We examined the effect of TLR4 silencing on the mRNA expression level of NFkB. We observed that silencing TLR4 in HMVRECs under high glucose significantly increased NFkB mRNA expression (Figure3.9), this increase was even higher than treating the cells with high glucose alone (fig 3.5) with ($p<0.001$). As well as, the NFkB mRNA significantly increased in TLR4 silenced control HMVRECs ($P<0.001$), increasing it even more than its expression level in non-transfected high glucose treated cell ($p<0.01$). The addition of irrelevant scrambled sequence siRNA control to high glucose treated cell and control cells did not result in any significant changes from their compared groups with no siRNA. Figure 3.9

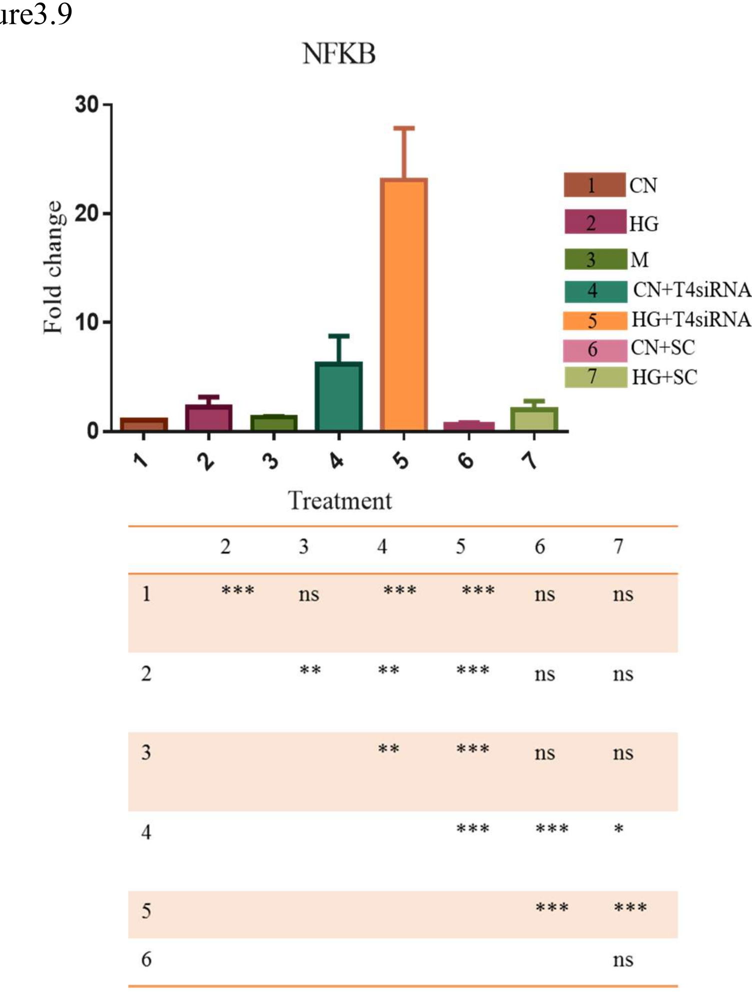


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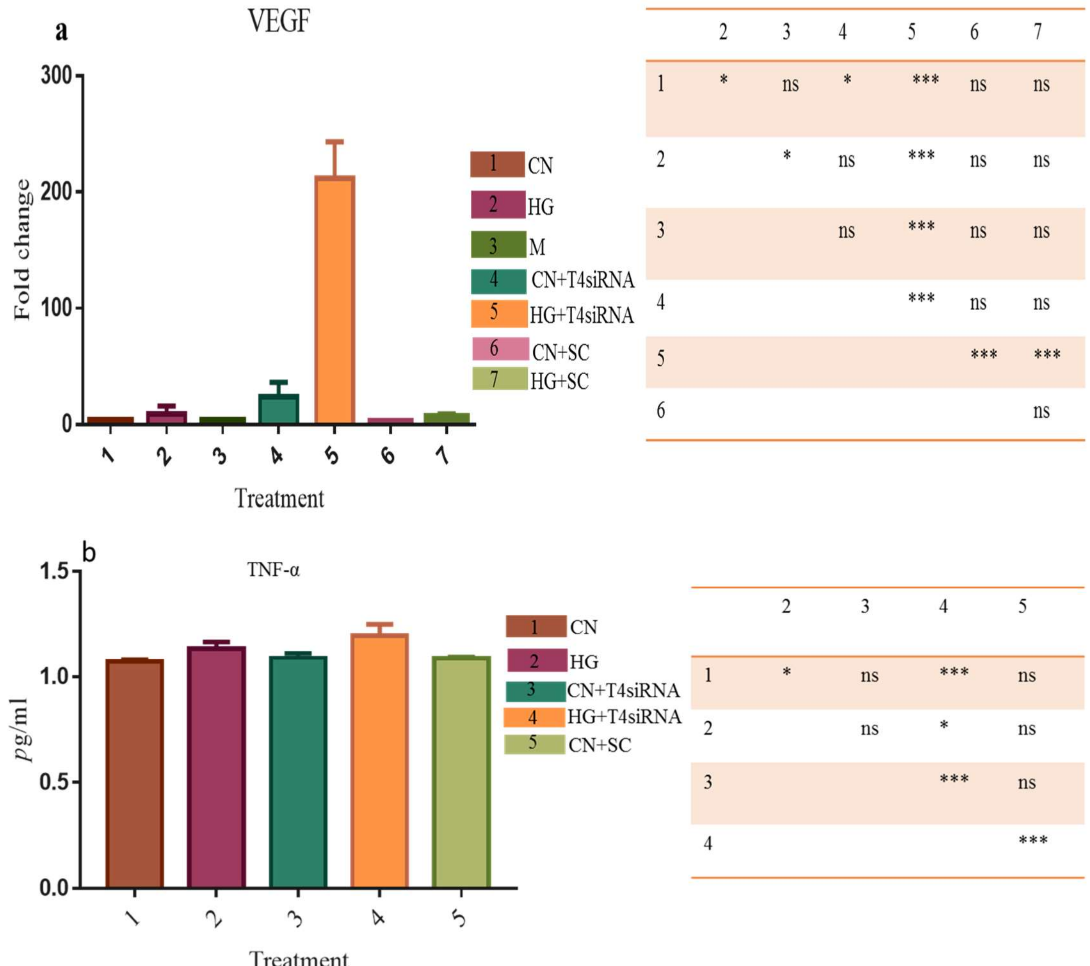
Figure 3.9: Increased NFkB mRNA expression in response to TLR4 silencing in HMVRECs. The bars show the relative quantity of NFkB mRNA in HMVRECs cell with TLR4 siRNA transfection. The mRNA expression normalized to β-actin under different treatment groups. After 6hrs of transfection, the cells were treated with 4 (5.5mM glucose (CN)), 5 (30mM High glucose (HG)), whereas SC added to 6 (control) cells and 7 (HG) treated cells as a negative transfection control. The data presented as means (SD) of 3-4 independent experiments, analyzed by One-way ANOVA with post-hoc Tukey-Kramer multiple comparisons test between individual groups. Two-tailed $p$ value is significant at $p<0.05$. *** $P<0.001$, **$P<0.01$, *$P<0.05$. Abbreviations: 5.5 normal glucose control (CN); Mannitol (M); 30mM high glucose (HG); TLR4 silenced Control cells (CN+T4siRNA); TLR4 silenced HG-treated cells (HG+T4siRNA); Scrambled sequence siRNA to control cells (CN+SC); and Scrambled sequence siRNA to HG-treated cells (HG+SC), and not significant (ns).

### 3.10: TLR4 siRNA failed to attenuate the increased inflammatory cytokine produced by HMVRECs cells in response to high glucose:

To investigate the effect of TLR4 on inflammation, we transfected the HMVRECs with TLR4 siRNA and examined the level of inflammatory biomarkers including TNF-α, INF-γ, IL-4 as well as angiogenesis marker VEGFA in HMVRECs cells under high glucose condition. The level of VEGFA mRNA after TLR4 siRNA transfection under high glucose was significantly increased compared with HG alone (Figure3.10a) with mean (SD) 212(31.45) ($p<0.001$). The mRNA expression of VGEFA growth factor in siRNA transfected control cells was also significantly increased compared to control cells alone 23.92(12.52) ($p<0.01$) (Figure 3.10a). We also measured secreted inflammatory biomediators TNF-α, IL-4 and IFN-γ protein in the cultural supernatant of HMVRECs cells by use of multiplexing ELISA assays. As shown before (Figure 3.4b), high glucose significantly increases TNF-α level; here we show that silencing TLR4 does not attenuate TNF-α ($p<0.001$) (Figure3.10b &c). It was noted that silencing TLR4 and treating the cells with 30mM glucose significantly increases the level of TNF-α in cells under high glucose when compared to high glucose alone ($p<0.05$) (Figure3.10b & c). On contrary to the 10mM NAC effect on 30mM high glucose treated cells (Figure 3.4b), there was no significant good effect of NAC treatment of TLR4 silenced high glucose treated cell (Figure3.10c). Whereas IL-4 and INF-γ were not affected significantly by silencing TLR4 (Figure3.10 d & e).

Figure 3.10(a-d)

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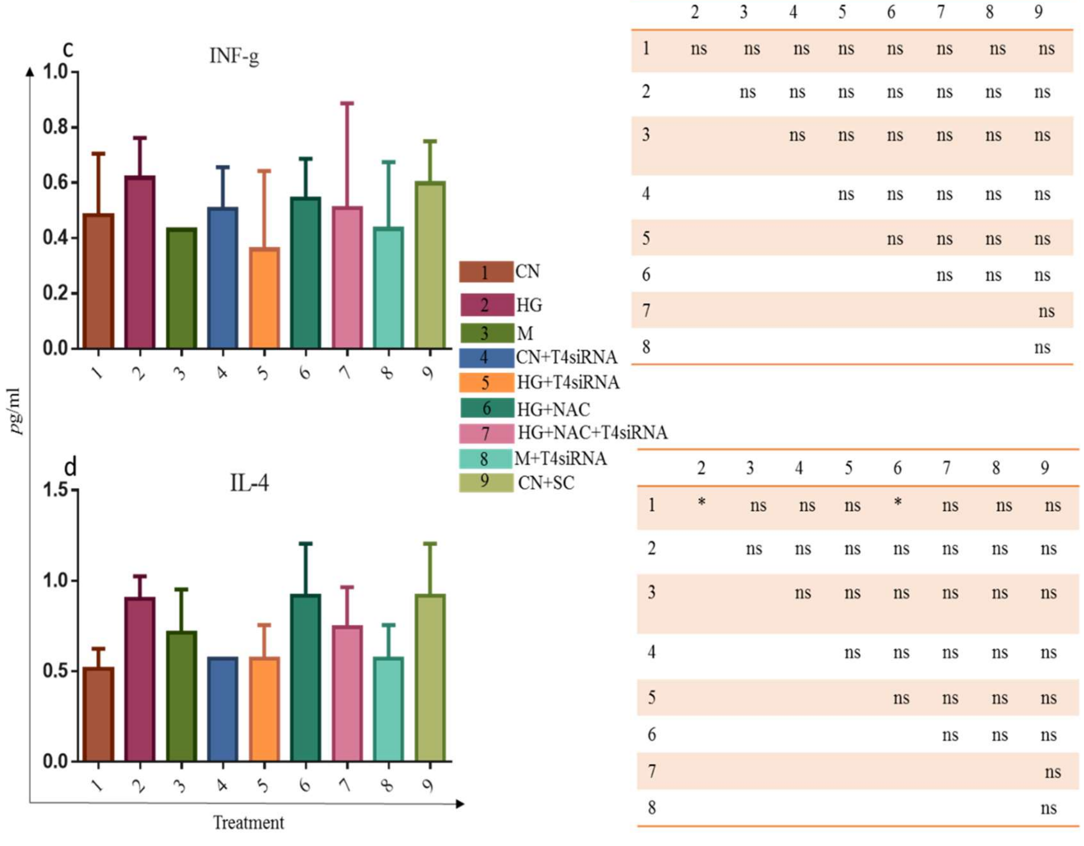


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Figure 3.10: Silencing TLR4 in HMVRECs increases VEGFA and TNF-α. After 6hrs of transfection, the cells were treated with 4 (5.5mM glucose (control), 5 (30mM glucose (HG)), 7(10mM N-acetyl cysteine (NAC) along with HG) and 8 (25mM mannitol) as an osmotic control, in addition to 9 (SC) and a negative transfection control. (a) The relative quantity of VEGFA mRNA in HMVRECs cell with TLR4 siRNA transfection. The mRNA expression normalized to β-actin under different treatment groups. (b) TNF-α protein in the supernatant of siRNA transfected HMVRECs after different treatment as indicated using multiplex ELISA. (c)INF-γ protein in the supernatant of siRNA transfected HMVRECs after different treatment as indicated using multiplex ELISA. (d) IL-4 protein in the supernatant of siRNA transfected HMVRECs after different treatment as indicated using multiplex ELISA. The data presented as means (SD) of 3-4 independent experiments, analyzed by One-way ANOVA with post-hoc Tukey-Kramer multiple comparisons test between individual groups. Two-tailed *p* value is significant at $p<0.05$. *** $P<0.001$, **$P<0.01$, *$P<0.05$. Abbreviations: 5.5 mM glucose (control); Mannitol (M); 30mM high glucose (HG)); and 10mM N- Acetyl Cysteine (NAC) + 30mM high glucose (NAC+HG). TLR4 silenced Control cells (CN+T4siRNA); TLR4 silenced HG treated cells (HG+T4siRNA); TLR4 silenced HG and antioxidant treated cells (HG+NAC+T4siRNA); TLR4 silenced Mannitol treated cells (M+T4siRNA); Scrambled sequence siRNA to control cells (CN+SC); and Scrambled sequence siRNA to HG treated cells (HG+SC), not significant (ns)

### 3.11 Silencing TLR4 with TLR4 siRNA reduced the TLR4 protein level compared to non-transfected group.

Since we have shown a decrease in the mRNA expression of TLR4 with TLR4 siRNA (Figure3.7), therefore to confirm similar effect on the TLR4 protein level after siRNA transfection we measured the TLR4 protein level and compared with the TLR4 proetin level in non-transfected group. We observed a difference in the TLR4 protein level between the two groups. As shown in Figure3.11. There was a 2.6 fold decrease (66.4%) in TLR4 protein level between transfected and non-transfected cells in the HG-treated group [0.4138(0.11) vs 0.1393(0.049)($p$>0.05)]. As well as, there is a significant difference between CN and CN siRNA transfected cells [0.551(0.2) vs 0.179(0.06) ($p$<0.05)] with 2.7 fold decrease (67.5%) in TLR4 protein in control TLR4 silenced cells. Figure 3.11.

