ROLE OF ENDOPLASMIC RETICULUM STRESS, OXIDATIVE STRESS AND INFLAMMATION ON EARLY AORTIC VASCULOPATHY OF TYPE 2 DIABETIC RAT MODEL

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by

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LIST OF SYMBOLS

α	Alpha
β	Beta
°	Degree
°C	Degree Celsius
±	Plus-minus
<	Less than
≥	Greater than or equal to
μL	Microliter
mL	Milliliter
Kg	Kilogram
G	Gram
mg/mL	Milligram/milliliter
ng/ml	Nanograms per millilitre
Pg/mL	Picogram/milliliter
pmol/ml	Picomoles per milliliter
mg/kg	Milligram/kilogram
%	Percentage
nmol/mg	Nanomoles/milligram
mmol/L	Millimoles/liter
mL/kg	Milliliter/kilogram
w/v	Weight/volume
Da	Dalton
KDa	Kilo-Dalton
M	Meter

O_2	Oxygen
CO_2	Carbon dioxide

LIST OF ABBREVIATIONS

ACh	Acetylcholine
Akt	Protein Kinase B
ANOVA	Analysis of variance
ATF-6	Activated transcription factor 6
ATP	Adenosine Triphosphate
BP	Blood Pressure
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CHOP	C/EBP homologous protein
cGMP	Cyclic guanosine monophosphate
COX	Cyclooxygenase
DM	Diabetes mellitus
ECD	Endothelial cell dysfunction
EDCF	Endothelium-derived contracting factor
EDHF	Endothelium-derived hyperpolarization factor
EDRF	Endothelium-derived relaxing factor
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ERS	Endoplasmic reticulum Stress
ET-1	endothelin-1
ETC	Electron transport chain
FAD	Flavin adenine dinucleotide
FBG	Fasting blood glucose

FMN	Flavin mononucleotide
HFD	High-fat diet
IDF	International Diabetes Federation
IL	Interleukins
IN	Insulin
IP_3	Inositol 1,4,5-triphosphate
IP_3R	Inositol 1,4,5-triphosphate receptor
IR	Insulin receptor
IRE-1α	Inositol-requiring kinase-1
IRS-1	Insulin receptor substrate-1
JNK	C Jun kinase
KCl	Potassium chloride
L-NAME	L-nitro-arginine methyl ester
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MLCP	Myosin light-chain phosphatase
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κβ	Nuclear factor kappa-light-chain-enhancer of activated
NO	Nitric oxide
ONOO-	Peroxynitrite
OX	Xanthine Oxidase
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PGI^2	Prostacyclin
PI3K	Phosphatidylinositol-3-kinase
PKC	Protein kinase C
PKG	Protein kinase G
PSS	Physiological saline solution

PVDF	Polyvinylidene difluoride
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SERCA	Sarcoplasmic/endoplasmic reticulum calcium ATPase
sGC	Soluble guanylyl cyclase
SOD	Superoxide dismutase
STZ	Streptozotocin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TBS	Tris Buffer Saline
TBST	Tris Buffer Saline/Tween-20
TNF-α	Tumor necrosis factor-alpha
TUDCA	Tauroursodeoxycholic acid
UDCA	Ursodeoxycholic acid
UPR	Unfolded protein response
USM	Universiti Sains Malaysia
VSMC	Vascular smooth muscle cell

LIST OF APPENDICES

PERANAN TEKANAN RETIKULUM ENDOPLASMA, TEKANAN OKSIDATIF DAN KERADANGAN KE ATAS VASKULOPATI AORTA PERINGKAT AWAL PADA MODEL TIKUS DIABETES JENIS 2

ABSTRAK

Tekanan retikulum endoplasma menyumbang kepada keadaan patologi lain seperti rintangan insulin, komplikasi makro dan mikrovaskular yang berkaitan dengan diabetes. Matlamat kajian ini adalah untuk menilai kesan tekanan retikulum endoplasma, rintangan insulin endothelial, tekanan oksidatif dan keradangan ke atas vaskulopati peringkat awal di dalam aorta tikus diabetes jenis 2. Tikus jantan jenis Sprague-Dawley berumur 8 – 10 minggu, dengan berat kira-kira 250 hingga 300 g telah digunakan untuk kajian ini. Tikus dibahagikan kepada dua kumpulan: kumpulan kawalan (n= 9) dan kumpulan diabetes (n= 18). Kumpulan diabetes diberikan diet tinggi lemak (HFD) selama 4 minggu. Mereka kemudiannya disuntik secara intraperitoneum (i.p) dengan satu dos 40 mg/kg streptozotocin yang dilarutkan dalam penimbal natrium sitrat 0.1M pH 4.5. Tikus yang mempunyai paras glukosa darah berpuasa melebihi 11.1mmol/l dianggap menghidap diabetes. Tikus kawalan diberikan diet biasa dan disuntik dengan isipadu natrium sitrat yang sama. Tekanan darah dipantau dalam keadaan hangat, sedar dan terkawal menggunakan kaedah kuf ekor bukan invasif melalui sistem pemantauan tekanan darah. Pada minggu ke-13, tikus diabetes dibahagikan secara rawak kepada dua kumpulan: tikus diabetes (n= 9) dan tikus diabetes yang menerima asid tauroursodeoxycholic (TUDCA) 150 mg/kg/hari secara i.p selama dua minggu (n= 9). Pada minggu ke-15, semua tikus dimatikan menggunakan campuran ketamin (300 mg/kg, i.p) dan xylazine (30 mg/kg, i.p). Selepas dimatikan, aorta diasingkan dan diletakkan di dalam alatan rendaman organ

untuk menentukan pengenduran berperantara- asetilkolin dan insulin serta mekanisma pengenduran dengan menggunakan perencat farmakologi. Penanda tekanan retikulum endoplasma, pengawalatur dan protein yang terlibat di dalam isyarat laluan pengeduran telah dinilai menggunakan Western blotting. Di dalam aorta tikus kawalan, pengenduran-berperantara asetilkolin dan insulin adalah melalui pengaktifan isyarat laluan eNOS/PI3K, NAD(P)H oksidase dan saluran K_{ATP}. Di dalam tikus diabetes, pengenduran-berperantara asetilkolin melalui pengaktifan laluan eNOS/PI3K dan NAD(P)H oksidase, manakala pengenduran-berperantara insulin melalui laluan eNOS/PI3K sahaja. Menariknya, rawatan dengan TUDCA ke atas tikus diabetes menunjukkan bahawa pengenduran-berperantara asetilkolin dan insulin dipulihkan melalui pengaktifan isyarat laluan eNOS/PI3K, NAD(P)H oksidase dan saluran K_{ATP}. Kajian fungsian ini disokong oleh peningkatan ekspresi protein vaskular (IRS-1, Akt dan eNOS) dan juga penurunan protein penanda tekanan retikulum endoplasma (IRE-1, BiP dan PERK) di dalam tikus diabetes yang menerima TUDCA. Oleh itu, data ini mencadangkan bahawa perencatan tekanan retikulum endoplasma boleh menjadi sasaran yang berpotensi untuk rawatan vaskulopati di dalam diabetes jenis 2.

