THE EFFECTS OF METHANOLIC EXTRACT OF *Clinacanthus nutans* L. (BELALAI GAJAH) ON ATHEROGENIC RISK MARKERS IN A TYPE 2 DIABETIC RAT MODEL

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by

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LIST OF ABBREVIATIONS

4-HNE	4-hydroxynonenal
ACh	Acetylcholine
AGEs	Advanced glycation end products
AI	Atherogenic index
AMPK	5’ AMP-activated protein kinase
ANOVA	Analysis of variance
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cGMP	Cyclic guanosine monophosphate
CNME	*Clinacanthus nutans* methanolic leaves extract
COX	Cyclooxygenase
CyPA	Cyclophilin A
DAG	Diacylglycerol
DM	Diabetes mellitus
DPPH	2,2-diphenyl-1-picrylhydrazyl
ECM	Extracellular matrix
ED	Endothelial dysfunction
EDCF	Endothelium-derived contracting factor
EDRF	Endothelium-derived relaxing factor
ELISA	Enzyme-linked immunosorbent assay

eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
FAD	Flavin adenine dinucleotide
FBG	Fasting blood glucose
FMN	Flavin mononucleotide
FRIM	Forest Research Institute Malaysia
GC-MS	Gas chromatography- mass spectrometry
GLUT2	Glucose transporter 2
GPx	Glutathione peroxidase
GSH	Glutathione
GTP	Guanosine triphosphate
HBA_{1C}	Glycated hemoglobin
HCl	Hydrochloric
HDL-C	High-density lipoprotein
HRP	Horseradish peroxidase
IACUC	Institutional of Animal Care and Use Committee
IDF	International Diabetes Federation
IgG	Immunoglobulins G
IL	Interleukins
IMT	Intima-media thickness
iNOS	Inducible nitric oxide synthase
IP_3	Inositol 1,4,5-triphosphate
IP_3R	Inositol 1,4,5-triphosphate receptor

KCl	Potassium chloride
L-NAME	L-nitro-arginine methyl ester
LDL-C	Low-density lipoprotein
LPS	Lipopolysaccharide
MCP-1	Monocytes chemoattractant protein-1
MDA	Malondialdehyde
MLC	Myosin light-chain
MLCK	Myosin light-chain kinase
MLCP	Myosin light-chain phosphatase
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHMS	National Health and Morbidity Survey
NIST	National Institute Standard and Technology
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
oxLDL-C	Oxidized low-density lipoprotein cholesterol
PKC	Protein kinase C
PKG	Protein kinase G
PSS	Physiological saline solution
PUFAs	Polyunsaturated fatty acids
PVDF	Polyvinylidene difluoride

RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of mean
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
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TBS	Tris Buffer Saline
TBST	Tris Buffer Saline/Tween-20
TC	Total cholesterol
TG	Triglycerides
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
TxA_2	Tromboxane A_2
USM	Universiti Sains Malaysia
VCAM	Vascular cell adhesion molecule
VDCC	Voltage dependent calcium channel

VSMC	Vascular smooth muscle cell
XO	Xanthine oxidase

LIST OF SYMBOLS

α	Alpha
β	Beta
°	Degree
°C	Degree celsius
γ	Gamma
±	Plus-minus
<	Less than
≥	Greater than or equal to
μM	Micrometer
μL	Microliter
mL	Milliliter
cm	Centimeter
kg	Kilogram
g	Gram
mg/mL	Milligram/milliliter
Pg/mL	Picogram/milliliter
mg/kg	Milligram/kilogram
%	Percentage
g/mol	Gram/molecule
nmol/mg	Nanomoles/milligram

mmol/L	Millimoles/liter
U/mg	Unit/milligram
mL/kg	Milliliter/kilogram
nm	Nanometer
w/v	Weight/volume
w/w	Weight/weight
U/mL	Unit/milliliter
eV	Electron-volt
Da	Dalton
m	Meter
O_2	Oxygen
CO_2	Carbon dioxide

KESAN EKSTRAK METANOL *Clinacanthus nutans* L. (BELALAI GAJAH) PADA PENANDA RISIKO ATEROGENIK DALAM MODEL TIKUS DIABETES JENIS 2