Figure 3.11

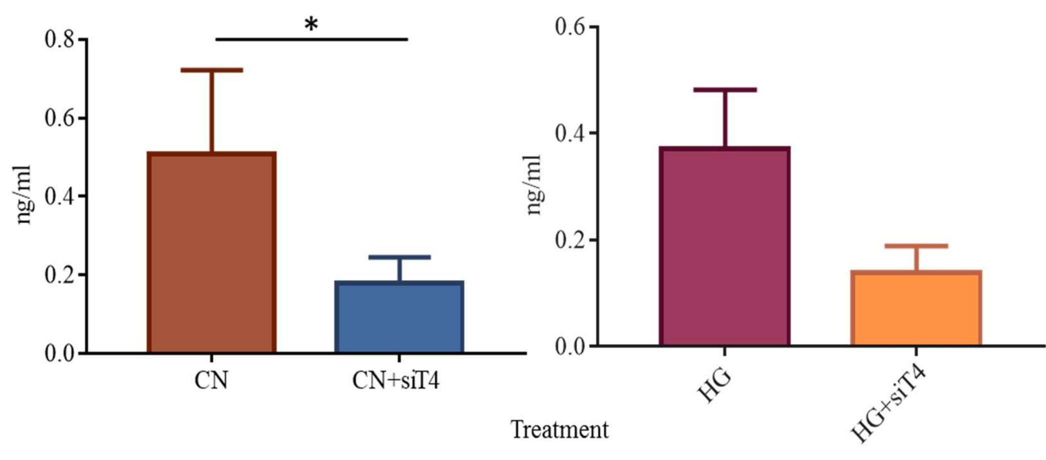


Figure3.11: Protein level in ng/ml of TLR4 attenuated by TLR4 siRNA technique. The bars show the quantity of TLR4 protein in HMVRECs with TLR4 siRNA transfection compared to non-transfected group. After 6hrs transfection, the cells were treated with 5.5mM glucose (control) and with 30mM high glucose (HG)). The data presented as means (SD) of 3-4 independent experiments, analyzed by One-way ANOVA with post-hoc Tukey-Kramer multiple comparisons test between individual groups. The two-tailed *p*-value is significant at $p<0.05$. *$P<0.05$ between Control and Control siRNA transfected (CN+siT4). Abbreviations: 5.5 mM glucose (control); 30mM high glucose (HG). TLR4 silenced Control cells (CN+ siT4); TLR4 silenced HG-treated cells (HG+T4siRNA).

### 3.12 Electric Cell-substrate Impedance Sensing (ECIS) provides real time supportive data

To provide support for TLR4 siRNA transfection effect on HMVRECs compared to non-transfected HMVRECs, we seeded the cells on (8W10E+) gold electrodes and measured the electric currents passing through fully confluent monolayers independently in each well by the Electrical Cell–Substrate Impedance Sensing (ECIS).

#### 3.12.A: High glucose treatment of HMVRECs causes barrier dysfunction:

The effect glucose treatment on HMVRECs barrier function was assessed on ESIC; we evaluated whether cell treated with 5.5mM glucose, 30mM glucose, and 10mM NAC along with 30mM glucose (HG+NAC) have different effects on the cell barrier function. We observed a significant decrease in transelectrical resistance (TER) in cells exposed to 30mM glucose compared to control( $p<0.05$). On the other hand, treating glucose challenged cells with 10mM NAC did not change the TER significantly compared to control cells as shown in Figure 3.12.A

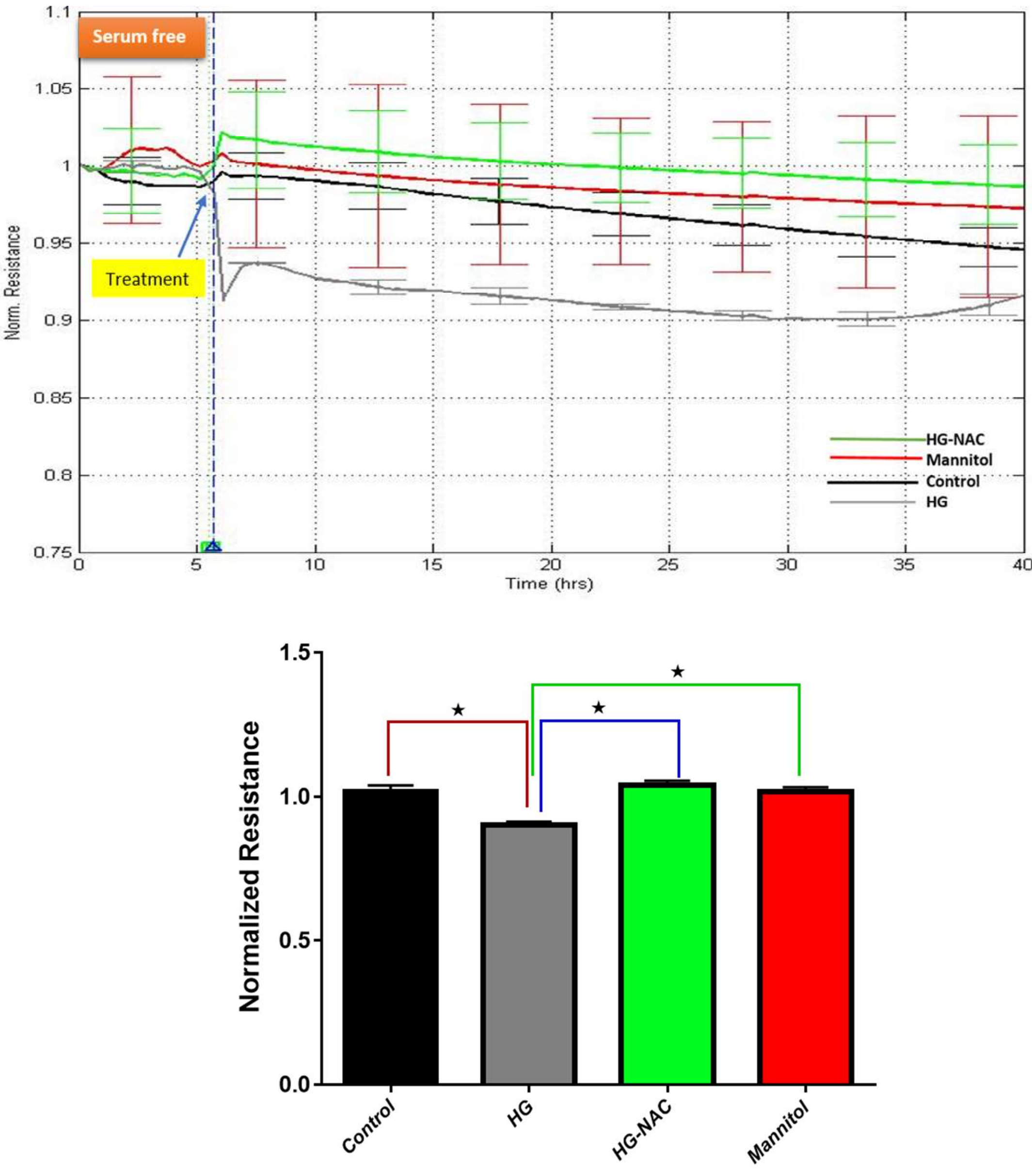


Figure 3.12A. Significant decrease in cell barrier function with HG treatment. Transelectrical resistance-barrier function of high glucose treated HMVRECs cells. 5.5mM glucose (Control), 30 mM high glucose (HG), 25mM Mannitol (M) and 30mM High glucose with 10mM Antioxidant (HG-NAC). Two-tailed *P*-value is significant <0.05. * p<0.05 significant difference between groups as shown in fig. Abbreviations: 5.5mM glucose (control); Mannitol (M); 30mM high glucose (HG)); and 10mM N- Acetyl Cysteine (NAC) + 30mM high glucose (NAC+HG).

### 3.12.B: siRNA transfection does not restores normal barrier function of high glucose treated cells:

To assess the TLR4 contribution to cell behavior, we transfected the HMVRECs with TLR4 siRNA and treated them with 5.5mM glucose, 30mM glucose, and 10mM NAC along with 30mM glucose. We observed a significant difference in resistance between the TLR4 silenced high glucose treated cells compared to TLR4 silenced control cells (figure 3.12.B.a) $p<0.0001$. Wherase treating the TLR4 silenced cells with antioxidant NAC did not significantly increase the resistance compared to the TLR4 silenced high glucose treated cells, in another word, NAC did not improve cell permeability, as shown in(figure 3.12.B.b), $p>0.05$.

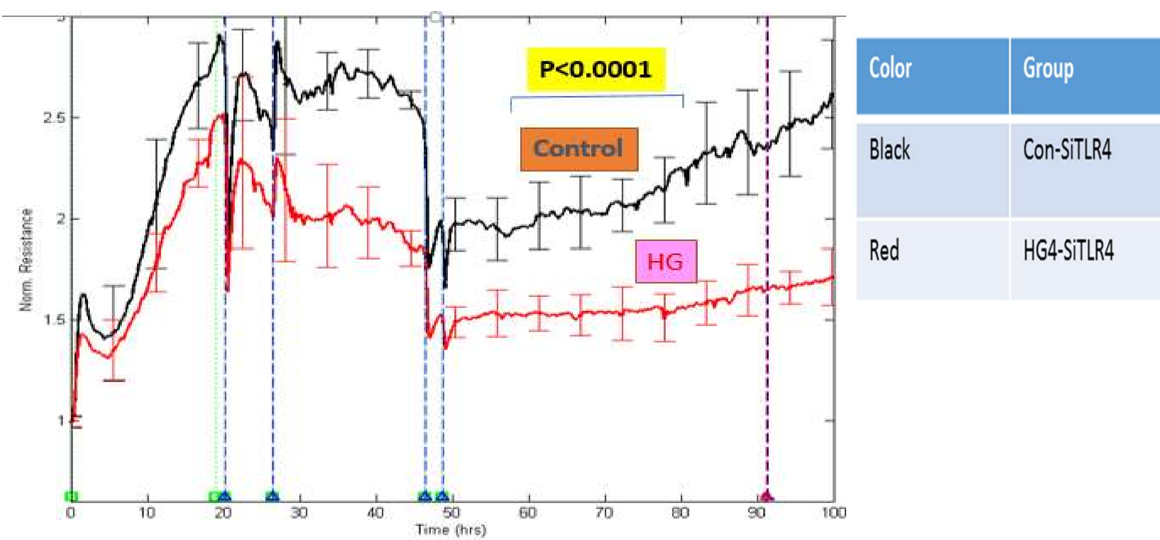


Figure3.12B.a: Difference resistance between the TLR4 silenced high glucose treated cells to TLR4 silenced control cells.Transelectrical resistance- barrier function of siTLR4-HMVRECs treated with 5.5mM glucose (control) and with 30 mM high glucose(HG). Two-tailed *P*-value is significant <0.05. *P*<0.0001 highly significant between Control vs. HG. Abbreviations: TLR4 silenced Control cells (Con-SiTLR4); TLR4 silenced HG-treated cells (HG4-SiTLR4).

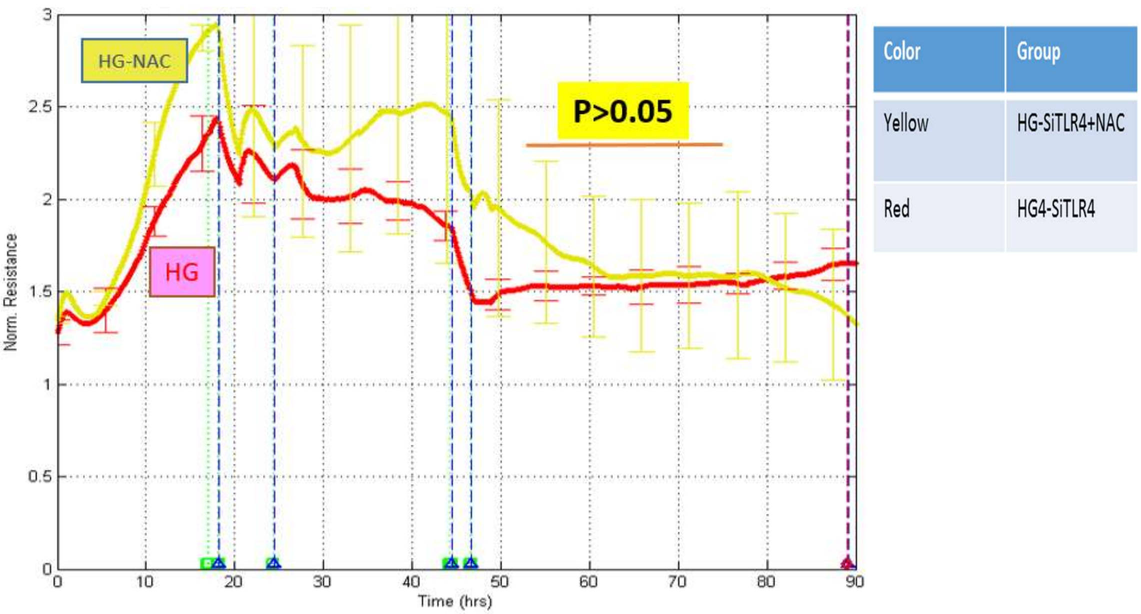


Figure 3. 12B.b: Antioxidant NAC treatment on HG cells does not restore the normal cell barrier function. Transelectrical resistance-barrier function of TLR4 silenced HMVRECs treated with 30mM high glucose (HG) and 30mM high glucose along with 10mM Antioxidant (HG-NAC). Two-tailed *P*-value is significant at <0.05. Abbreviations: TLR4 silenced HG-treated cells (HG4-SiTLR4) and TLR4 silenced HG with NAC treated cells(HG-SiTLR4+NAC)

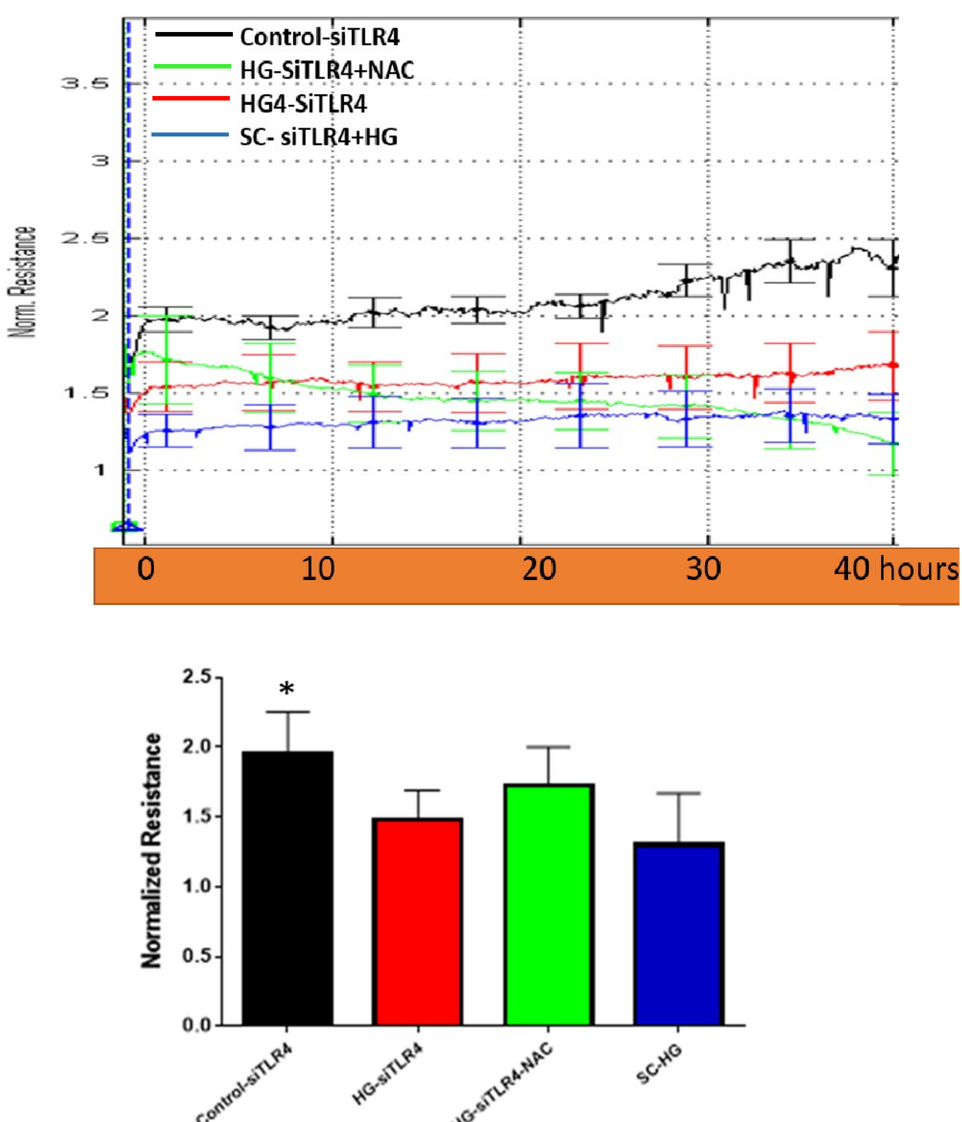


Figure3.12B.c: The treatment hours for TLR4 silenced cells (0-40hrs). Transelectrical resistance-barrier function of siTLR4-HMVRECs treated with 5.5mM glucose (Control), 30mM high glucose (HG) and SC with30mM high glucose (SC-HG) and 30mM high glucose along with10mM Antioxidant (HG-NAC) shows a significant difference in resistance between the TLR4 silenced control group compared to other groups. Two-tailed *P*-value is significant at <0.05. Abbreviations: TLR4 silenced Control cells (Con-SiTLR4); TLR4 silenced HG-treated cells (HG4-SiTLR4), TLR4 silenced HG with NAC treated cells (HG-SiTLR4+NAC) and scrambled sequence with HG(SC-siTLR4+HG).