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ABSTRACT

Endoplasmic reticulum (ER) stress contributes to other pathological conditions such as insulin resistance, macro- and microvascular complications associated with diabetes. The aim of this study was to elucidate the role of ER stress, endothelial insulin resistance, oxidative stress and inflammation on early vasculopathy in aorta of type 2 diabetic rats (T2DM). The male Sprague-Dawley rats of 8 – 10 weeks old, weighing about 250 to 300 g were employed for this study. The rats were divided into two groups: control group (n= 9) and diabetic group (n= 18). The diabetic group were placed on high-fat diet (HFD) for 4 weeks. They were then injected intraperitoneally (i.p) with 40 mg/kg of single dose streptozotocin dissolved in 0.1 M sodium citrate buffer of pH 4.5. Rats with fasting blood glucose greater than 11.1 mmol/l were considered to be diabetic. The control rats were on the normal diet and injected with equal volume of sodium citrate. Blood pressure was monitored in a warm, conscious and restrained state using the non- invasive tail-cuff method via a blood pressure monitoring system. At the 13th week, the diabetic rats were randomly divided into two groups: diabetic rats (n= 9) and diabetic rats receiving tauroursodeoxycholic acid (TUDCA) 150 mg/kg/day i.p for two weeks duration (n= 9). At the 15th week, all rats were sacrificed using a mixture of ketamine (300 mg/kg, i.p) and xylazine (30 mg/kg, i.p). After sacrifice, aortas were isolated and mounted on the organ bath to determine acetylcholine- and insulin- mediated relaxations as well as the relaxation mechanisms using pharmacological inhibitors. ER stress marker, regulators and associated

relaxation signaling pathways proteins were assessed using Western blotting. In the aorta of control rats, acetylcholine- and insulin-mediated relaxation through activation of eNOS/PI3K, NAD(P)H oxidase and K_{ATP} channels pathways. In diabetic rats, acetylcholine- mediated relaxation through activation of eNOS/PI3K and NAD(P)H oxidase pathways, whereas insulin-mediated the relaxation through eNOS/PI3K pathway only. Interestingly, treatment with TUDCA in diabetic rats showed that the relaxation mediated by acetylcholine and insulin were reverted through activation of eNOS/PI3K, NAD(P)H oxidase and K_{ATP} channels pathways. The functional study was supported with an enhanced expression of vascular proteins (IRS-1, Akt and eNOS) with corresponding downregulation of ER stress proteins (IRE-1, BiP and PERK) in the diabetic rats receiving TUDCA. Therefore, these data suggested that the inhibition of ER stress could be a potential target for the management of T2DM vasculopathy.

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Diabetes mellitus (DM) is a chronic metabolic disturbance that arises from increased blood glucose, such that the body is unable to synthesize any insulin or release sufficient insulin or utilize the insulin (IDF, 2019). Insulin is a macromolecule (hormone) that is produced in the pancreas. It enables circulating glucose to reach the cells of the body (Viegas and Neuhauss, 2021). Also, insulin plays a vital role in protein and fat metabolism. Insulin deficiency, or cells failure to appropriately respond to insulin, contributes to elevated blood glucose levels, which is a sign of diabetes. Deficiency of insulin for a long-term might leads to diabetes complications like nephropathy, retinopathy, neuropathy and cardiovascular diseases. There is a greater risk of morbidity and mortality in individuals with diabetes relative to the overall population (Asrafuzzaman *et al.*, 2017). In recent years, the global incidence of diabetes in adults has been growing.

The global incidence of diabetes was anticipated at 151 million in 2000, 194 million in 2003, 246 million in 2006, 285 million in 2009, 366 million in 2013, 382 million in 2011, 415 million in 2015, 425 million in 2017 and 463 million in 2019 (IDF, 2019). It was projected by 2045 about 700 million people would be affected by diabetes. The prevalence of diabetes diagnosed in adults in Malaysia is 7.2% in 2011, 8.3% in 2015 and 9.4% in 2019 (MHM, 2019). The age range of diagnosed adults from 40 to 49 years has the highest prevalence of diabetes in Malaysia at 12.4% (MHM, 2019). It is an indication that if diabetes is not put under control, it will lead to a severe global health crisis, if not pandemic.

Vasculopathy refers to any disease conditions that affect the blood vessels while early vasculopathy refers to the initial signs of any disease to the blood vessels. The hallmarks of early vasculopathy are early endothelial cell apoptosis, reduced nitric oxide (NO) bioavailability and vascular leakage (Bruni *et al*., 2018). During the course of the disease, vascular leakage is an early event that causes one of the earliest signs (vascular tone failure, persistent tissue hypoxia and diminished capillary blood flow) (Bruni *et al*., 2018; Matucci-Cerinic *et al*., 2017). Also, early vasculopathy is accompanied by increased production of pro-angiogenic factors and endothelial cell dysfunction (ECD) (Matucci-Cerinic *et al*., 2017).

Diabetic-vasculopathy is a disorder triggered by increased blood glucose levels in the vasculatures that results in organ-specific complications. In diabetes, organ-specific vascular complications include microvascular and macrovascular complications. Microvascular complications in diabetic-vasculopathy include retinopathy, neuropathy and nephropathy, while macrovascular complications in diabetic-vasculopathy include peripheral disease, ischemic heart disease, atherosclerosis, cerebrovascular disease (Forbes and Cooper, 2013). Hyperglycemia contributes to the development of reactive oxygen species (ROS) involved in the pathophysiological process of vascular complications (Fiorentino *et al*., 2013; Hammes, 2018).

The endothelium is a cell layer that protects the walls of all vasculatures. They run from the heart to the capillaries. In adults, endothelial cells have around 10^{13} cells spanning about 4000 m^2 of surface area (Jamwal and Sharma, 2018; Zhang *et al*., 2018). The endothelium was first regarded as an inert barrier that surrounds all vasculatures. In fact, systematic research has led to a breakthrough in the dynamic functions and their roles in maintaining cardiovascular stability. The vascular-

endothelium performs functions like maintaining vascular tone, coagulation, development of new vasculature, and inflammation (Endemann and Schiffrin, 2004). It is now defined as a fundamental unit in the endocrine system rather than viewing it as an inert barrier (Maamoun *et al*., 2019a). The ensdothelial cells are known to produce many vascular mediators like endothelium-derived relaxing factor (EDRF), endothelium-derived contracting factor (EDCF), and endothelium-derived hyperpolarizing factor (EDHF) to maintain the haemodynamic of the vasculature. EDRF exist in the form of nitric oxide (NO) that are produced by endothelial nitric oxide synthase (eNOS). NO diffuses into smooth muscle cells to stimulate soluble guanylate cyclase and enhance cyclic guanylate monophosphate in the smooth muscle to cause vascular relaxation.