ABSTRAK

Diabetes mellitus dikaitkan dengan disfungsi endotelium (ED); ia menyebabkan kerosakan progresif vaskular. *Clinacanthus nutans* telah didokumentasikan mempunyai sifat antioksida, hipoglisemik, hipolipidemik dan anti-radang; sifat-sifat ini berpotensi meningkatkan fungsi endotelium dan mencegah perkembangan aterosklerosis. Pertama, kajian ini bertujuan untuk menilai fungsi endotelium, perubahan struktur awal vaskular, tekanan oksidatif dan keradangan vaskular pada model tikus diabetes jenis 2 (T2DM) yang disebabkan oleh diet tinggi lemak (HFD) dan streptozotocin (STZ) dos rendah (STZ). Kedua, untuk mengetahui kesan ekstrak methanol daun *C. nutans* (CNME) pada parameter dan model yang sama. **Bahagian pertama kajian**: Tikus Sprague-Dawley jantan dibahagikan kepada kumpulan bukan diabetes dan diabetes (n = 12 setiap kumpulan). Kumpulan diabetes diberi HFD selama 4 minggu sebelum suntikan intraperitoneal STZ; dan dikorbankan pada minggu 15. Tahap glukosa darah puasa (FBG) dan profil lipid diukur sebelum pengorbanan tikus. Setelah dikorbankan, aorta diasingkan; pengenduran dan pengecutan berperantara- dan tidak berperantara-endotelium aorta toraks ditentukan menggunakan organ bath. Ekspresi enzim eNOS aorta dinilai melalui pemblotan Western. Superoxide dismutase (SOD), malondialdehyde (MDA), factor nekrosis

tumor factor-alpha (TNF-α) aorta, perubahan histopatologi dan ketebalan intima-media aorta (IMT) juga diukur. **Bahagian kedua kajian:** Tikus Sprague-Dawley jantan dibahagikan kepada kumpulan bukan diabetes dan diabetes; diabetes teraruh oleh pemakanan HFD dan STZ dos rendah. Selepas tujuh minggu teraruh diabetes, tikus dibahagikan kepada lima kumpulan (n = 12 setiap kumpulan): kawalan bukan diabetes (C), bukan diabetes yang dirawat dengan CNME selama 4 minggu (500 mg/kg/hari) (C+CNME), tikus diabetes yang tidak dirawat (DM), diabetes yang dirawat dengan metformin (300 mg/kg/hari)(DM+Met) dan diabetes yang dirawat dengan CNME (500 mg/kg/hari) (DM+CNME). Tikus dikorbankan setelah 4 minggu rawatan dan parameter eksperimen serupa dengan bahagian 1 kajian diambil. **Hasil; Bahagian pertama:** Tikus diabetes mempunyai FBG, jumlah kolesterol (TC), trigliserida (TG), kolesterol lipoprotein berketumpatan rendah (LDL-C) dan indeks aterogenik (AI) yang lebih tinggi berbanding tikus bukan diabetes. Pengenduran berperantara-endotelium menurun, sementara pengecutan berperantara- dan tidak berperantara-endothelium meningkat pada tikus diabetes. Ekspresi eNOS lebih rendah pada tikus diabetes. Tahap IMT, MDA dan TNF- α meningkat sementara aktiviti SOD lebih rendah pada tikus diabetes berbanding dengan tikus bukan diabetes. **Bahagian kedua:** Kedua-dua kumpulan DM+CNME dan DM+Met telah mengurangkan tahap FBG berbanding kumpulan DM. Rawatan dengan CNME dan metformin pada tikus diabetes menunjukkan TC, TG, LDL-C dan AI yang lebih rendah berbanding dengan tikus diabetes yang tidak dirawat. Kedua-dua kumpulan diabetes yang dirawat dengan CNME dan metformin meningkatkan pemulihan kerosakan vasopengenduran berperantara-endotelium; ini dikaitkan dengan

peningkatan ekspresi protein eNOS aorta. Rawatan dengan CNME dan metformin juga mengurangkan pengecutan berperantara dan tidak berperantara-endotelium aorta pada tikus diabetes. Tahap IMT, MDA dan TNF-α dalam aorta dikurangkan sementara aktiviti SOD lebih tinggi dalam kedua-dua rawatan CMNE dan metformin pada tikus diabetes. Hasil kajian ini menunjukkan bahawa model T2DM yang teraruh oleh HFD dan STZ dos rendah mempunyai ED yang berkaitan dengan perubahan awal struktur vaskular, dan peningkatan tekanan oksidatif dan keradangan vaskular. Rawatan dengan ekstrak *C. nutans* meningkatkan vasopengenduran berperantara-endotelium, pengurangan pengecutan berperantara- dan tidak berperantara-endotelium, peningkatan ekspresi eNOS dan SOD, tahap AI, MDA, TNF-α yang lebih rendah, dan pengurangan IMT pada aorta tikus T2DM; semua kesan ini setanding dengan tikus diabetes yang dirawat metformin. Oleh itu, ekstrak metanol daun *C. nutans* berpotensi untuk diterokai lebih lanjut sebagai tambahan dalam rawatan T2DM untuk mencegah atau mengurangkan keparahan diabetes yang disebabkan oleh aterosklerosis.