## Chapter4. Discussion

Diabetic Retinopathy is a progressive retinal microvascular alteration leading to retinal ischemia, retinal permeability, retinal neovascularization and macular edema (3). Several biochemical changes due to hyperglycemia affect the microvasculature of the retina (5), in addition to an increase in the capillaries permeability, decrease in endothelial elasticity and neovascularization (6). Recently it has been indicated that the activation of the innate immune system and inflammation during diabetes is associated with TLRs activation, this led to an increased interest to TLRs signaling pathways and subsequent inflammatory reactions (1, 8, 23, 44-46).

Several studies linked TLR4 to inflammation during diabetes and the association with diabetic retinopathy (1, 51, 59). Thus, the primary aim of this study was to investigate the role of TLR4 in the pathogenesis of DR using primary human microvascular retinal endothelial cells (HMVREC) treated with HG, and by application of siRNA technique to silence TLR4. The small interfering RNA specifically targets the transcribed TLR4 mRNA and breaks it down, therefore, the TLR4 is silenced. In this study, we found that cells under high glucose condition had an increased mRNA expression of both TLR4 and TLR2 with upregulation of the inflammatory cytokine production. Importantly, when TLR4 was silenced, the expression level of TLR4 decreased (control vs. transfected control & HG vs. transfected HG) in both mRNA and protein level. In addition to the FITC conjugated control showing transfection efficiency; the transfection efficiency can also be confirmed with the decrease in TLR4 mRNA and protein level when comparing

transfected with non-transfected cells. The mRNA expression decreased with 72% and protein level decrease with 67%. Treating the transfected cells with HG showed a blunted response of TLR4 mRNA expression (p-value>0.05) compared to the marked increase in TLR4 mRNA expression in response to HG in non-transfected cells (p-value <0.0001). On the contrary, of TLR4; TLR2 mRNA expression significantly increased in HG-treated cells when TLR4 was silenced (p-value<0.0001). Another finding of this study was that NFkB and the inflammatory biomediators were not attenuated by TLR4 inhibition in hyperglycemic tretated cells. Importantly, the transfected cell exposed to HG did not correct the permeability disorder observed in non-transfected cells exposed to high glucose; similarly, treatment with antioxidant NAC did not increase cell resistance and did not improve the barrier disorder.

These findings are discussed in the following sections, and the impact of TLR4 in retinal associated barrier dysfunction in response to hyperglycemia is highlighted.

In this study, we observed that both TLR4 and TLR2 were significantly increased in HG (30mM) treated HMVRECs cells compared to normal glucose control (5.5mM). This observation is in agreement with previously reported studies that demonstrated upregulation of TLR4 and TLR2 in response to hyperglycemia whether in different cell types (54, 73-75) or similar cells as used in this study (1, 51) and diabetic mice (59, 76). Rajmani and Jailal et al., (2014) showed that treating the human microvascular retinal endothelial cells (HMVREC) with high glucose significantly increases the mRNA and protein expression of TLR2 and TLR4 compared to control (1). In addition to the study

by Rajamani and Jailal (2014), another study reported by Wang et al. (2015), both of them used different concentrations of glucose; 15mmol/l and 25mmol/l of glucose. The results of both studies demonstrated that increased expression either of TLR2 or TLR4 in response to different glucose concentrations used, also lead to an increase in the inflammatory biomediators (1, 77). In the current study, we used the same cell type but different glucose concentration.

We also reported in this study that treating the HMVRECs with HG increases VEGFA, NFkB, IL-4, and TNF-α, whereas INF-γ was not affected significantly. Vascular endothelial growth factor (VEGF) is the most potent factor for stimulating angiogenesis. Increase in VEGF during DR leads to neovascularization, but these blood vessels are leaky causes hemorrhage, complicating the disease more (6). Wang et al., (2015) demonstrated that the expression of proangiogenic factor VEGF is enhanced when the HMEC-1 cells were treated with 15mmol/l and 25mml/l glucose (77). Another study on STZ induced diabetes mice reported that the expression of VEGF in TLR4 WT/WT mice is much higher than in Mut/Mut mice (76). Also, the above research (77) reported a significant increase of TNF-α and NFkB in high glucose treated cells compared to control. The importance of TNF-α in DR was stated by Huang et al. (78) that: "TNF-alpha is required for late BRB breakdown in diabetic retinopathy, and its inhibition prevents leukostasis and protects vessels and neurons from apoptosis.", - BRB stands for blood retinal barrier-. Both MyD88 dependent and TRIF pathways lead to translocation of NFkB to the nucleus therefore transcription of proinflammatory genes and cytokine

release; a property of TLR4 is that it can single through both pathways activating NFkB and increasing the inflammatory cytokines (7).

As well as, we reported a non-significant increase in INF-γ in HMVRECs under high glucose challenge compared to control, on the other hand, most of the studies relating TLRs with DR repeatedly included same cytokines, chemokines and adhesion molecules (IL-1b, IL-6, IL-8, TNF-a, IFN-β, MCP-1, MCP-2) (see table4).

Turning to TLR4 siRNA transfection to breakdown TLR4 mRNA, gave interesting various results when compared to other similar studies (Table 4). We reported here that TLR4 siRNA significantly decreases TLR4 mRNA expression in all groups of interest. This finding is supported by Dasu et al. 2008 (73), Rjamani & Jailal 2014 (1) and by Wang et al2015 (77). Those investigators applied siRNA against TLR4 and reported similar findings in the present study (Table 4). Whereas Rajamani & jailal and Wang et al. used human microvascular retinal cells, Dasu et al. applied the siRNA against Human monocytes (THP-1 cells).

Clearly, we also reported that silencing TLR4 significantly increased TLR2 mRNA expression in both transfected controls and transfected HG group. This was not reported before- up to our knowledge- in HMVRECs. Indeed, the studies used siRNA technique to silence TLR2 and or TLR4 did not report any change in either receptor when one of them is silenced (1,77). Similar to our results, but on different cells, Dasu et al. 2008, reported a significant increase in TLR2 mRNA level when transfected the high glucose

treated THP-1 cells with TLR4 siRNA alone, in addition, no change was observed in the TLR4 mRNA level when the cells were transfected with TLR2 siRNA (73).

Moreover, we reported that silencing TLR4 did not attenuate the increase in NFkB, VEGFA, and TNF-α in high glucose treated HMVRECs. In contrast to our finding, Rajamani & Jailal 2014(1), and Wang et al. 2015 (77) both reported that TLR4 siRNA attenuates high glucose-mediated inflammation. Although, silencing TLR4, increases TLR2 expression, the high glucose-mediated inflammation in THP-1 cells was reduced (73). However, we contribute the upregulation in NFkB, VEGFA, and TNF-α to the increased expression of TLR2; this might be a compensatory mechanism and/, or other unknown factors might contribute to this observed effect on TLR4 silencing. On a clinical base, a molecular study on chinses population concluded that, TLR4 polymorphisms associated with diabetes type 2 and with susceptibility to DR (85).

The antioxidant N-Acetyl-L-cysteine (NAC) is an ROS scavenger; 10mM of NAC used in this study to negate the effect of oxidative stress in high glucose treated cells, here we used 10mM of NAC for as long as 40 hrs. Moreover, we observed the NAC improved the permeability in HMVRECs exposed to high glucose but not in silence TLR4 HMVRECs cells exposed to high glucose. This finding supports that TLR4 is needed to maintain normal barrier function and highlights the importance of the integrity of immune receptor expression in HMVRECs and its response to anti-oxidant. Our data showed that NAC treatment in TLR4 silenced cells significantly upregulates TNF-α, in contrary to its effect in normal cells expressing TLR4. This could also be explained by the upregulation of

TLR2 in this study when TLR4 is silenced and as a compensatory mechanism for silenced TLR4 and its consequence on the inflammatory pathway and the barrier dysfunction. A study reported the time dependency of NAC effect, as they stated that long time NAC treatment at low concentration might have a proinflammatory effect (79). These observations need further studies to elucidate the mechanism of this observation.

ESIC is used for monitoring monolayer cell behavior and cell permeability or barrier function; it gives quantitative real time data. Reported here for TLR4 siRNA-transfected cells that provided more supportive data than other methods. This is the first time to report such real time data on TLR4 silenced HMVRECs. The current data showed that silenced TLR4 in HMREC treated with high concentration of glucose (30mM) demonstrated increased permeability with barrier dysfunction compared to control cells. This finding indicates that silencing TLR4 alone has no protective or improvement effect against the barrier dysfunction in HRMEC in response to high concentration of glucose.

There is no doubt that in vivo techniques get data that are more powerful compared to in vitro data, as the cells in culture do not completely mimic the cell behavior in its original in vivo environment, but this does not negate the credibility of cell culture methods for studying human diseases. Prior studies were either in vitro (80) or only observational studies on rats (1) or in the fibrovascular membrane of PDR patient (77), all showing increased TLR4 expression in hyperglycemic condition. While Observational studies can give association between TLR4 and DR, they do not explain pathogenesis. Consequently, by looking in a study like the one conducted by Wang et al. (77) in 2015, we can support

our study with a more mechanistic approach by introducing siRNA to block TLR4 expression in primary HMVRECs. Above all, this study provides a real time data on HMVRECs cell barrier function when TLR4 blocked by seeding the cells on gold electrodes in ECIS-an electrical biosensor- and recording cell activity throughout time. As the previous study showed an association, however, now we can say that TLR4 alone is not sufficient to cause pathogenesis of DR and further mechanistic studies in vivo are needed.

Table 4. Studies onTLR2 and TLR4 expression induced by high glucose:

| Method | TLR 2/4 expression | Target | Downstream signaling targets | Cytokines/chemokine release | Other biomarkers | Reference |
|---|---|---|---|---|---|---|
| **High Glucose(HG) 15 or 25 mM** | ↑ TLR2 & TLR4(mRNA &protein) | Human Microvascular Retinal Endothelial Cell line | ↑MyD88, TRIF, ↑ ↑IRF3 ↑NF-$\kappa$B | ↑IL-1$\beta$, ↑IL-8, ↑TNF-$\alpha$, ↑MCP-1 | ↑ICAM-1, ↑VCAM-1 | (1) |
| **HG+ TLR-4/2 Inhibition (TAK242, TIP, TLR-2 neutralizing antibody)** | N/A | | ↓NF-$\kappa$B p65 | ↓IL-1$\beta$, ↓IL-8, ↓TNF-$\alpha$, ↓MCP-1 | ↓ICAM-1, ↓VCAM-1 | |
| **siRNA against TLR-2 and TLR-4** | ↓ 50% TLR-4/2 (protein) | | ↓NF-$\kappa$Bp65 | ↓TNF-$\alpha$ ↓IL-8 ↓THP-1 | | |
| **Streptozotocin-induced diabetes** | ↑TLR4(mRNA & protein) | Retina of Diabetic Rats | ↑MyD88, IRAK, ↑TRAF, TRAM, ↑TRIF ↑ ↑NF-κB | ↑TNF-α, ↑IL-1β, ↑IFN-β | | (80) |
| **TAK-242 treatment** | ↓TLR4 (mRNA to68.7 %, protein to 63.6%) | | | ↓TNF-α, ↓IL-1β, ↓IFN-β | | |
| **10-25mM glucose (HG)** | ↑TLR4/2(mRNA, protein) | Human monocytes (THP-1 cells) | ↑NF-KB | | | (73) |
| **HG+TLR2 siRNA** | No change TLR4 (mRNA) | | ↓48%NF-KB | ↓IL-1b (80%), ↓ ↓IL-6 (42%), ↓MCP-1 (60%), TNF-alpha (85%), | | |
| **HG+TLR4 siRNA** | ↑ TLR2(mRNA) | | ↓45%NF-KB | ↓IL-1b (55%), | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | ↓IL-6(51%),<br>↓MCP-1(65%),<br>↓TNF-alpha (89%) | | |
| **HG+TLR4/2 siRNA** | N/A | | ↓76% NF-KB | N/A | | |
| **Diabetic retinopathy patients** | ↑TLR4(immunofluorescence staining) | Pre-membrane of diabetic retinopathy patients(fibrovasculature) | N/A | N/A | | (77) |
| **STZ-induced diabetes in mice** | ↑TLR4(immunofluorescence staining) | diabetic retina | N/A | N/A | | |
| **25 & 15 mmol/l glucose** | ↑ TLR4 (mRNA, protein, flow cytometry) | HMEC-1 cell line | ↑MyD88 | ↑IL-1β, | ↑VEGF,<br>↑bFGF | |
| **HG+TLR4 siRNA** | ↓TLR4 (protein) | | N/A | ↓IL-1β | | |
| **HG+TLR4 antagonist (*Rhodobacter sphaeroidesLPS)*** | N/A | | N/A | ↓IL-1β | | |
| **HG+TLR4 deficient mice** | N/A | MRECs from TLR4 deficient mice | N/A | ↓IL-1β | | |
| **STZ-induced diabetes in mice** | N/A | TLR4 WT/WT mice | N/A | ↑↑TNF-α,<br>↑↑IL-1β,<br>↑↑MIP-2 | ↑↑↑VEGF<br>↑HIF-1α | (81) |
| | | TLR4 WT/Mut mice | | ↑TNF-α,<br>↑IL-1β,<br>↑MIP-2 | ↑VEGF<br>↑HIF-1α | |
| | | TLR4 Mut/WT mice | | ↓TNF-α,<br>↓IL-1β,<br>↓MIP-2 | ↓VEGF<br>↓HIF-1α | |
| | | TLR4 Mut/Mut mice | | ↓↓TNF-α,<br>↓↓↓IL-1β,<br>↓MIP-2 | ↓↓↓VEGF<br>↓HIF-1α | |

**Conclusion:**

In this study, we investigated the expression of TLR4 in the microvascular endothelial cells under high glucose, shedding light to its contribution in the genesis of diabetic retinopathy. We clearly demonstrated that high glucose (30mM) induces the TLR4 expression, and increases the inflammatory cytokine TNF-α, NFkB and angiogenesis marker VEG-A, whereas silencing TLR4 increased the expression of such biomediators even more. Silencing of TLR4 in HMVRECs exposed to high glucose (30mM) did not ameliorate or reverse the permeability and barrier disorder indicating that TLR4 alone did not contribute to the pathogenesis of DR and improvement of the barrier function. In addition to decrease in both mRNA and protein level when cells are transfected with TLR4 siRNA. The current study highlights that the immune system receptors “TLRs” may act as one unit, and not as an individual basis that could play an important role in the regulation of inflammation, as well being an early event in the eye and retinal vascular disorders. Thus, indicating the importance of TLR4 to prevent an exaggerated immune response, which might be an effect of a compensatory mechanism by other TLRs for the loss of TLR4 or presence of other unknown markers that increase the inflammation.

Limitation:

A limitation of this study is the application of only on a method for knocking out the expression of TLR4; the use of siRNA. Admittedly, additional methods like Lentiviral vectors, plasmids, shRNA or specific chemical blockers against TLR4 like TAK 242 would give a more insight and further validate our results.