Endothelium impairment refers to the imbalance between EDRF and EDCF or reduced bioavailability of NO (Jamwal and Sharma, 2018). The endothelium impairment might also be due to ROS's excessive production, enhanced generation of growth factor, adhesion molecules, amplified thrombosis and alters fibrinolysis, increased production of proinflammatory cytokines and chemokines. eNOS uncoupling or enhanced breaking down of NO by superoxide anion/NO reduction is among the early signs of endothelial cells impairments (Cade, 2008; Incalza *et al*., 2018). Superoxide interacts with NO to produce peroxynitrite (ONOO-) (Landmesser *et al*., 2003; Potenza *et al*., 2009; Wolin *et al*., 2011). ONOO- facilitates nitration of proteins, leading to endothelial cell impairment and apoptosis (Mathews and Berk, 2008; Radi, 2004). The generation of superoxide anion is achieved by enzymes like NADPH oxidases (NOX), mitochondria, xanthine oxidase (OX), uncoupled eNOS, cytochrome P-450 oxygenase and cyclooxygenase (COX). The increased production of superoxide anion by these sources will profoundly affect vascular oxidative stress.

Superoxide anion is the first ROS to be formed during endothelial cell impairment, and it is responsible for the generation of other products like hydrogen peroxide.

Preclinical animal studies that appropriately reflect the pathogenesis of human illness like diabetes are essential because of their nature (Podell *et al*., 2017). Many animal studies used toxic chemicals to induce diabetes. These toxic chemicals like streptozotocin (STZ) generate free radical species that damage the beta cells in the pancreas. STZ is the most common chemical used in the development of diabetes in animal studies. A moderate dose of STZ induces a minor deficiency in insulin production, similar to the final phases of type 2 diabetes (T2DM) (Reed *et al*., 2000). An animal study that put together both a high-fat diet (HFD) and a moderate dose of STZ will cause peripheral insulin dysfunction and beta-cell failure. These conditions will excellently mimic T2DM's pathogenesis in human (Asrafuzzaman *et al*., 2017; Reed *et al*., 2000). Insulin dysfunction, hyperinsulinemia, dyslipidaemia and obesity are produced by animals treated with HFD (Eleazu *et al*., 2013). HFD can lead to attenuation of autophosphorylation of the insulin receptor, insulin receptor substrate-1 (IRS-1), phosphatidylinositol-3-kinase (PI3K), and protein kinase B (Akt) (Grundleger and Thenen, 1982; Kim *et al*., 2000; Youngren *et al*., 2001; Zierath *et al*., 1997). Also, HFD causes inflammatory genes to be overexpressed, decrease gene expression of liposynthetic and protein uncoupling (Moraes *et al*., 2003). HFD induces insulin resistance and facilitates the development of T2DM via impairment of the IRS/PI3K/Akt cascade, which modifies insulin action in target tissues (Kim *et al*., 2000; Youngren *et al*., 2001; Zierath *et al*., 1997).

Diabetes, endothelial impairment, and cardiovascular disorders, have been reported to be related to prolonged endoplasmic reticulum (ER) stress activation (Maamoun *et al*., 2019a). Prolonged ER stress may occur in diabetes due to an increase

in blood glucose in the vasculature, which amplified the demand for protein production (Flamment *et al.*, 2012; Hu *et al*., 2017). These rise protein load on the ER, which increases the risk of unfolded or misfolded protein accumulation in the ER lumen causing mispairing of cysteine residues in the process of protein production (Lisa *et al*., 2012). This will activate a coping mechanism called unfolded protein response (UPR), which tries to address the problem via the three canonical arm sensors [Inositol-requiring kinase 1α (IRE1α), protein kinase-like ER kinase (PERK) and activating transcription factor 6 (ATF6)] and binding immunoglobulin protein (BiP) that function closely with each other. These three canonical arm sensors activates processes like ER-associated degradation (ERAD), alters the rate of translation to delay the entry of new proteins into the ER lumen, amplified gene expression of molecular chaperones to facilitate and enhance the proper folding of proteins in order to reverse the condition (Flamment *et al*., 2012).

Prolonged ER stress can also contribute to endothelial cell impairment due to the accumulation of misfolded or unfolded proteins leading to insulin resistance and ROS generation. Insulin resistance is one of the vital factors for endothelial cell impairment in *in-vivo* and *in-vitro* studies (Cersosimo and DeFronzo, 2006; Duncan *et al*., 2008; Muniyappa and Sowers, 2013). Also, under insulin resistance conditions like hyperglycaemia and hyperinsulinaemia; they have been shown to upregulate ER stress and UPR (Boden *et al*., 2014; Ye, 2013). ER stress results in impairment of insulin signaling cascade (IRS/PI3K/Akt/eNOS) via the activation of proinflammatory cytokines (Amen *et al*., 2019) and increased ROS production (Qatanani and Lazar, 2007). The IRS-1 and IRS-2 receptors' ablation via the excessive expression of inflammatory cytokines leads to the attenuation of IRS/PI3K/Akt/eNOS with the outcome of insulin resistance. The UPR also produces ROS under prolonged ER stress

(Santos *et al*., 2009), indicating that ER stress is linked to endothelial cell impairment and NO concentration reduction (Dong *et al*., 2016). Therefore, insulin resistance mediates ER stress activation, but prolonged ER stress can enhance insulin resistance (Hummasti and Hotamisligil, 2010). ER stress mediates an increase in oxidative stress, but ROS stimulate ER stress (Malhotra and Kaufman, 2007). These indicate that a vicious cycle exists in the ER stress mechanisms involved in the development and progression of T2DM. ER stress has emerged as a powerful mediator of metabolic abnormalities in obesity and T2DM models in the last decade (Hotamisligil, 2010a).

Chemical chaperones aid in the refolding of misfolded or unfolded polypeptides by increasing the stability of native proteins. Chemical chaperones like tauroursodeoxycholic acid (TUDCA) and 4-phenylbutyric acid are the most promising chaperones in managing diseases. TUDCA is a secondary bile acid formed by bacteria in the intestine. TUDCA has been shown to have a beneficial impact on various diseases, such as diabetes, vascular diseases, neurodegenerative diseases, and osteoarthritis, according to recent research (Castro-Caldas *et al*., 2012; Cho *et al*., 2015; Liu *et al*., 2015; Zhou *et al*., 2016).

1.2 Justification of the Study

Diabetes mellitus is a serious health problem with increasing cases in Malaysia and worldwide. Diabetes in 2010 has affected around 285 million persons globally and is anticipated to rise by 1.5 fold (about 439 million persons) in 2030 (Shaw *et al*., 2010). There will be a 20% rise in persons or adults with diabetes in developed countries while a 69% rise in developing countries between 2010 to 2030 (Shaw *et al*., 2010).

Diabetes mellitus is associated with endothelial impairment, which stimulates the release of superoxide and activates ER stress, leading to increased insulin resistance and oxidative stress. Endothelial impairment leads to a hemodynamic imbalance between the EDRF and EDCF needed for the cell's normal physiological function (Kang, 2014). Recent studies have implicated ER stress as the new frontier mechanism in the initiation and progression of diabetes. Our study may provide a clearer understanding of ER stress's molecular mechanism in relation to vascular dysfunction and insulin signaling pathways (IRS/PI3K/Akt/eNOS) in a T2DM model. In our study, TUDCA, a human endogenous molecule also known as a chemical chaperone is used to access the mechanisms through which ER stress have an impact on the aorta of T2DM rats' model, which may leads to endothelial ECD. There have been no studies to date on the role of ER stress in early vasculopathy, oxidative stress and inflammation in the aorta of T2DM, necessitating this study.