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ABSTRACT

Diabetes mellitus is associated with endothelial dysfunction (ED); causing progressive vascular damage. *Clinacanthus nutans* has been documented to have antioxidant, hypoglycemic, hypolipidemic and anti-inflammatory properties; properties with the potential to improve endothelial function and prevent atherosclerosis development. Firstly, this study aims to evaluate endothelial function, early vascular structural changes, vascular oxidative stress and inflammation in a model of type 2 diabetes (T2DM) rat induced by high-fat diet (HFD) and low-dose streptozotocin (STZ). Secondly, to determine the effects of *C. nutans* methanolic leaves extract (CNME) on the above parameters on the same model. **First part of study**: Male Sprague-Dawley rats were divided into non-diabetic and diabetic groups (n=12 per group). Diabetic groups were fed 4 weeks of HFD before intraperitoneal injection of STZ; and sacrificed at week 15. Fasting blood glucose (FBG) and lipid profile were measured prior to sacrifice. Upon sacrifice, the aorta was isolated; endothelial-dependent and -independent relaxations and contractions were determined using the organ bath. Aortic endothelial nitric oxide synthase (eNOS) expression was assessed via Western blotting. Aortic superoxide dismutase (SOD), malondialdehyde (MDA), tumor necrosis factor-alpha (TNF-α), histopathological

changes and aortic intima-media thickness (IMT) were also measured. **Second part of study:** Male Sprague-Dawley rats were divided into non-diabetic and diabetic groups. After seven week of diabetes induction, rats were divided into five groups (n=12 per group): non-diabetic control (C), non-diabetic treated with 4 weeks of CNME (500 mg/kg/daily)(C+CNME), untreated diabetic rats (DM), diabetic treated with metformin (300 mg/kg/daily)(DM+Met) and diabetic treated with CNME (500 mg/kg/daily)(DM+CNME). Rats were sacrificed after 4 weeks of treatment and experimental parameters similar to part 1 of study were performed. **Results; First part:** Diabetic rats have higher FBG, total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C) and atherogenic index (AI) compared to non-diabetic rats. Endothelium-dependent relaxation was decreased while endothelial-dependent and -independent contractions were increased in diabetic rats. eNOS expression was lower in diabetic rats. IMT, MDA and TNF-α levels were increased while SOD activity lower in diabetic rats. **Second part:** Both DM+CNME and DM+Met groups reduced FBG levels compared to the DM group. Treatment with CNME and metformin in diabetic rats showed lower TC, TG, LDL-C and AI compared to untreated diabetic rats. Both diabetic-treated with CNME and metformin groups significantly improved the impairment in endothelium-dependent vasorelaxation; this was associated with increased expression of eNOS. Treatment with CNME and metformin also reduced endothelium-dependent and -independent contractions in diabetics. Aortic IMT, MDA and TNF-α levels were reduced while SOD activity was higher in both CMNE and metformin treated diabetic rats. These results demonstrated that the T2DM model induced by HFD and low-dose STZ has

ED associated with early vascular structural changes, and increased vascular oxidative stress and inflammation. Treatment with *C. nutans* extract improved endothelial-dependent vasodilatation, reduced endothelial-dependent and -independent contractions, increased eNOS expression and SOD levels, lower AI, MDA, TNF-α levels, and reduced IMT in aorta of T2DM rats; all these effects were comparable with metformin-treated diabetic rats. Thus, the methanolic extract of *C. nutans* leaves has the potential to be further explored as an adjunct in the treatment of T2DM to prevent or reduce severity of diabetes induced atherosclerosis.

CHAPTER 1

INTRODUCTION

1.1. Background of the Study

Diabetes mellitus is a metabolic disorder characterized by altered glucose and lipid metabolism, resulting in persistent hyperglycemia. It is a known risk factor for atherosclerosis and cardiovascular diseases (Tabit *et al*., 2010). The prevalence of diabetes mellitus has increased globally in recent years. According to the International Diabetes Federation, there are about 463 million adults living with diabetes mellitus, accounting for about 9.3% of the global population. This figure is predicted to rise to 528 million by the year 2030 and 700 million by the year 2045 (IDF, 2019). There is an alarming increase in the prevalence of diabetes mellitus in Malaysia, as reported by the National Health and Morbidity Survey (NHMS). Also, based on the NHMS 2019 report, there are about 3.9 million diabetic patients in Malaysia, which accounts for about 18.3% of the Malaysian population. This was an increase compared to the findings of 2011 and 2015 of 11.2% and 13.4% respectively (Ministry of Health Malaysia, 2019).