Moreover, assessment of other proinflammatory cytokines like IL-1, IL-6, IL-8, and chemokines, in addition to assessment of adhesion molecules like ICAM-1 and VCAM-1, and application of other antioxidants would enforce our findings more.

Prospective: These results guide us to future research on the role of the immune metabolism and immune, vascular systems with the pathogenesis of DR. for example, studies of TLRs pathway, by use of blockers for TLR4 alone, TLR2 alone, and both TLR2/4, we can assess the role of these receptors in DR development. Also, we can apply MyD88 inhibitor; we can evaluate the effect of all TLRs except TLR3 as it signals through the non-MyD88 pathway, thus assess the abnormalities that develop in the retina about diabetic retinopathy concerning TLRs. Use of in vivo animal model would help to understand the role of TLRs in DR with more details.

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Appendix A: IRB approval

Qatar University
Institutional Bio-safety Committee

**To: Dr. Nasser Rizk**
Department of Health Sciences
College of Arts and Science

4th June, 2015

**Dear Dr. Rizk,**

**Subject: Research grant: QUST-CAS-SPR-14\15-39**

**Ref: Project titled "Identifying the Contribution of Toll-Like Receptors in Pathogenesis of Diabetic Retinopathy in Human Microvascular Retinal Endothelial Cells in vitro"**

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 14/14-15**. Please refer to this approval number in all your future correspondence pertaining to this research.

Best wishes.

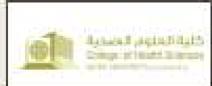
**Dr. Marawan Abu-Madi PhD, MLS(ASCP)cm**
Chairperson, QU-IBC
Department of Health Sciences
College of Arts & Sciences
Qatar University
Tel: +974 4403 4791
abumadi@qu.edu.qa

Institutional Bio-safety Committee
(IBC)
Office Of Academic Research

Appendix B: Acknowledgments of this study

The thesis was presented orally in the HMC Fifth Assembly of the Research Forum on Diabetes, Obesity and Metabolism toke place on 26th of May 2016, by the Metabolic Institute in Qatar, and won the first prize for graduate poster award in Qatar university annual research forum 2016 toke place on 3rd of May 2016. The certificate is attached. It also awarded the first prize in the first annual Health Research Retreat of Qatar University (HRR/QU) toke place on 4th of June 2016.

Appendix C: Poster



Postdoc and Graduate Students, Biomedical and Health Sciences

# The Contribution of Toll-Like Receptors in the Pathogenesis of Diabetic Retinopathy in Human Microvascular Retinal Endothelial Cells

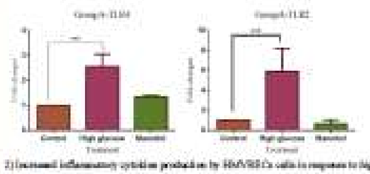
Fadheela DadBakhsh Mohammed, Dr. Nasser Rizk
Department of Biomedical Science, College of Health Science, Qatar University
QUST-CAS-SPR-14/15-39

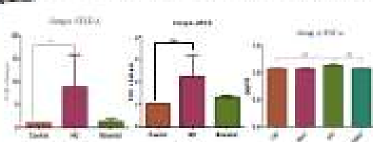
## ABSTRACT

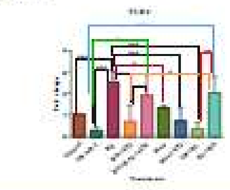
## INTRODUCTION

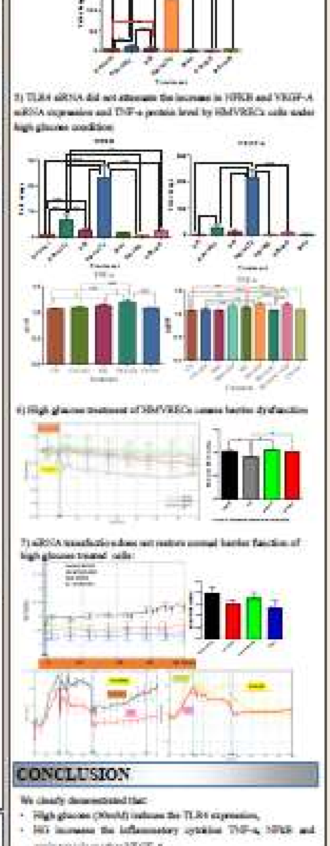
## Hypothesis

## RESULTS







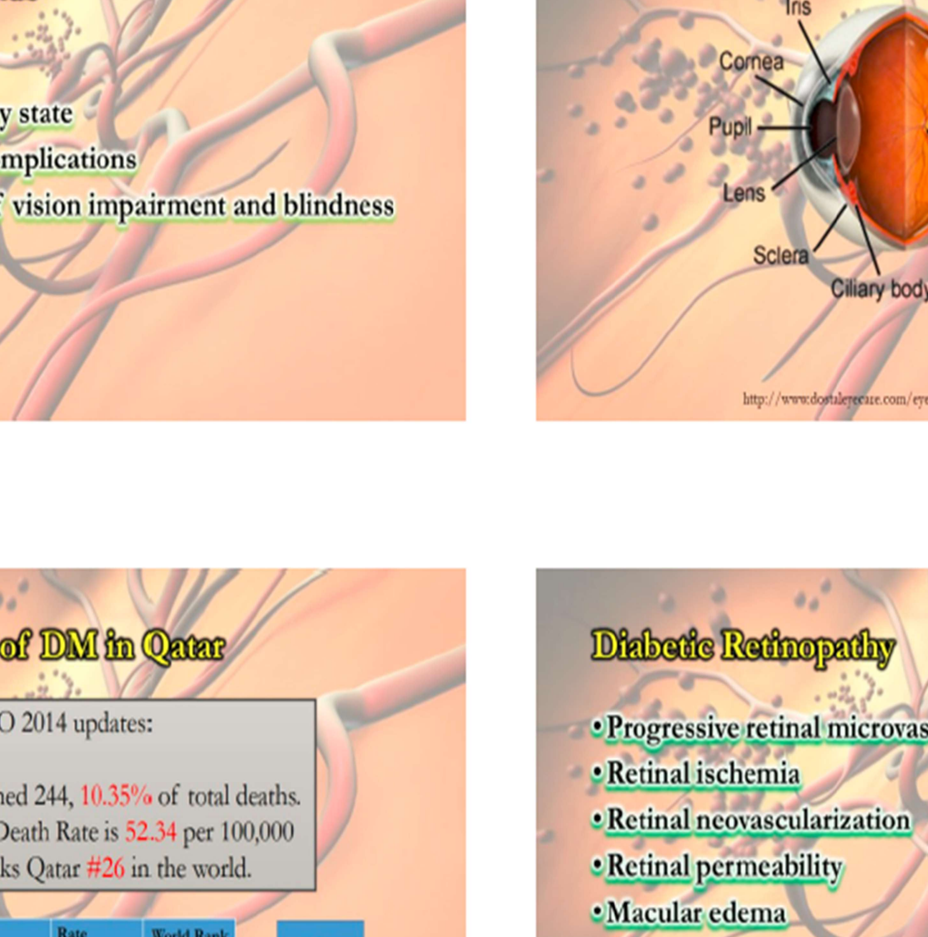


## METHODOLOGY

## CONCLUSION

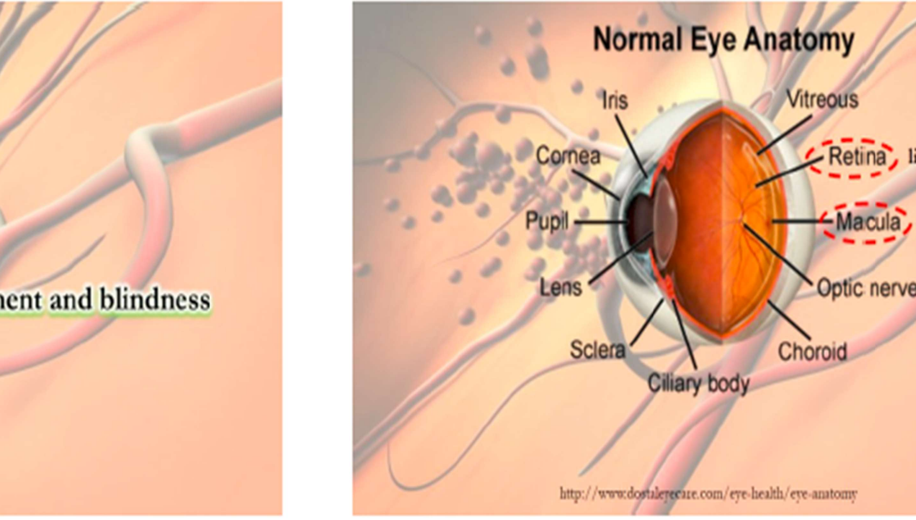
## ACKNOWLEDGMENT

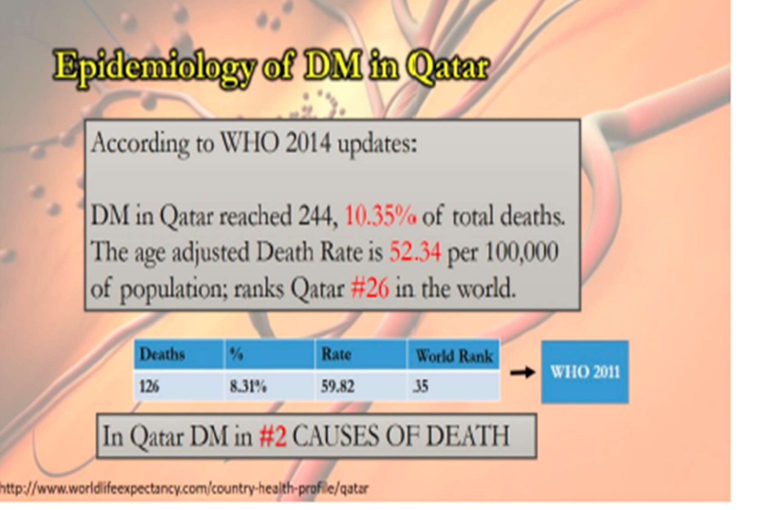
Appendix D: Power point presentation



## Diabetes Mellitus

- Hyperglycemia
- Pro-inflammatory state
- Microvascular complications
- Leading cause of vision impairment and blindness
- Global epidemic



## Epidemiology of DM in Qatar

According to WHO 2014 updates:

DM in Qatar reached 244, 10.35% of total deaths. The age adjusted Death Rate is 52.34 per 100,000 of population; ranks Qatar #26 in the world.

| Deaths | % | Rate | World Rank |
|---|---|---|---|
| 126 | 8.31% | 59.82 | 35 |

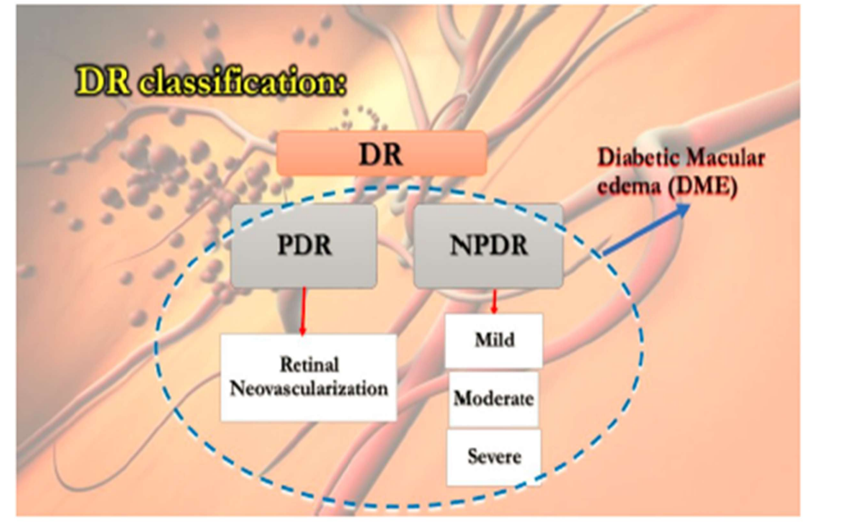
→ WHO 2011

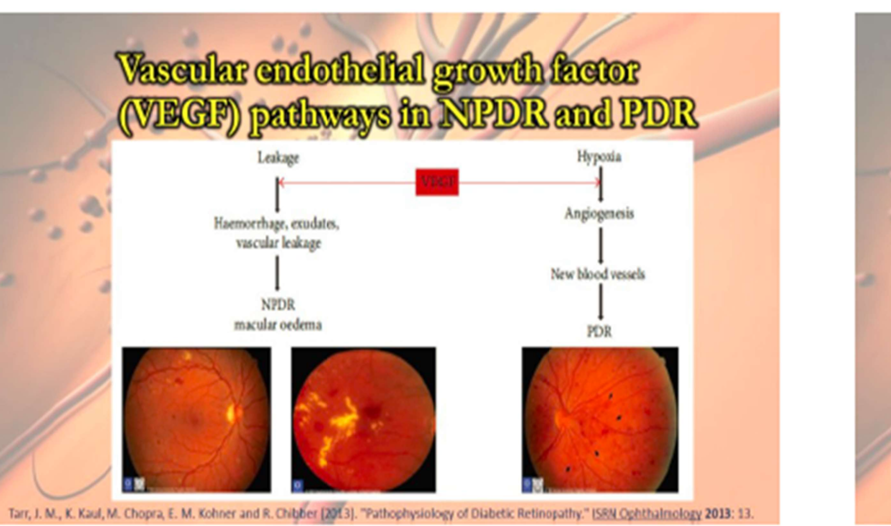
In Qatar DM in #2 CAUSES OF DEATH

http://www.worldlifeexpectancy.com/country-health-profile/qatar

## Diabetic Retinopathy

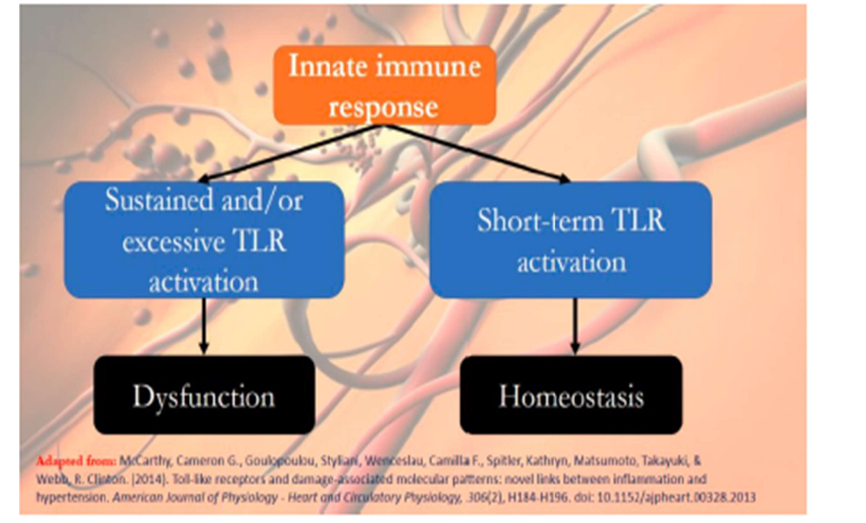
- Progressive retinal microvascular alterations
- Retinal ischemia
- Retinal neovascularization
- Retinal permeability
- Macular edema