1.3 Objectives

1.3.1 General Objective

To elucidate the role of ER stress on early vasculopathy, endothelial insulin resistance, oxidative stress and inflammation in aorta of T2DM rats.

1.3.2 Specific Objectives

1. To determine ER stress sensors expression (BiP, PERK and IRE-1 proteins) in the aorta of control, T2DM and TUDCA supplementation of T2DM (DMT).

2. To determine endothelium-dependent relaxation in the aorta of control and T2DM rats and the effect of ER stress inhibition on DMT.

3. To determine insulin-mediated relaxation in the aorta of control and T2DM rats and the effect of ER stress inhibition on DMT.

4. To determine the expression of IRS-1, Akt, eNOS proteins in the aorta of control and T2DM rats and the effect of ER stress inhibition on DMT.

5. To assess oxidative stress markers (SOD activity and MDA level) in the aorta of control and T2DM rats and the effect of ER stress inhibition on DMT.

6. To determine the level of inflammation markers (TNF-alpha and IL-6 levels) in the aorta of control and T2DM rats and the effect of ER stress inhibition on DMT.

7. To determine the effects of ER stress on food consumption, systolic blood pressure, body weight and fasting blood glucose changes on control, T2DM and DMT.

CHAPTER 2

LITERATURE REVIEW

2.1 The Vasculature

2.1.1 The Architecture of the Blood Vessel Wall

The peripheral vasculature is made up of all the blood vessels which are located outside of the heart (Mustapha *et al*., 2020). The peripheral vasculature is divided into the following categories: the aorta and branches, arterioles, capillaries, venules and veins. Each of the above sections of the peripheral vasculature has a different architecture and function based on which organ it serves. Blood vessels, with the exception of capillaries, are composed of three layers: tunica intima, tunica media and tunica adventitia (Figure 2.1) (Nathaniel *et al*., 2009). The tunica intima also called the tunica's inner layer, is made up of an endothelial lining that allows blood to flow freely. The adventitia is the vessel's outer layer, which provides structural support and form. The tunica media, also known as the middle layer, consists of elastic and muscular tissue that governs the vessel's inner diameter (Nathaniel *et al*., 2009). Based on the vessel's size and position, the amount of muscle and collagen fibrils in each layer differs. Arteries are responsible for providing blood and nutrients to tissues (Nathaniel *et al*., 2009). Since elastin is present in large blood vessels, it allows the blood vessels to expand and change in diameter (Nathaniel *et al*., 2009).

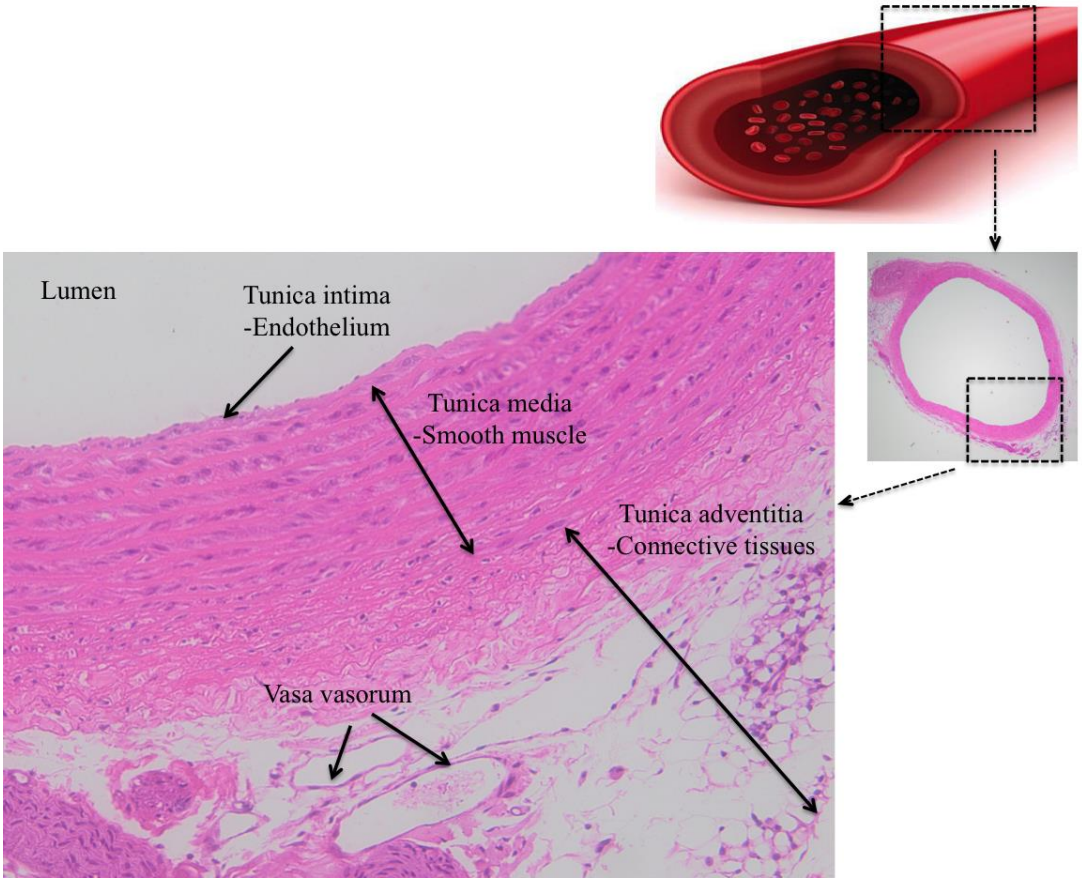

Figure 2.1 Classification and structure of blood vessel. It is modified from https://image.shutterstock.com/image-illustration/blood-vessel-sliced

2.1.2 The endothelium

The endothelium is a layer of cells that coats all blood vessel walls that run from the heart to the thinnest capillaries. For decades, the endothelium was viewed first as an inert barrier, but extensive studies led to a breakthrough in the dynamic roles and their function in sustaining cardiovascular stability. Endothelial cells have about 10^{13} cells in adult covering around 4000 m^2 surface area (Jamwal and Sharma, 2018; Zhang *et al.*, 2018). The endothelial cell is averagely around 20-40 uM long, 0.1- 0.5 uM thick, and 10-15uM in width (Cahill and Redmond, 2016). The endothelium plays an essential role in physiological and pathological conditions involving vasculogenesis, angiogenesis, inflammation, vascular permeability, and vascular tone (Endemann and Schiffrin, 2004). Endothelial cells have metabolic activity and

function like endocrine or paracrine glands. As a result, some vasoactive mediators such as EDRFs and EDCFs are released from endothelial cells to control vascular tone (Jamwal and Sharma, 2018). The EDRFs consist of NO, prostacyclin (PGI_2) and EDHFs, while the EDCFs consist of endothelin-1 (ET-1), ROS, angiotensin II (Ang II), prostaglandin H_2 and thromboxane A_2 (TxA_2) (Kang, 2014). Any conditions that alter these tightly controlled systems of EDFRs and EDCFs result in endothelial dysfunction.