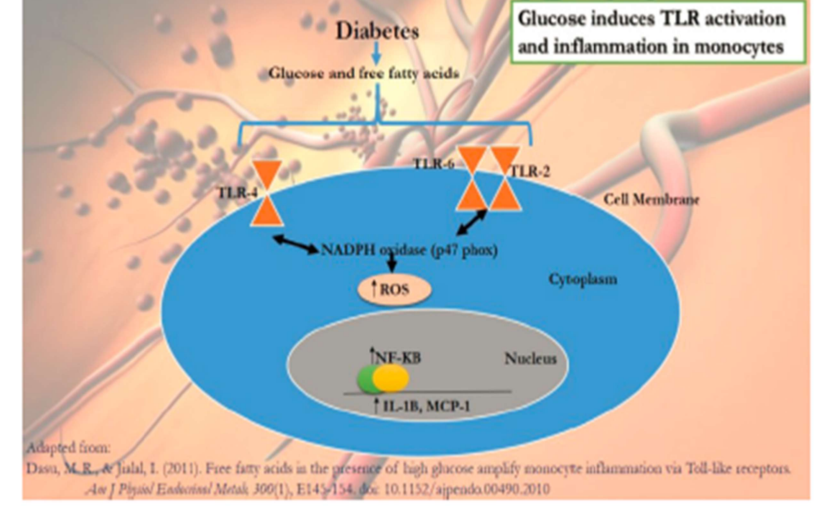


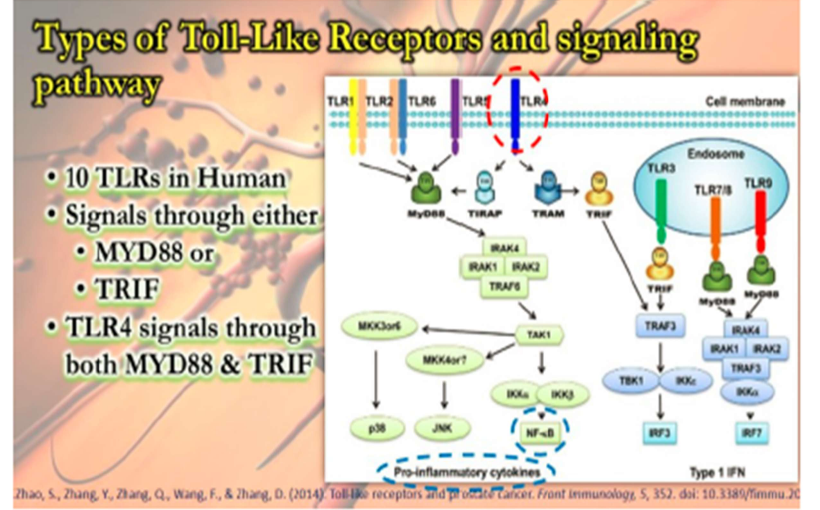


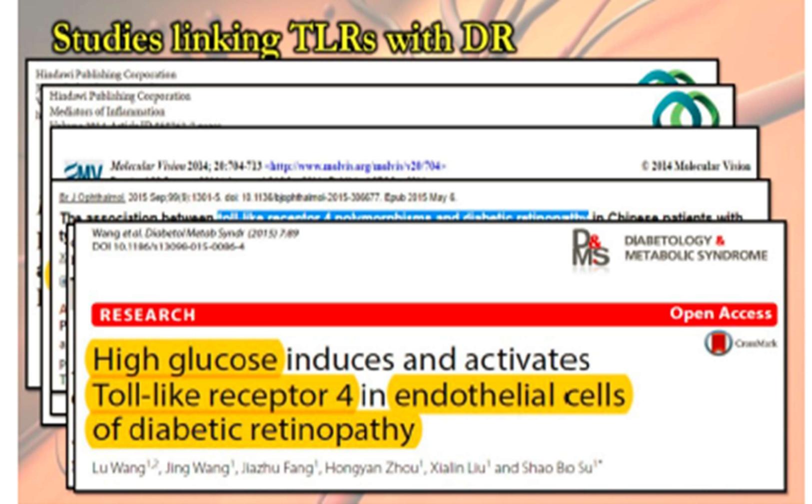

## Toll-Like Receptors

- Key receptors in the innate immune system recognizes the molecular patterns of different microbial infections (Pattern recognition receptors)
- Recognize conserved pathogen-associated molecular patterns (PAMPs) and
- Damage-associated molecular patterns (DAMPs), endogenous ligands released during tissue damage and inflammation

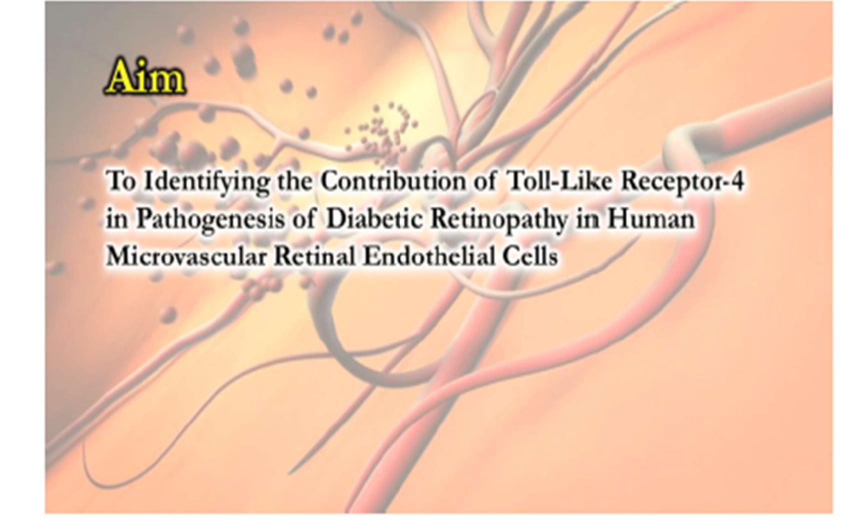

Innate immune response
Sustained and/or excessive TLR activation
Short-term TLR activation
Dysfunction
Homeostasis
Adapted from: McCarthy, Cameron G., Goulopoulou, Styliani, Wenceslau, Camilla F., Spitler, Kathryn, Matsumoto, Takayuki, & Webb, R. Clinton. (2014). Toll-like receptors and damage-associated molecular patterns: novel links between inflammation and hypertension. American Journal of Physiology - Heart and Circulatory Physiology, 306(2), H184-H196. doi: 10.1152/ajpheart.00328.2013

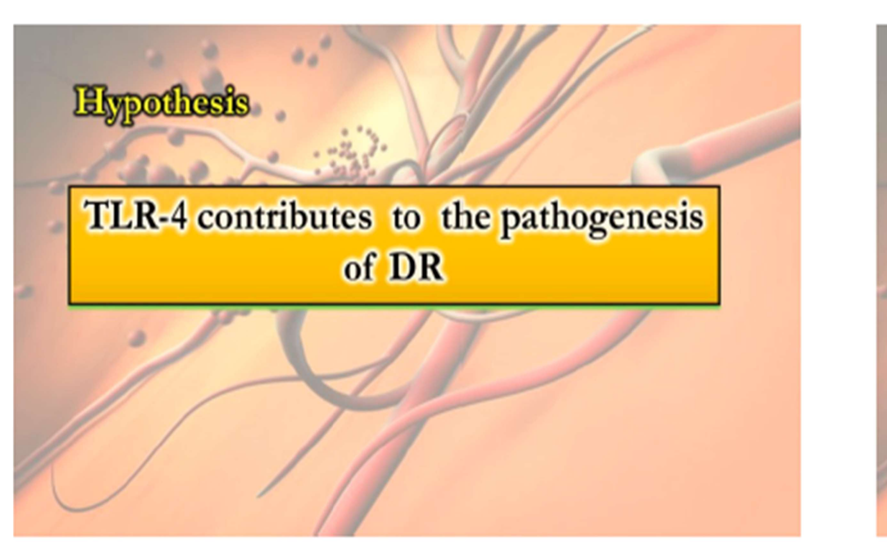

Glucose induces TLR activation and inflammation in monocytes
Diabetes
Glucose and free fatty acids
TLR-4
TLR-6
TLR-2
Cell Membrane
NADPH oxidase (p47 phox)
ROS
Cytoplasm
NF-KB
Nucleus
IL-1B, MCP-1
Adapted from:
Dasu, M. R., & Jialal, I. (2011). Free fatty acids in the presence of high glucose amplify monocyte inflammation via Toll-like receptors. Am J Physiol Endocrinol Metab, 300(1), E145-154. doi: 10.1152/ajpendo.00490.2010

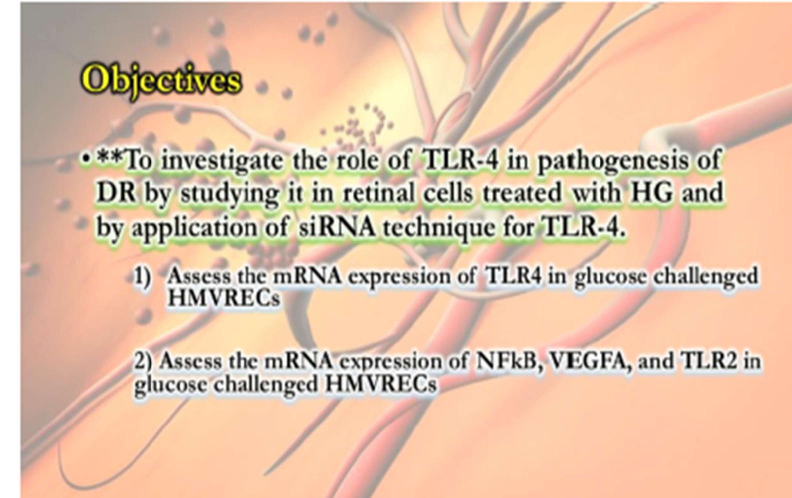

Types of Toll-Like Receptors and signaling pathway
• 10 TLRs in Human
• Signals through either
• MYD88 or
• TRIF
• TLR4 signals through both MYD88 & TRIF
Cell membrane
Endosome
Pro-inflammatory cytokines
Type 1 IFN
Zhao, S., Zhang, Y., Zhang, Q., Wang, F., & Zhang, D. (2014). Toll-like receptors and prostate cancer. Front Immunology, 5, 352. doi: 10.3389/fimmu.20

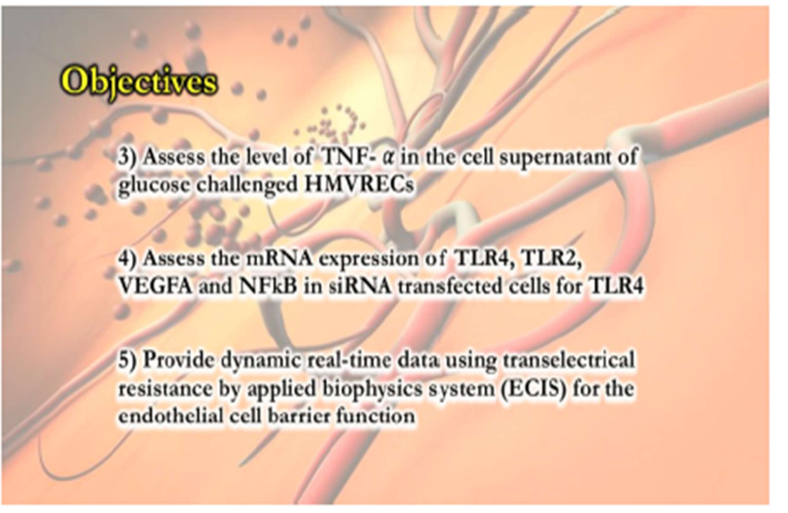

Studies linking TLRs with DR
Hindawi Publishing Corporation
Mediators of Inflammation
Molecular Vision 2014; 20:704-713
© 2014 Molecular Vision
DIABETOLOGY & METABOLIC SYNDROME
RESEARCH
Open Access
High glucose induces and activates Toll-like receptor 4 in endothelial cells of diabetic retinopathy
Lu Wang, Jing Wang, Jiazhu Fang, Hongyan Zhou, Xialin Liu and Shao Bo Su

Master Project


Aim
To Identifying the Contribution of Toll-Like Receptor-4 in Pathogenesis of Diabetic Retinopathy in Human Microvascular Retinal Endothelial Cells


Hypothesis
TLR-4 contributes to the pathogenesis of DR


Objectives
• **To investigate the role of TLR-4 in pathogenesis of DR by studying it in retinal cells treated with HG and by application of siRNA technique for TLR-4.
1) Assess the mRNA expression of TLR4 in glucose challenged HMVRECs
2) Assess the mRNA expression of NFkB, VEGFA, and TLR2 in glucose challenged HMVRECs




# Methodology

Qatar University
Institutional Bio-safety Committee

To: Dr. Nasser Rizk
Department of Health Sciences
College of Arts and Science

6th June, 2013

Dear Dr. Rizk,

Subject: Research grant: [illegible]

Ref: Project titled "Identifying the Contribution of Toll-Like Receptors in Pathogenesis of Diabetic Retinopathy in Human Microvascular Retinal Endothelial Cells in vitro"

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:

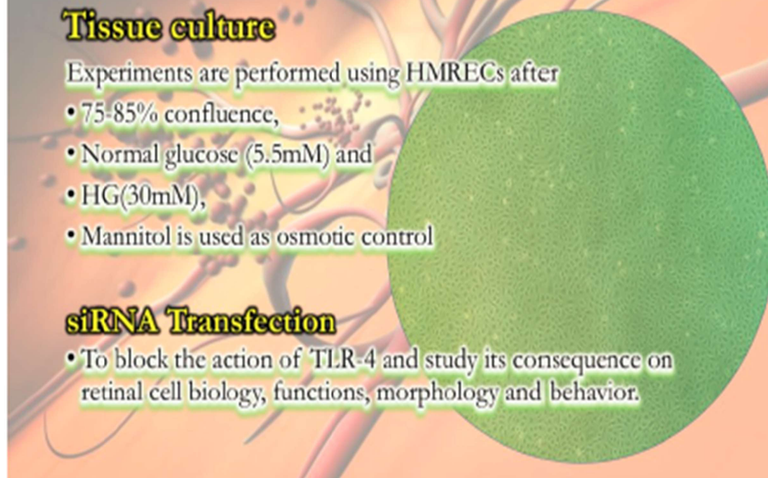
- Ensuring compliance with QU Safety Plans and applicable national and international regulations.
- Ensuring experiments that require prior IBC approval are not conducted until IBC approval is obtained and making initial determination of containment levels required for experiments.
- Notifying the IBC 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.

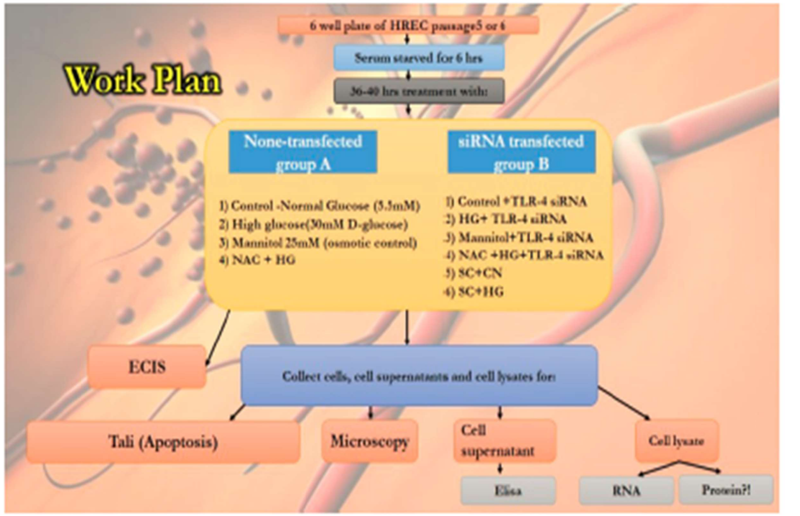
Your research approval No. is: **QU-IBC [illegible]**. Please refer to this approval number in all your future correspondence pertaining to this research.

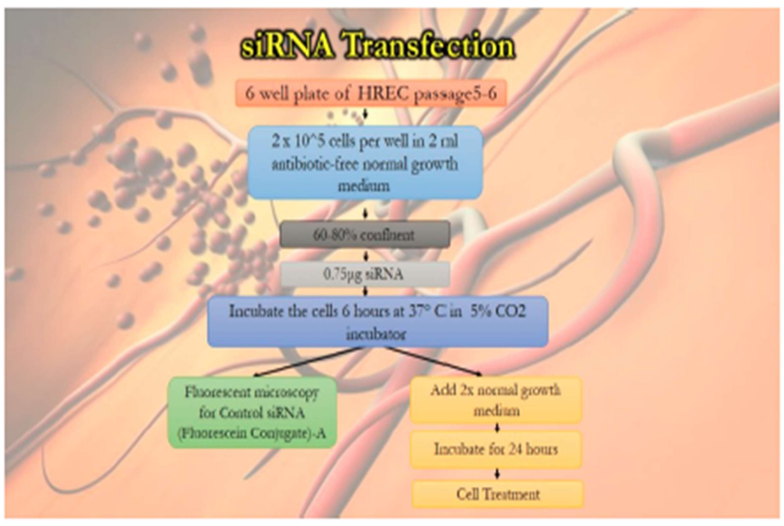
Best wishes,

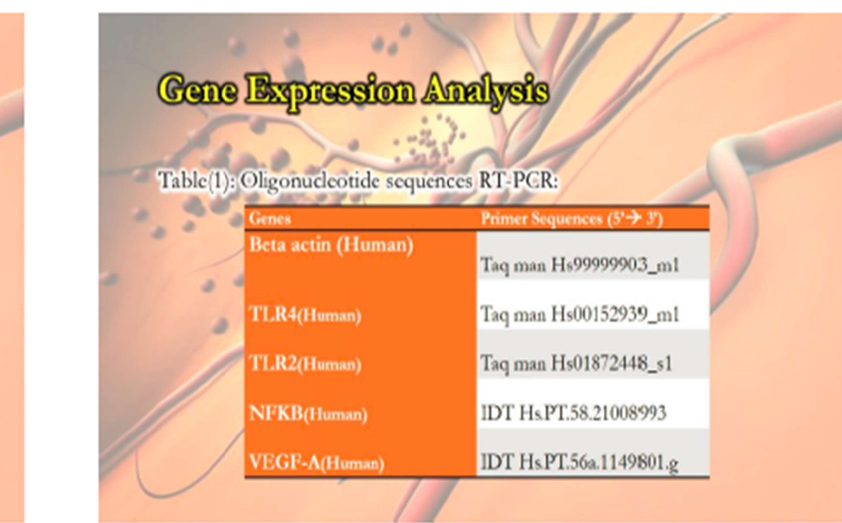
Dr. Marawan Abu-Madi PhD, MLS(ASCP)
Chairperson, QU-IBC
Department of Health Sciences
College of Arts & Sciences
Qatar University
Tel: +974 [illegible]

Institutional Bio-safety Committee (IBC) Office Of Academic Research





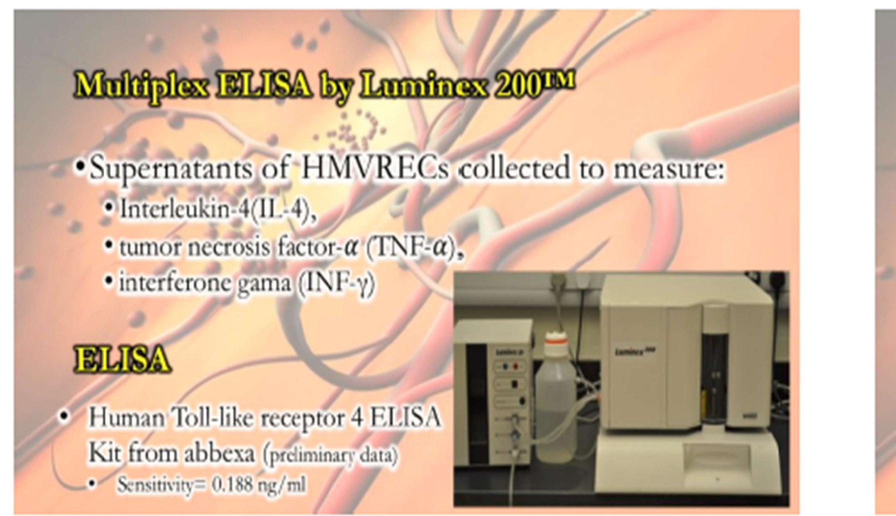


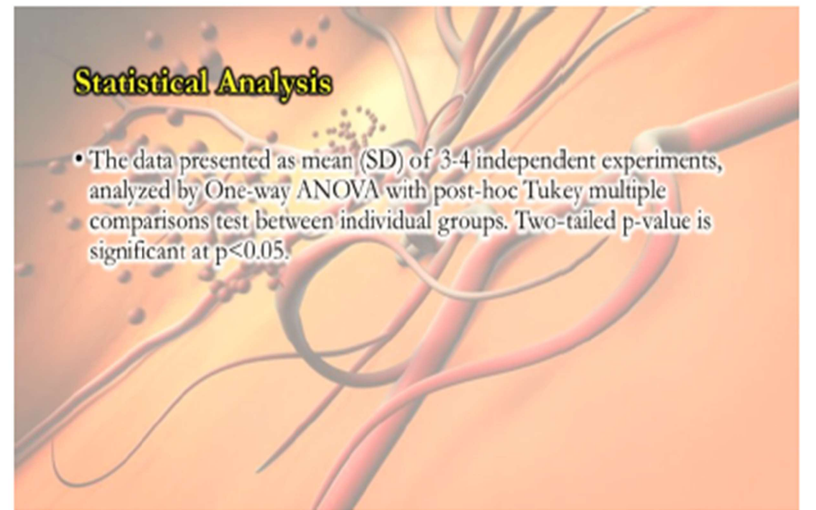




| Genes | Primer Sequences (5'→ 3') |
| --- | --- |
| Beta actin (Human) | Taq man Hs99999903_m1 |
| TLR4(Human) | Taq man Hs00152939_m1 |
| TLR2(Human) | Taq man Hs01872448_s1 |
| NFKB(Human) | IDT Hs.PT.58.21008993 |
| VEGF-A(Human) | IDT Hs.PT.56a.1149801.g |

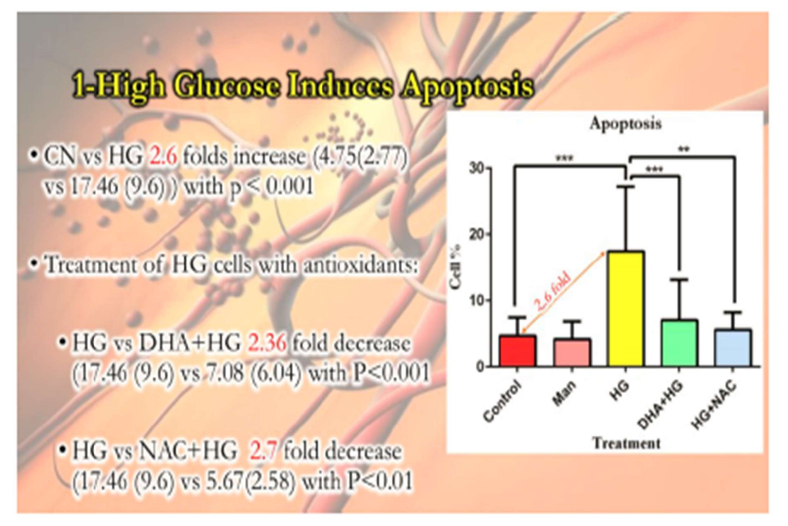
Apoptosis
• Tali Image Cytometer
Green-fluorescent Annexin V–Alexa Fluor® 488 conjugate
Red-fluorescent Propidium iodide (PI)

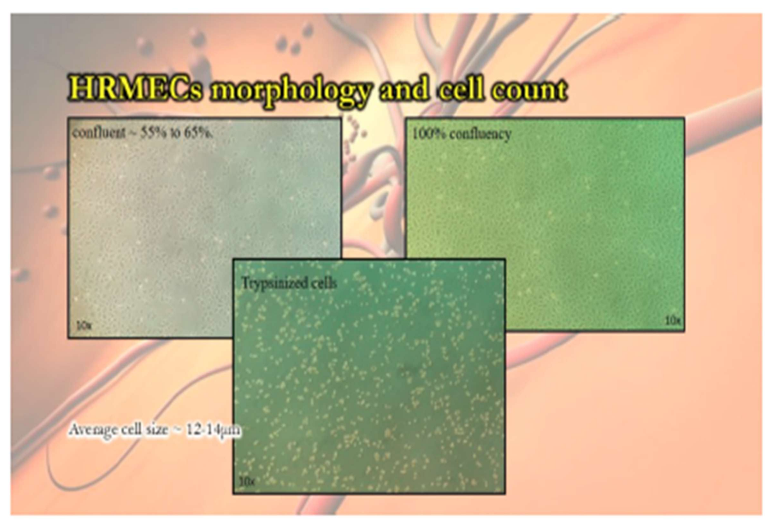
Electric Cell-substrate Impedance Sensing (ECIS)
• Cell culture and treatments were conducted on a plate of the Electrical Cell–Substrate Impedance Sensing to analyze the barrier function
• ECIS 8W10E+ culture plates coated with gold electrodes
• L-cysteine
• attachment factor
• Cells
• 18-24hrs
• siRNA/ Serum free treatment
• Treatment

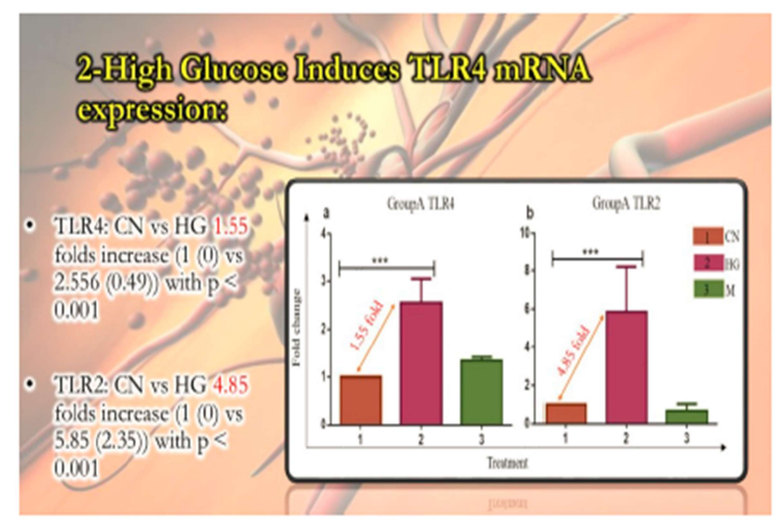

Multiplex ELISA by Luminex 200™
• Supernatants of HMVRECs collected to measure:
• Interleukin-4(IL-4),
• tumor necrosis factor-α (TNF-α),
• interferone gama (INF-γ)
ELISA
• Human Toll-like receptor 4 ELISA Kit from abbexa (preliminary data)
• Sensitivity= 0.188 ng/ml

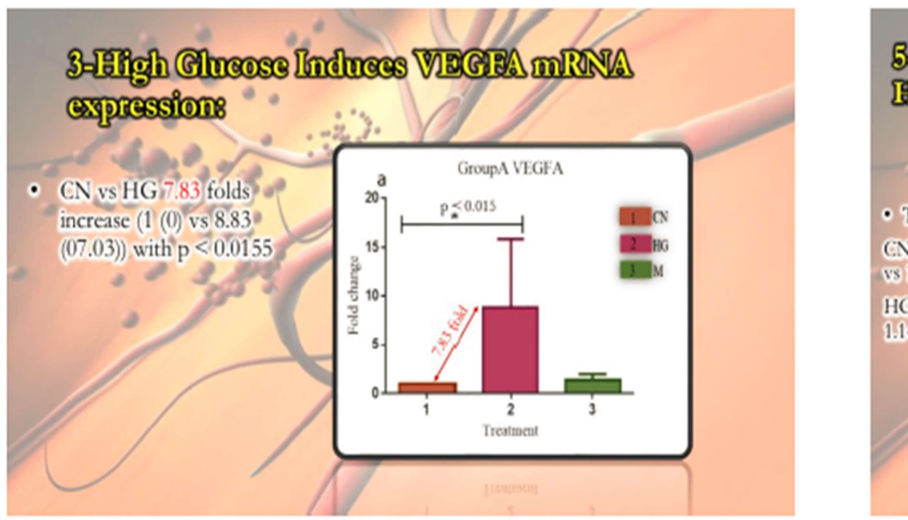

Statistical Analysis
• The data presented as mean (SD) of 3-4 independent experiments, analyzed by One-way ANOVA with post-hoc Tukey multiple comparisons test between individual groups. Two-tailed p-value is significant at p<0.05.

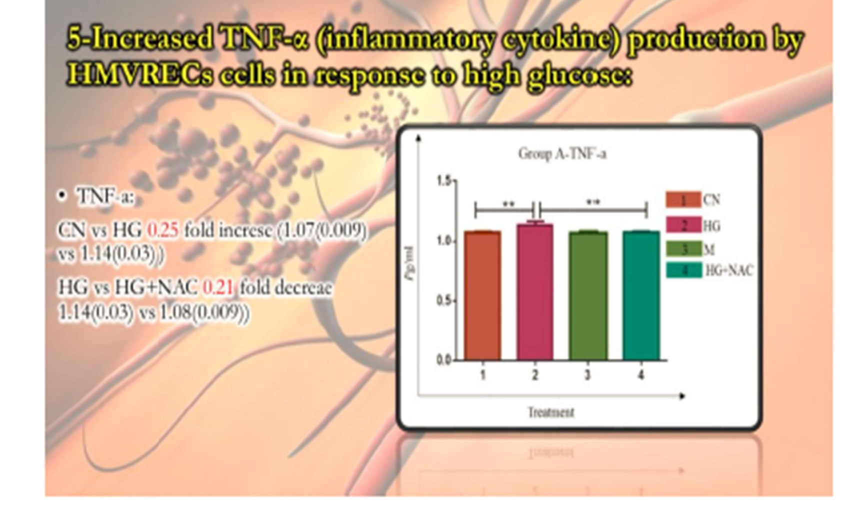
Results and
Discussion

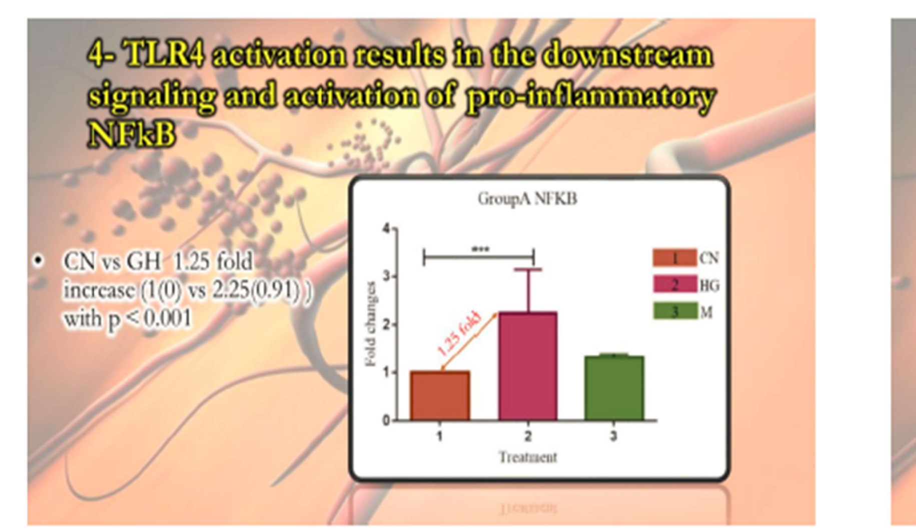

1-High Glucose Induces Apoptosis
• CN vs HG 2.6 folds increase (4.75(2.77) vs 17.46 (9.6) ) with p < 0.001
• Treatment of HG cells with antioxidants:
• HG vs DHA+HG 2.36 fold decrease (17.46 (9.6) vs 7.08 (6.04) with P<0.001
• HG vs NAC+HG 2.7 fold decrease (17.46 (9.6) vs 5.67(2.58) with P<0.01
Apoptosis
Cell %
2.6 fold
Control
Man
HG
DHA+HG
HG+NAC
Treatment