2.1.2(a) Endothelium-derived relaxing factors

2.1.2(a)(i) Nitric oxide (NO)

NO is a lipid-permeable free radical molecule that is small, labile, and unstable. A series of studies published in the 1980s revealed NO's endogenous formation and its physiological importance as an EDRF (Furchgott and Zawadski, 1980). NO can regulate apoptosis and cellular differentiation, autonomic and central neural systems, genes expression and epigenetic outcome, immune responses and vascular tone (Ghimire *et al*., 2017; Kim *et al*., 2000; Malyshev *et al*., 1999; Moncada *et al*., 1991; Socco *et al*., 2017; Vanhoutte, 2018; Vasudevan *et al*., 2016; Zhou and Brune, 2005). NO prevents platelet activity and vascular smooth muscle cell proliferation, adhesion, and migration at the smooth muscle level, which lead to vasodilation, decreased systemic blood pressure and increased blood flow (Sato *et al*., 2008). NO is the major vasodilator agent and its production and bioavailability are essential for the normal function of endothelium (Roberts and Porter, 2013). However, the onset of vascular disorders such as diabetes, atherosclerosis, hypertension, and diabetes is signaled by a decreased in NO bioavailability.

2.1.2(a)(ii) The NO synthesis

The three different types of nitric oxide synthase (NOS) isozymes generate NO to carry out a wide range of biological functions. These three NOS isozymes include NOS-I;eNOS, inducible nitric oxide synthase (NOS-II; iNOS) and neuronal nitric oxide synthase (NOS-III; nNOS). The main subtype that controls vascular function is eNOS. Therefore, several triggers such as shear stress, acetylcholine, bradykinin, histamine, and 17β-estradiol, can activate eNOS and increase NO synthesis, both in calcium(Ca^{2+})-dependent as well as Ca^{2+}-independent ways (Bae *et al*., 2003; Kellogg *et al*., 2005; Kolluru *et al*., 2010; Miyazaki-akita *et al*., 2007; Mondillo *et al*., 2009). For example, acetylcholine bind to the M3 muscarinic receptor on the endothelial cells to amplified calcium concentration, which the latter then attaches to the calmodulin and results in the stimulation of the calmodulin-binding site on the eNOS to produce NO in the presence of co-factors such as tetrahydrobiopterin (4BH), flavin mononucleotide (FMN), nicotinamide adenine dinucleotide phosphate (NADPH) and flavin adenine dinucleotide (FAD) as depicted in Figure 2.2.

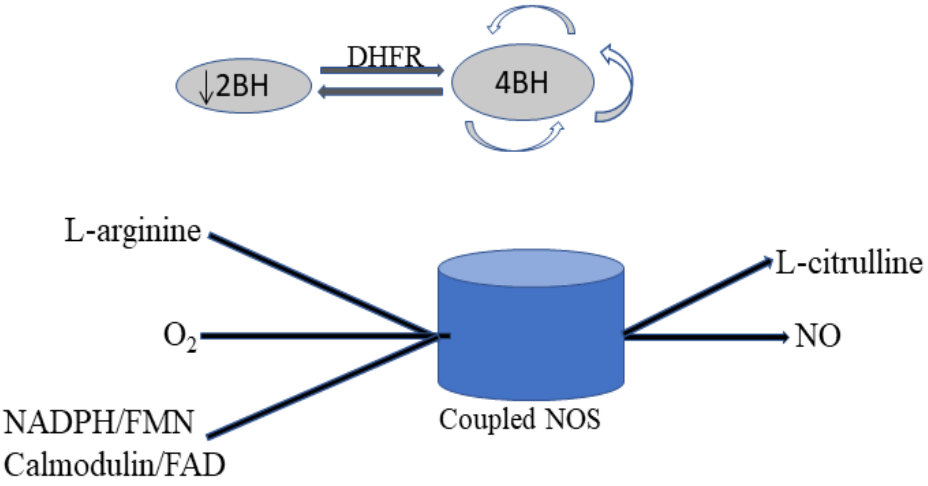

Figure 2.2 The synthesis of nitric oxide. Abbreviation: BH2: dihydrobiopterin; BH4: tetrahydrobiopterin; DHFR: dihydrofolate reductase; NO: nitric oxide; NOS: nitric oxide synthase; NADPH: nicotinamide adenine dinucleotide phosphate; FMN: flavin mononucleotide; FAD: flavin adenine dinucleotide

Within the physiological context, the expression of iNOS is low, but once there is overexpression of iNOS, this leads to NO indefinite production independent of Ca^{2+} concentration. Tumours, chronic inflammation and infection are the most common causes of iNOS induction (Kroncke *et al*., 1998). The expression of iNOS due to inflammation in the endothelium causes vascular dysfunction by restricting the bioavailability of 4BH (Gunnett *et al*., 2005).

nNOS generates NO in both the peripheral and central nervous systems, and it plays a role in cellular signaling (Toda *et al*., 2012; Toda and Nakanishi-toda, 2011). nNOS generates NO that is involved in controlling neuronal firing and excitability. The release of neurotransmitters like acetylcholine, serotonin and histamine are controlled by nNOS (Prast and Philippu, 2001; Straub *et al*., 2007).

2.1.2(a)(iii) The NO pathways

Substances such as acetylcholine and insulin binds their receptors on endothelial cells and activate NO pathway.

a) Acetylcholine-induced NO-mediated relaxation

Acetylcholine binds to M3 muscarinic receptors leads to the synthesis of NO, which is catalysed by eNOS. NO then diffuses through the endothelial cell membrane into the vascular smooth muscles cells (VSMC), where it promotes the conversion of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP) by soluble guanylyl cyclase (sGC). Protein kinases such as protein kinase G (PKG), phosphodiesterases, tyrosine kinases, phospholipase C (PLC), tyrosine phosphatases, and ion channels are all modulated by cGMP, both explicitly and implicitly. cGMP triggers protein kinase G (PKG), which avert Ca^{2+} entry through voltage-dependent Ca^{2+} channel (VDCC) and release of Ca^{2+} from ER receptor called inositol 1,4,5-triphosphate receptor (IP_3R). PKG can trigger the re-uptake of cytoplasmic Ca^{2+} by Sarco/endoplasmic reticulum calcium ATPase (SERCA). This process reduced the amount of Ca^{2+} in the cytoplasm. Also, PKG stimulates the myosin light-chain phosphatase (MLCP) and dephosphorylates smooth muscle myosin. Such a process induces vasorelaxation by preventing the contractile units' tonic contraction (Dudzinski *et al.*, 2006) (Figure 2.3).