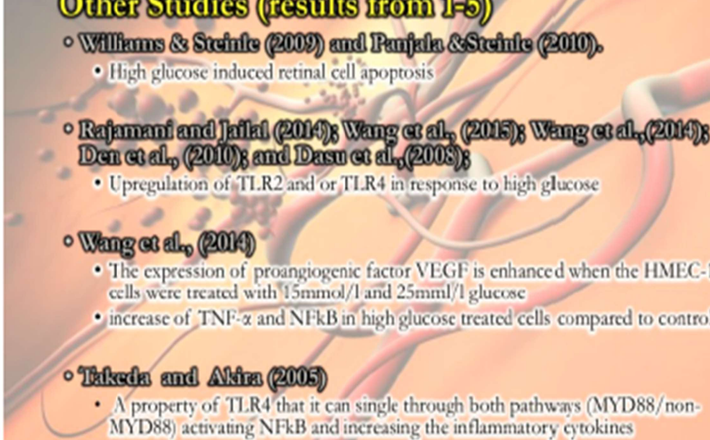

HRMECs morphology and cell count
confluent ~ 55% to 65%.
100% confluency
Trypsinized cells
Average cell size ~ 12-14μm
10x

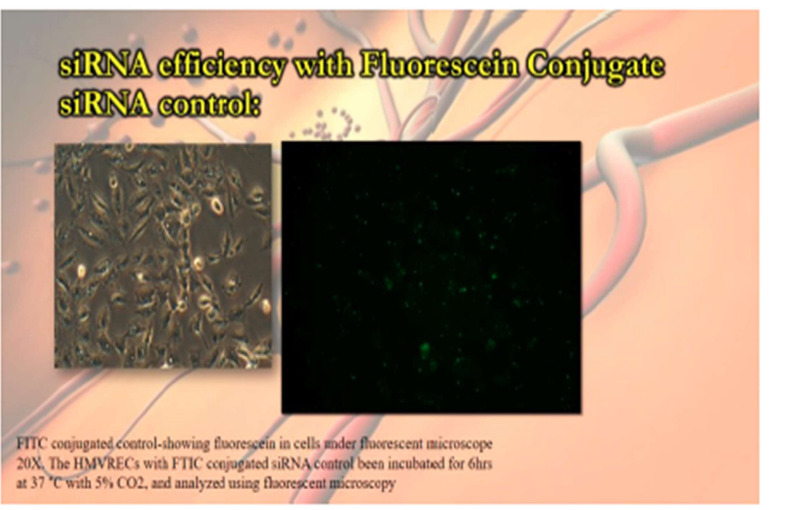

2-High Glucose Induces TLR4 mRNA expression:
• TLR4: CN vs HG 1.55 folds increase (1 (0) vs 2.556 (0.49)) with p < 0.001
• TLR2: CN vs HG 4.85 folds increase (1 (0) vs 5.85 (2.35)) with p < 0.001
GroupA TLR4
GroupA TLR2
Fold change
1.55 fold
4.85 fold
CN
HG
M
Treatment

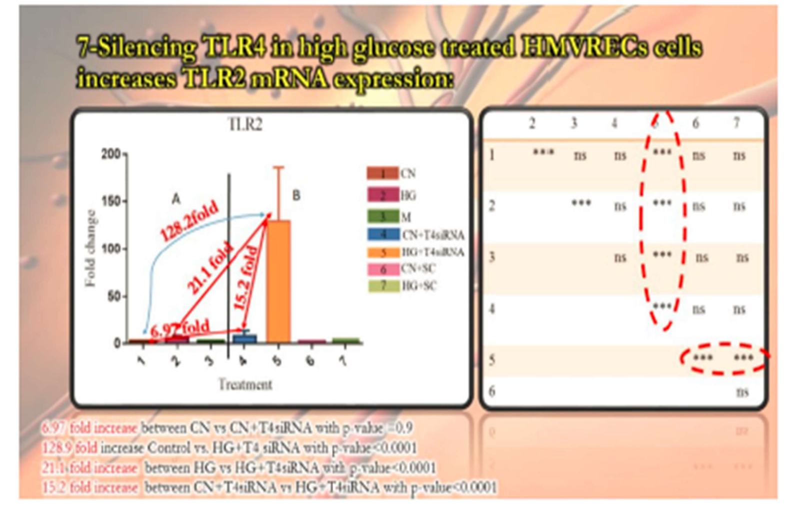

3-High Glucose Induces VEGFA mRNA expression:
• CN vs HG 7.83 folds increase (1 (0) vs 8.83 (07.03)) with p < 0.0155
GroupA VEGFA
a
p < 0.015
*
7.83 fold
Fold change
20
15
10
5
0
1
2
3
Treatment
1 CN
2 HG
3 M

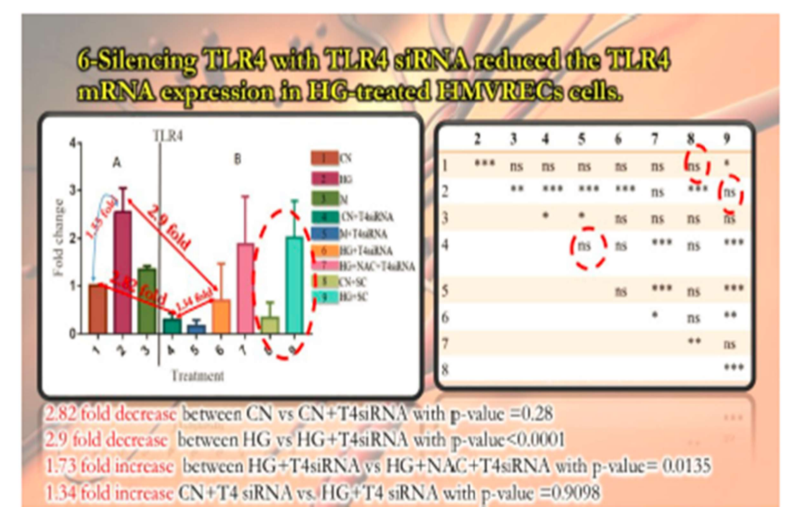

5-Increased TNF-α (inflammatory cytokine) production by HMVRECs cells in response to high glucose:
• TNF-a:
CN vs HG 0.25 fold increse (1.07(0.009) vs 1.14(0.03))
HG vs HG+NAC 0.21 fold decreae 1.14(0.03) vs 1.08(0.009))
Group A-TNF-a
**
**
Pg/ml
1.5
1.0
0.5
0.0
1
2
3
4
Treatment
1 CN
2 HG
3 M
4 HG+NAC

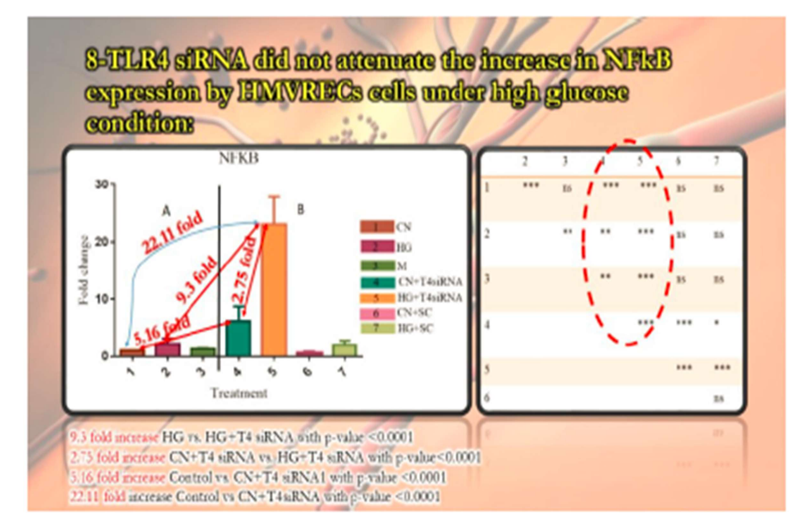

4- TLR4 activation results in the downstream signaling and activation of pro-inflammatory NFkB
• CN vs GH 1.25 fold increase (1(0) vs 2.25(0.91) ) with p < 0.001
GroupA NFKB
***
1.25 fold
Fold changes
4
3
2
1
0
1
2
3
Treatment
1 CN
2 HG
3 M

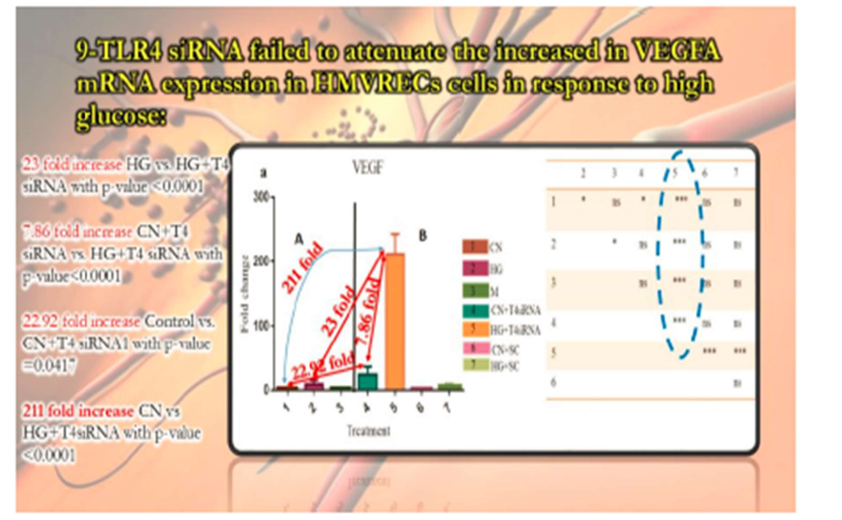

Other Studies (results from 1-5)
• Williams & Steinle (2009) and Panjala &Steinle (2010).
• High glucose induced retinal cell apoptosis
• Rajamani and Jailal (2014); Wang et al., (2015); Wang et al.,(2014); Den et al., (2010); and Dasu et al.,(2008);
• Upregulation of TLR2 and or TLR4 in response to high glucose
• Wang et al., (2014)
• The expression of proangiogenic factor VEGF is enhanced when the HMEC-1 cells were treated with 15mmol/l and 25mml/l glucose
• increase of TNF-α and NFkB in high glucose treated cells compared to control.
• Takeda and Akira (2005)
• A property of TLR4 that it can single through both pathways (MYD88/non-MYD88) activating NFkB and increasing the inflammatory cytokines

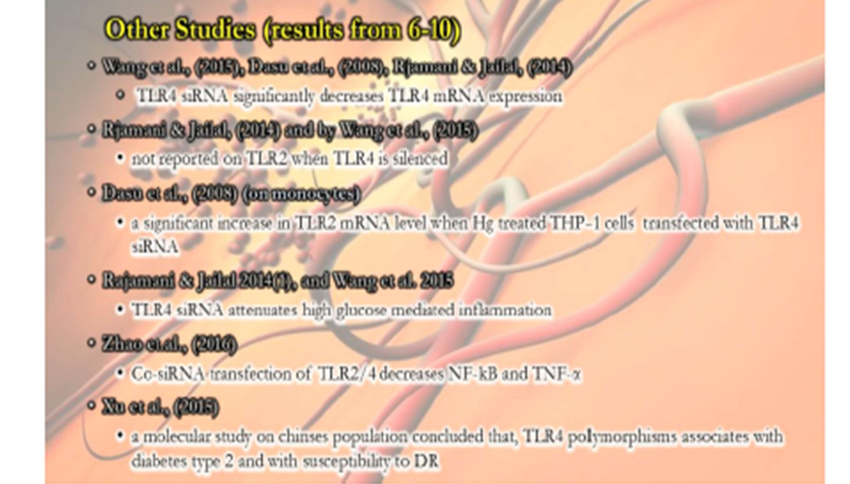

siRNA efficiency with Fluorescein Conjugate siRNA control:
FITC conjugated control-showing fluorescein in cells under fluorescent microscope 20X. The HMVRECs with FTIC conjugated siRNA control been incubated for 6hrs at 37 °C with 5% CO2, and analyzed using fluorescent microscopy

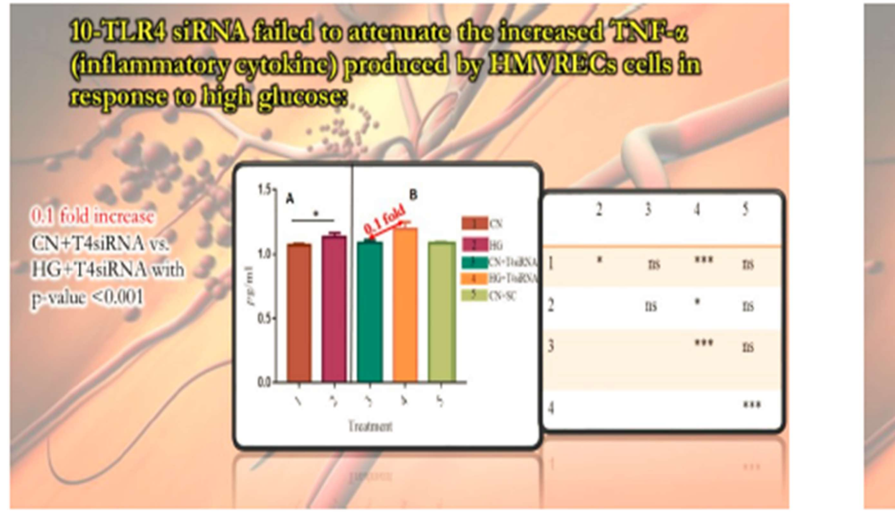

7-Silencing TLR4 in high glucose treated HMVRECs cells increases TLR2 mRNA expression:
TLR2
Fold change
Treatment
128.2fold
21.1 fold
15.2 fold
6.97 fold
CN
HG
M
CN+T4siRNA
HG+T4siRNA
CN+SC
HG+SC
6.97 fold increase between CN vs CN+T4siRNA with p-value =0.9
128.9 fold increase Control vs. HG+T4 siRNA with p-value<0.0001
21.1 fold increase between HG vs HG+T4siRNA with p-value<0.0001
15.2 fold increase between CN+T4siRNA vs HG+T4siRNA with p-value<0.0001

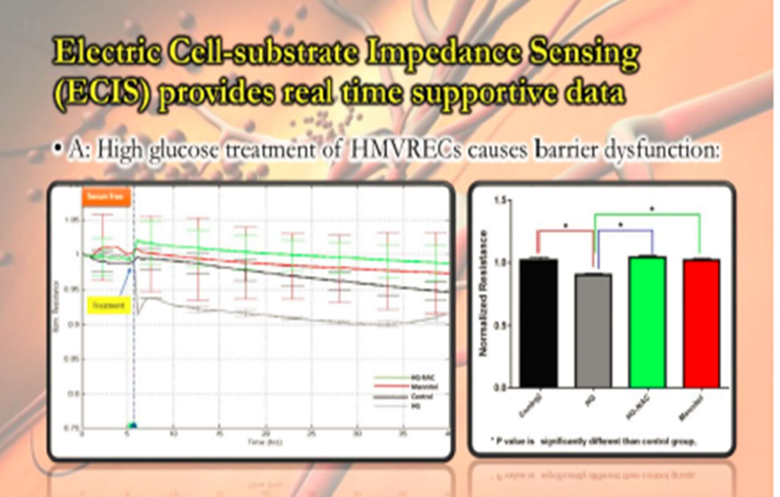

6-Silencing TLR4 with TLR4 siRNA reduced the TLR4 mRNA expression in HG-treated HMVRECs cells.
TLR4
Fold change
Treatment
2.9 fold
CN
HG
M
CN+T4siRNA
M+T4siRNA
HG+T4siRNA
HG+NAC+T4siRNA
CN+SC
HG+SC
2.82 fold decrease between CN vs CN+T4siRNA with p-value =0.28
2.9 fold decrease between HG vs HG+T4siRNA with p-value<0.0001
1.73 fold increase between HG+T4siRNA vs HG+NAC+T4siRNA with p-value= 0.0135
1.34 fold increase CN+T4 siRNA vs. HG+T4 siRNA with p-value =0.9098

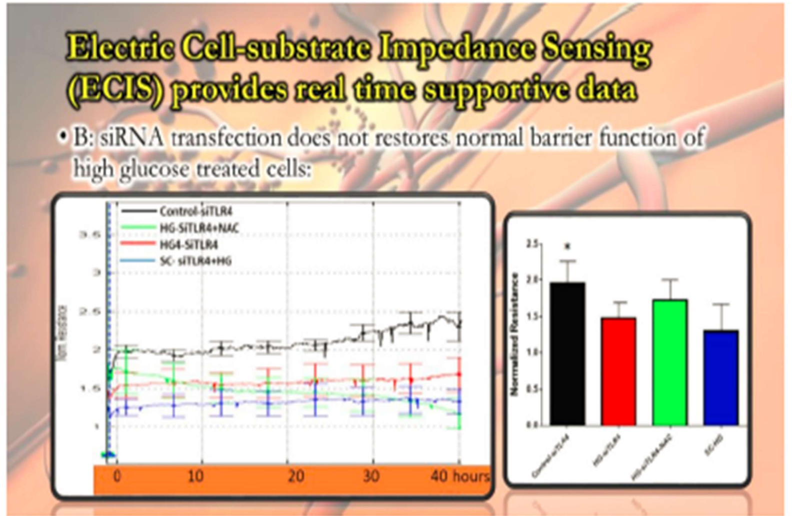

8-TLR4 siRNA did not attenuate the increase in NFkB expression by HMVRECs cells under high glucose condition:
NFKB
Fold change
Treatment
22.11 fold
9.3 fold
2.75 fold
5.16 fold
CN
HG
M
CN+T4siRNA
HG+T4siRNA
CN+SC
HG+SC
9.3 fold increase HG vs. HG+T4 siRNA with p-value <0.0001
2.75 fold increase CN+T4 siRNA vs. HG+T4 siRNA with p-value<0.0001
5.16 fold increase Control vs. CN+T4 siRNA1 with p-value <0.0001
22.11 fold increase Control vs CN+T4siRNA with p-value <0.0001

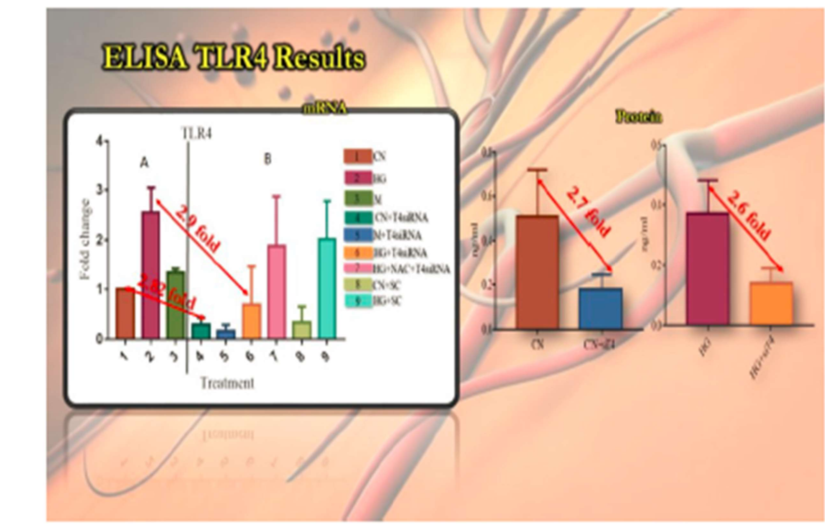

9-TLR4 siRNA failed to attenuate the increased in VEGFA mRNA expression in HMVRECs cells in response to high glucose:
23 fold increase HG vs. HG+T4 siRNA with p-value <0.0001
7.86 fold increase CN+T4 siRNA vs. HG+T4 siRNA with p-value<0.0001
22.92 fold increase Control vs. CN+T4 siRNA1 with p-value =0.0417
211 fold increase CN vs HG+T4siRNA with p-value <0.0001
VEGF
211 fold
23 fold
7.86 fold
22.92 fold
Fold change
Treatment
CN
HG
M
CN+T4siRNA
HG+T4siRNA
CN+SC
HG+SC

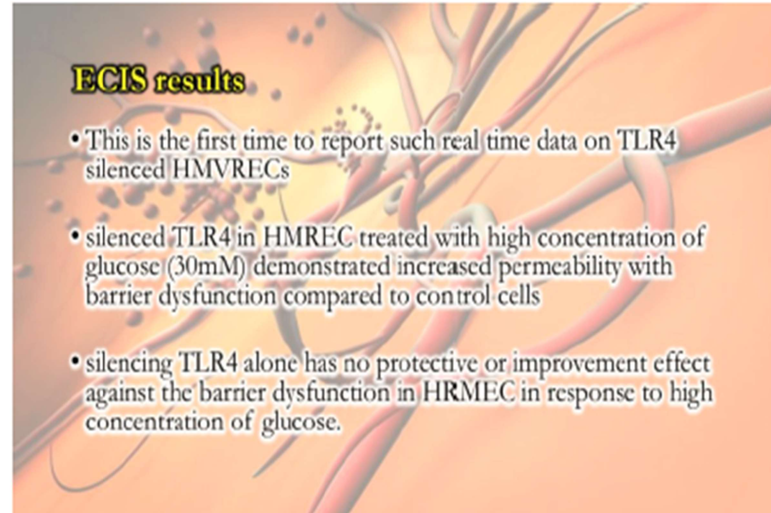

Other Studies (results from 6-10)
Wang et al., (2015), Dasu et al., (2008), Rjamani & Jailal, (2014)
TLR4 siRNA significantly decreases TLR4 mRNA expression
Rjamani & Jailal, (2014) and by Wang et al., (2015)
not reported on TLR2 when TLR4 is silenced
Dasu et al., (2008) (on monocytes)
a significant increase in TLR2 mRNA level when Hg treated THP-1 cells transfected with TLR4 siRNA
Rajamani & Jailal 2014(1), and Wang et al. 2015
TLR4 siRNA attenuates high glucose mediated inflammation
Zhao et.al., (2016)
Co-siRNA transfection of TLR2/4 decreases NF-kB and TNF-α
Xu et al., (2015)
a molecular study on chinses population concluded that, TLR4 polymorphisms associates with diabetes type 2 and with susceptibility to DR


10-TLR4 siRNA failed to attenuate the increased TNF-α (inflammatory cytokine) produced by HMVRECs cells in response to high glucose:
0.1 fold increase
CN+T4siRNA vs. HG+T4siRNA with p-value <0.001
0.1 fold
pg/ml
Treatment
CN
HG
CN+T4siRNA
HG+T4siRNA
CN+SC


Electric Cell-substrate Impedance Sensing (ECIS) provides real time supportive data
A: High glucose treatment of HMVRECs causes barrier dysfunction:
Normalized Resistance
* P value is significantly different than control group.


Electric Cell-substrate Impedance Sensing (ECIS) provides real time supportive data
• B: siRNA transfection does not restores normal barrier function of high glucose treated cells:
Control-siTLR4
HG-SiTLR4+NAC
HG4-SiTLR4
SC- siTLR4+HG
0
10
20
30
40 hours
Normalized Resistance


ELISA TLR4 Results
mRNA
TLR4
A
B
Fold change
Treatment
2.9 fold
2.82 fold
Protein
2.7 fold
2.6 fold
CN
HG


ECIS results
• This is the first time to report such real time data on TLR4 silenced HMVRECs
• silenced TLR4 in HMREC treated with high concentration of glucose (30mM) demonstrated increased permeability with barrier dysfunction compared to control cells
• silencing TLR4 alone has no protective or improvement effect against the barrier dysfunction in HRMEC in response to high concentration of glucose.

Conclusion

## Major Findings and Conclusion

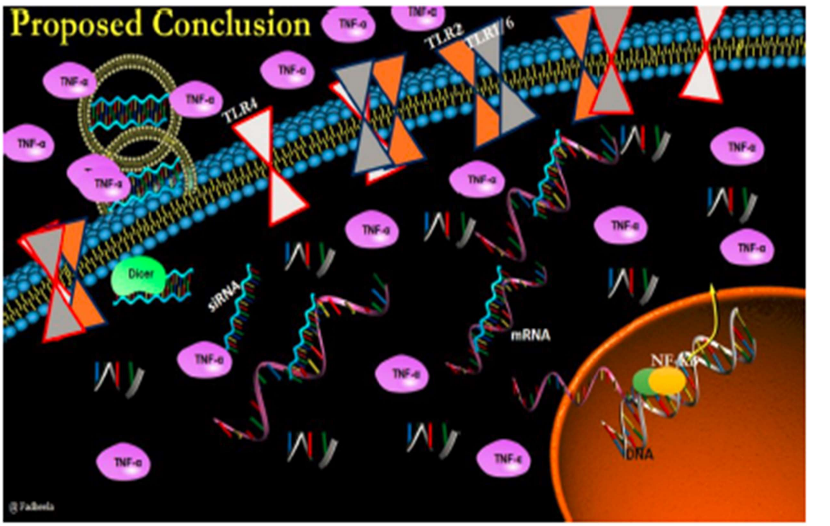
1. HG increased cell apoptosis which was decreased near normal after treatment with antioxidant
2. HG induced TLR4 expression and induced,TLR2, NFkB, VEGFA and TNF-α
3. siRNA Transfection decreased expression TLR4 ~72% mRNA, 67% protein
4. silencing TLR4 in HG treated cells increased the expression of TLR2, NFkB, VEGFA and TNF-α
5. silencing TLR4 alone has no protective or improvement effect against the barrier dysfunction in HRMEC in response to HG
6. TLR4 alone is not enough to cause pathogenesis of DR and further mechanistic studies in vivo are needed.

## Limitations

- Use of only on method for knocking out the expression of TLR4(siRNA)
- Inability to to assess other proinflammatory cytokines like IL-1β, IL-6, IL-8 and chemokines and adhesion molecules like ICAM-1 and VCAM-1

## Future Directions

- Assess IL-1β, IL-6, IL-8, ICAM-1 and VCAM-1
- Studies of TLRs pathway in relation to DR (MYD88)
- Use of in vivo animal model would help to understand the role of TLRs in DR with more details


Proposed Conclusion
TLR4
TLR2
TLR1/6
TNF-α
Dicer
siRNA
mRNA
DNA

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College of Health Sciences
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Dr. Christopher Triggle
Dr. Shahrad Taheri
Ms.Mooza alkhinji
Dr.Abdulrahman aljamal

Acknowledgment

Thank You
Q?

Appendix E: Curriculum vitae

Name : Fadheela Dad Bakhsh Mohammed

Nationality : Pakistani

Place of Birth : Doha-Qatar

Address : P.O. Box 5415, Doha, Qatar

Mobile : +974 66245334

Email : fadheela@qu.edu.qa ; fadheela55@outlook.com

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Education:

High school certificate 94.5% 2008

Bachelor in biomedical science from Qatar University GPA 3.54 Spring 2013

Attended the "First Regional Nutrition Conference: Nutritional Challenges in the East Mediterranean Region" held at Qatar university on March 2011

Basic Life Support course (BLS) in HMCMarch 2013

Co-investigator in Graduation Project entitled" association of single nucleotide polymorphisms with coronary artery disease in Qatar" between Qatar university and HMC from 2011-2013 [UREP 10-030-3-007]

Attended the "Aging and Health forum" held at Qatar University on April 2012

Attended the "First Aid lecture" on March 2011

Attended the reorientation session on Hazardous Material and Waste Management in HMC 2013

Attended the reorientation session on Fire Safety in HMC on February 2013

Completed the Hamad Medical Corporation Corporate Orientation Program on February 2013 Laboratory work skills in Qatar University Laboratories and five month training course in HMC laboratories

Attended the CAS safety awareness week workshop started 23rd of February 2014

Completed a course in SPSS program on March 2014

Attended the Biological safety principles and practices form at HMC on May 2014

Attended Spill kit training on 25th Jun 2014

Attended Epidemics Preparedness and Hajj Symposium on 30th August 2014

Completed IATA course for dangerous goods transport on December 2014 [certified dangerous goods shipper]

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Experience:

Volunteered in Qatar University in swine flu campaign in 2009

Volunteered in Qatar University in the organization of the Science collage open day on May 2010

Participated in the scientific day of college of art and science QU 2011

Participated in the" World AIDS Day 2011: Stigma and discrimination in health care settings" held at Qatar university on December 2011

Participated in the scientific day of college of art and science QU 2013

Participated in the First Youth Scientific Forum 2013

Participated in Junior Scientists Symposium 2013

Worked onwith EZ1 advanced instrument for blood DNA extractionand on real time PCR applied bio-system 7500 [allelic discrimination] on 4 SNPs[UREP 10-030-3-007] and additional9 different SNPs with Dr. Nasser Rizk

Collected blood samples from Heart Hospital and Hamad outpatient children clinic [personal interaction with patients]

Participated with poster presentation in Qatar Foundation Annual Research Conference 2013[ARC'13. Citation: Fadheela Bakhsh. (2013). Associations Of Single Nucleotide Polymorphisms(Rs2383207) On Chromosome 9P21 With Coronary Artery Diseases In Qatar. Qatar Foundation Annual Research Forum Proceedings: Vol. , BIOSP 035. DOI: 10.5339/qfarf.2013.BIOSP-035

[Research assistant]Worked on 150 samples with flow cytometer on a project entitled "Immunophenotyping of Peripheral Blood Lymphocyte Subsets in Healthy Qatari Adults" 2013

[Research assistant]Working as research assistant in project "Gold Nanopartical-Based Assays for Direct and Cost Effective Detection of High Burden Infections"[NPRP 4-1215-3-317], as collected 100 HCV samples for RNA extraction and 110 TB samples for DNA extraction; optimizing a gold nanopartical assay for detection of both HCV and TB; September 2013- September 2015

Laboratory technician in Qatar University Biomedical Research center Science October 2015

Gave Oral presentation in the HMC Fifth Assembly of the Research Forum on Diabetes, Obesity and Metabolism toke place on 26th of May 2016, by the Metabolic Institute in Qatar.

Poster Presentation in Qatar University research forum 2016

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Awards:

Enrolled in the dean list college of art and science Spring 2011

Enrolled in the dean list college of art and science Spring 2012

Enrolled in the dean list college of art and science Spring 2013

Won the 1stprize in First Youth Scientific Forum 3013

Won the 1stprize in Junior Scientists Symposium 2013

Won the best poster award for graduate poster in Qatar university annual research forum 2016 toke place on 3rd of May 2016

Won the 1st prize in the first annual Health Research Retreat of Qatar University (HRR/QU)

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Publication:

The cardiovascular implication of single nucleotide polymorphisms of chromosome 9p21 locus among Arab population" Journal of Research in Medical Sciences.[pubmed]

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Languages:

Fluent in English, Arabic and Urdu/Hindi oral and written

Fluent in Baluchi (native language)

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Extra Perfection:

Assimilative and good interpersonal skills

Ability to work under pressure

General computer skills,Proficient in Word, PowerPoint, Excel and can solve minor computer software problems

Excellent written and verbal communication skills

Ability to work alone and in team environment

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Hobbies:

Reading,navigatingInternet, volunteering, seeking more computer skills and learning new languages

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