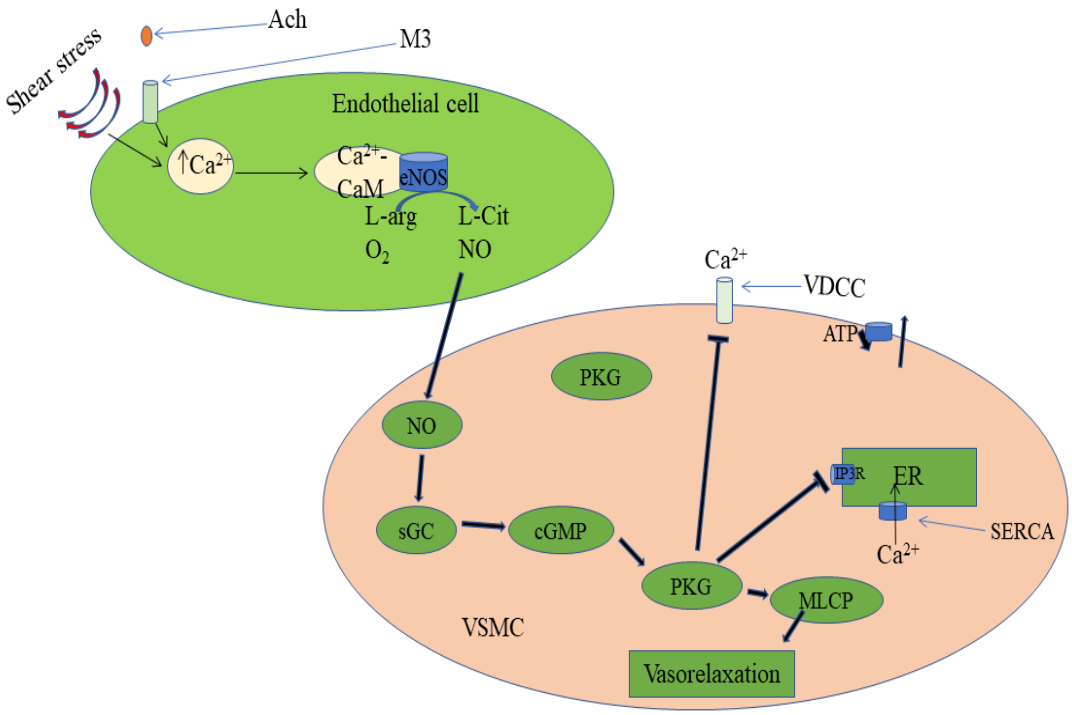

Figure 2.3 A potential mechanism of NO-mediated response in the blood vessel is depicted in the above diagram. When endothelial cells are activated by ACh or shear stress, the intracellular Ca^{2+} concentration rises, and NO is generated. NO enters the VSMC and activates the sGC, causing cGMP to be generated. The generation of cGMP activates PKG, which result in vasorelaxation. The figure was modified from Jamwal and Sharm (Jamwal and Sharma, 2018)

SERCA (sarco/endoplamic reticulum Ca^{2+} ATPase), ER (endoplasmic reticulum), ATP (adenosine triphosphates), M3 (muscarinic three receptor), CaM (calmodulin), ATP (adenosine triphosphates), PKG (protein kinase G), MLCK (myosin light chain phosphatase), IP3R (inositol 1,4,5-triphosphate receptor).

b) Insulin-induced NO-mediated relaxation

The insulin pathways

When insulin binds to insulin receptors (IR), it results in intrinsic kinase activity (tyrosine) within the receptor and active three main signaling pathways. Among the pathways activated is the insulin receptor substrate (IRS)/phosphatidylinositol-3-kinase (PI3K) pathways (Figure 2.4), which trigger insulin's metabolic functions and vasorelaxation effects (Mustapha *et al.*, 2021).

Insulin increases signal transduction along metabolic and mitogenic/gene-regulatory pathways.

IRs are located on the cell membrane of insulin-responsive cells. The IR has two extracellular subunits known as the alpha peptide and two transmembrane subunits, also known as beta-peptides (Mustapha *et al*., 2020). The four subunits are linked to each other by a disulfide bond between them. The transmembrane beta units contain intrinsic tyrosine kinase activity. When insulin bind to the alpha subunit of the IR, which will then activate it. The alpha subunit's activation will activate and autophosphorylation of the beta subunit by the tyrosine kinase enzyme. The phosphorylated IRS stimulates the downstream signaling cascade by activating and recruiting PI3K which then leads to the recruitment and phosphorylation of protein kinase B (Akt) to the plasma membrane of a cell (Mustapha *et al*., 2021). The phosphorylated Akt bind to eNOS, which leads to oxidation of L-arginine to L-citrulline and NO production (Alkaitis and Crabtree, 2012). Akt is like most other protein kinases that has been at the nexus of apoptosis and survival. Its plays an important role in a variety of interrelated cell signaling pathways involved in apoptosis inhibition, cell growth and metabolism. Diabetes, cardiovascular, neurological disorders and cancer have all been linked to perturbations in the Akt-regulated pathways (Nitulescu *et al*., 2018).

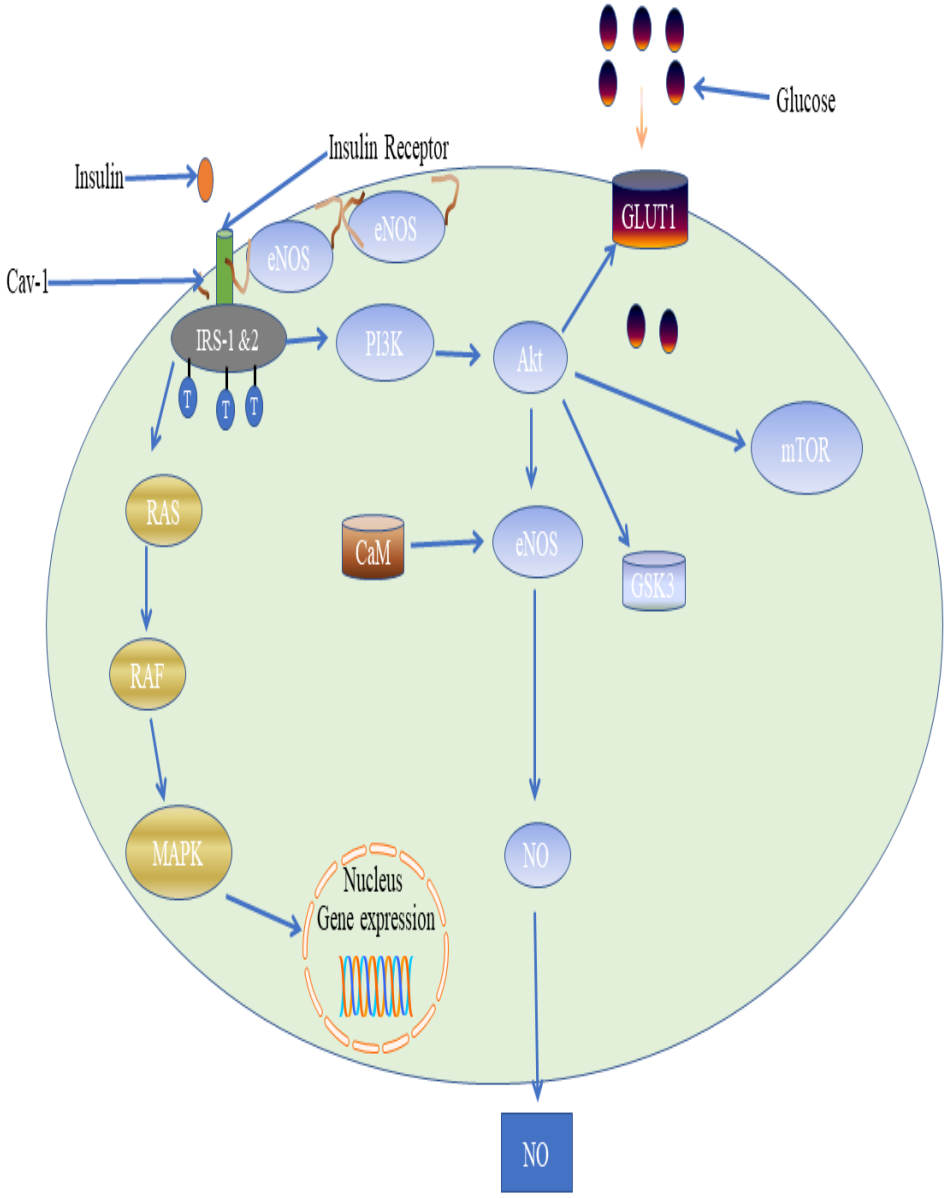

Figure 2.4 A potential mechanism of insulin-mediated response is depicted in the above diagram via PI3K/Akt/eNOS pathways. Insulin activates its receptors on the endothelial cells through tyrosine autophosphorylation; this marks the beginning of insulin downstream signaling cascade. This process leads to activation and phosphorylation of Akt, among other pathways. The Akt phosphorylates eNOS to produce NO. The figure was modified from Barvitenko *et al*. (Barvitenko *et al*., 2021)

2.2 Diabetes Mellitus

2.2.1 Definition and classification

Diabetes mellitus (DM) is a metabolic condition characterized by elevated blood glucose levels due to improper insulin production, insulin function, or even both. DM can be classified into four such as type 1 DM (T1DM), T2DM, gestational diabetes (GDM), as well as other specific types (ADA, 2019).

T1DM, also called juvenile diabetes or insulin-dependent diabetes, occurs when the immune system destroys the pancreas' beta cells that contain insulin, usually resulting in absolute insulin deficiency (ADA, 2019). The origins of this devastating mechanism are not well known, but the possible theory is that the autoimmune reaction is triggered by a mixture of genetic damaged and an environmental stimulus (viral infection). This metabolic disorder occurs at any age, but T1DM develops more commonly in children and adolescents. T1DM constitutes around 5-10% of the overall number of cases of diabetes.

T2DM, initially known as non-insulin-dependent diabetes, constitutes approximately 90-95% of the overall number of diabetic subjects. It is triggered by a mixture of progressive β - cell failure and dysfunction in target tissues along with insulin resistance (ADA, 2019). T2DM is associated with a more significant number of chronic comorbid conditions that can compromise patients' quality of life and contribute to cardiovascular disease progression. Indeed, premature death is associated with T2DM, triggered primarily by coronary artery disease, renal failure and stroke (Zheng *et al*., 2018). T2DM is linked to insulin resistance and many related health complications like obesity, atherosclerosis, and drop in testosterone levels (Burgos-moron *et al*., 2019). Insulin resistance is associated with activation of the ER stress

response, contributing to lipid metabolism disturbance and amplified insulin resistance (Flamment *et al*., 2012; Ozcan *et al*., 2004). Insulin resistance is defined by increased blood glucose level (hyperglycemia) and hyperinsulinemia, high glycosylated haemoglobin (HbA1c) levels, reduced adiponectin production, glucose intolerance, hyperlipidemia, reduced postprandial infusion rate of glucose and increased plasma inflammatory markers (Ye, 2013). β-cells appear to generate more insulin in response to increased blood glucose levels to counteract the disruption via a positive feedback mechanism, promoting a hyperinsulinemia disorder. A predominant attribute of T2DM and insulin resistance is hyperinsulinemia. Insulin resistance occurs due to impairment of insulin signaling cascade, which affects the normal physiological response to insulin via phosphatidylinositol-3-kinase (IRS/PI3K/Akt/eNOS) and mitogen-activated protein kinase (MAPK) signaling cascades (Villalobos-Labra *et al*., 2017).

GDM was previously identified as any degree of glucose intolerance discovered during pregnancy. However, GDM is now recognized as a metabolic disorder irrespective of whether the disease existed prior to birth or continued thereafter. This concept aided in developing a standardized strategy for detecting and classifying GDM, but it was constrained by inaccuracy. With the current obesity and diabetes crisis, more childbearing age women are developing T2DM, with a rise in expectant mothers with undiagnosed T2DM (ADA, 2019).

Other specific types of diabetes other than those mentioned above may be predisposed, like (i) pancreatitis and cystic fibrosis, (ii) monogenic diabetes syndromes, and (iii) drug or chemical-induced diabetes.

2.2.2 Diagnosis and symptoms

DM can indeed be diagnosed using plasma glucose parameters, such as the fasting plasma glucose (FPG) or 2-hour plasma glucose (2-h PG) values obtained throughout a 75-gram oral glucose tolerance test (OGTT) or HbA1c guidelines as shown in Table 2.1 (ADA, 2019). DM is confirmed after repeating any of the above tests in Table 2.1 on a different day. The American Diabetic Association (ADA) guidelines in 2010 have added another parament called glycated haemoglobin (HbA1c) after standardizing the method (ADA, 2014). Based on the guidelines, it has shown that when an individual has fasting blood glucose (FBG) ≥ 126 mg/dL or 7.0 mmol/L, ≥ 200 mg/dL or 11.1 mmol/L during a 2-hour oral glucose tolerance test (OGTT), ≥ 6.5% or 48 mmol/mol of HbA1c and random plasma glucose (RPG) ≥ 200 mg/dL or 11.1 mmol/L can confirm individuals have diabetes (ADA, 2019). In clinics, the FBG level is preferable because it saves time and easy to measure compared to the OGTT that takes 2 hours to be completed (Saydah *et al*., 2001).

Table 2.1 Diagnosis Criteria for Diabetes

Diagnosis	**FBG**		**2-h OGTT**	
	mg/Dl	**mmol/L**	**mg/dL**	**mmol/dL**
Normal	< 140	< 7.8	< 110	< 6.0
Glucose tolerance impaired	≥ 140	≥ 7.8	< 126	< 7.0
Fasting glycaemia impaired	< 140	< 7.8	≥ 110 < 126	≥ 6.1 < 7.0
DM	≥ 200	≥ 11.1	≥ 126	≥ 126
Glycated haemoglobin				
Diagnosis	**%**		**mmol/L**	
Pre-DM	5.7 ≤ 6.4		42 ≤ 47	
DM	≥ 6.5		≥ 48	

DM: Diabetes mellitus, FBG: fasting blood glucose and OGTT: oral glucose tolerance test

Apart from carrying out a particular test for DM diagnosis, it is equally essential to take cognizance of DM's signs and symptoms like extreme thirst, abrupt weight loss, lack of energy, blurred vision, increased urination, weakness, bedwetting and constant hunger. DM that goes undiagnosed as well as untreated can cause serious vascular complications and even death (IDF, 2019).

The primary goal of DM medications is to maintain good glycaemic levels by achieving normal blood sugar levels. Bad and insufficient glycaemic regulation in DM patients is an important public health concern and a more considerable risk for diabetes complications. Personal care, such as blood glucose control, medication adherence and diet modifications, exercise, foot-care, and symptoms identification, are vital components for early detection (Padma *et al*., 2012).

Gut endocrine cells produce incretin hormones, such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) during food intake, and they play critical roles in glycaemic control, rendering them desirable targets in the management of diabetes (Drucker, 2007). Exogenous treatment of GLP-1 analogues is used as a complementary therapy.

Intravenous insulin treatment with daily monitoring of the patient's blood sugar levels is a traditional approach for managing T1DM. Also, insulin therapy has become a standard part of T2DM management. Medications used in DM management aim to bring down insulin resistance, minimize glucose reabsorption, increase endogenous insulin secretion, and reduce hepatic glucose production, among other strategies. Anti-diabetic medications target a variety of receptors and are widely available on the market. The anti-diabetic medications are classified into the following: sulfonylureas (e.g. gliclazide), meglitinides (e.g. nateglinide), biguanides (e.g. metformin),

thiazolidinediones (TZD) (e.g. pioglitazone), alpha-glucosidase inhibitor (e.g. acarbose), dipeptidyl-peptidase-4 (DPP-4) inhibitors (e.g. linagliptin), glucagon-like peptide-1 (GLP-1) agonist (e.g. exenatide) and sodium-glucose cotransporter 2 (SGLT2) inhibitors (e.g. canagliflozin). Even though a handful of small molecules with anti-diabetic potential have been identified, their clinical utility progress is now being assessed in various stages of clinical trials (Kerru *et al*., 2018). These smaller molecules are liked with different side effects and need to develop new potent hypoglycaemic drugs with improved pharmacological profiles. The identified targets for these smaller molecules are as follows: Protein tyrosine phosphatase 1 B (PTP1B) [e.g. 2-(4-fluorophenylimino)-4-thiazolidinedione], Dipeptidyl peptidase-4 (DPP-4) (e.g. evogliptin and omarigliptin), Free fatty acid receptor 1 (FFAR1) (e.g. 3-methyl derivative of phenoxyacetamide), G protein-coupled receptor (GPCR), Peroxisome proliferator-activated receptor (PPAR), Sodium-glucose co-transporter (SGLT), α-Glucosidase, Aldose reductase (ALR)**,** Glycogen phosphorylase (GP), Fructose-1,6-bisphosphatase (FBPase), Glucagon receptor (GCGr) and Phosphoenolpyruvate carboxykinase (PEPCK) (Kerru *et al*., 2018).

2.2.3 Diabetes and endothelial dysfunction

DM is often linked to endothelial cell dysfunction (ECD), especially reducing EDRF, which is thought to be crucial in developing diabetes-related vascular complications (De Vriese *et al*., 2000). ECD is an essential factor in the pathogenesis of diabetes-related vascular complications, affecting both micro and macro-vasculatures. The micro-vasculature include neuropathy, nephropathy and retinopathy, while macro-vasculature has peripheral disease, ischemic heart disease, atherosclerosis, and cerebrovascular disease (Gupta *et al*., 2020). The perturbation of

endothelium involves other molecular mechanisms like ER stress, inflammation, apoptosis and oxidative stress, attenuating the metabolic cascades within the endothelium (Cimellaro *et al*., 2016; Incalza *et al*., 2018).

Endothelial dysfunction is defined as functional and structural impairment of the vascular endothelial layer, marked by decreased NO bioavailability/or eNOS activation and abnormal angiogenesis (Jamwal and Sharma, 2018). The earliest and most essential event that marks endothelial dysfunction is the decreased production, release, and activity of endothelium-derived NO (Liao, 2013). The reduced NO bioavailability is one of the leading causes of the onset of endothelial dysfunction (Incalza *et al*., 2018). Endothelial dysfunction is a major contributor to the pathogenesis of diabetes-related cardiovascular problems. These problems might be due to impaired eNOS activity or decreased eNOS protein expression, triggered by uncoupling, insulin resistance and hyperglycemia (Incalza *et al*., 2018; Mokhtar *et al*., 2016). Hyperglycemia, a common symptom of diabetes, has been linked to the development of endothelial dysfunction (Maamoun *et al*., 2019b).

Endothelial insulin resistance is defined as a lack of insulin's ability to perform its metabolic functions in the endothelium. Insulin sensitivity in the peripheral tissues is reduced when the PI3-K/Akt pathway is disrupted. When the PI3-K/Akt pathway is dysregulated, the MAPK pathway is significantly activated, resulting in the production of inflammatory mediators (Sanches *et al*., 2020). The lack of balance between the PI3-K/Akt and MAPK pathways leads to endothelial dysfunction, defined by a decreased synthesis of NO and enhanced production of ET-1 in endothelial cells.

2.2.4 High fat diets and STZ induced type 2 animal model

Animal models that fully mimic human DM's pathophysiology are critical due to the complex nature of the disease. Toxic chemicals are employed to damage beta-cells in the pancreas in animal models to induce diabetes. Toxic chemical like alloxan was initially employed in 1943, which produces free radicals with the resultant effect of beta-cells damage, but it can lead to kidney damage; as a result, it's rarely used (Ghasemi *et al*., 2014; Szkudelski, 2001). Another toxic chemical was introduced called STZ in 1963. STZ is by far the most prevalent chemical used to cause diabetes; it was employed to induce either T1DM or T2DM (Szkudelski, 2001). STZ in high doses disrupts insulin secretion significantly, a symptom identical to T1DM. However, such a dosage of STZ can cause ketonuria, a symptom of unregulated T1DM (Kashfi *et al*., 1995). STZ low doses can induce a mild deficiency of insulin secretion, which mimics T2DM's advanced stages more closely (Srinivasan *et al*., 2005). It is important to note that STZ low doses do not lead to insulin dysfunction, and it is a common condition in T2DM patients (Zhang *et al*., 2008). Earlier studies have shown that animals fed with a high-fat diet (HFD) develop insulin dysfunction for an extended period (Binh *et al*., 2013; Zhang *et al*., 2016). An animal model that put together both HFD and then follows by STZ low dose to trigger peripheral insulin dysfunction and beta-cell damage, respectively, will closely resemble both the pathogenesis and phenotype of human T2DM (Gheibi *et al*., 2017a).