The chronic nature of diabetes mellitus requires long-term monitoring to minimize related secondary complications. Hence, the management of diabetes mellitus constitutes an ever-increasing proportion of global as well as Malaysia national healthcare budgets. According to IDF 2019, the global annual diabetes

treatment cost constituted USD 700 billion, which was equivalent to the 10% of global health expenditure. The cost is expected to increase to USD 845 billion by year 2045 (IDF, 2019). In Malaysia, the annual expenditure for diabetes treatment was reported to be USD 3.6 billion in year 2019 and the cost is expected to increase to USD 5 billion by year 2045 (IDF, 2019). The economic burden brought by this pendemic disease could undermine the development advancement of the nation worldwide.

According to the World Health Organization 2019, it was reported that diabetes mellitus is ninth in the world top ten leading cause of death (WHO, 2019). In addition, IDF 2019 also indicated that diabetes mellitus constituted 11.3% of global all-cause mortality, causing 4.2 million deaths globally in year 2019 (IDF, 2019). Cardiovascular diseases were reported to be the main contributor to the mobidity and mortality in diabetic patients (Leon and Maddox, 2015); cardiovascular diseases account for an overwhelming 65-75% of deaths in people with diabetes (Ali *et al*., 2010). Prolonged hyperglycemia in diabetic individuals caused a significant risk of secondary cardiovascular complications. Therefore, the prevention of these diabetes-related cardiovascular complications, occurring at the macro- and microcirculation, will be effective means in the management of diabetes mellitus (Bate and Jerums, 2003).

Type 2 diabetes mellitus (T2DM) is associated with a marked increase in the risk of atherosclerotic vascular disorders, including coronary, cerebrovascular, and peripheral artery diseases (Yamagishi, 2011). Atherosclerosis is characterized by

endothelial dysfunction (ED), vascular oxidative stress and inflammation and the build-up of lipid, cholesterol, calcium and cellular debris within the intima of the vessel wall; all these factors are implicated in plaque formation and vascular remodelling (Kampoli *et al*., 2009; Tibaut *et al*., 2019). Abnormal lipid metabolism, ED, vascular oxidative stress, inflammation, and vascular structural changes are factors that contribute to initiate and accelerate atherosclerosis, thus changes in these parameters can be used as markers for vascular damage in diabetes (Kampoli *et al*., 2009: Niki, 2018; Steven *et al*., 2019; Ruparelia and Choudhury, 2020). In this study, these atherogenic markers were determined in a T2DM rat model induced by high-fat diet and low-dose streptozotocin.

The endothelium consists of a monolayer of cells that run through the inner lining of the blood vessels. It regulates vascular tone and maintains vascular homeostasis. This layer can be disturbed resulting in its dysfunction termed endothelial dysfunction (ED). ED refers to diminished availability of endothelium-derived relaxing factor (EDRF) or nitric oxide (NO), and increased production of vasoconstrictors (Tabit *et al*., 2010). ED occurs early before morphological changes become visible in the blood vessel wall. NO is formed in the endothelial cells via the enzymatic action of endothelial nitric oxide synthase (eNOS). ED plays a pivotal role in the pathogenesis of atherosclerosis in diabetes mellitus, and other cardiovascular diseases.

Chronic hyperglycemia in diabetes mellitus is associated with long-term micro- and macro-vascular complications, which contributes to organ dysfunction

and failure, especially to the eyes, kidneys, nerves, heart, and blood vessels (Panigrahi *et al*., 2016). In the diabetes state, exposure of arterial tissue to high glucose concentration induces superoxide production and increased oxidative stress. Superoxide anion reacts with NO to form peroxynitrite, which eliminates the biological activity of NO (Sharma *et al*., 2012a). Superoxide anion alters the catalytic activity of eNOS in endothelial cells, thus impairing NO production. Reduction in NO production leads to ED.

It has been observed that disorders induced by high-fat diet feeding resemble the human metabolic syndrome closely, with implications for cardiovascular health. Obesity and dyslipidemia are the components of metabolic syndrome, that are commonly seen in T2DM. High-fat diet intake causes obesity, which leads to insulin resistance. Insulin resistance and hyperglycemia increases diabetic complications such as atherosclerosis. High fat diet also leads to hyperlipidemia. Hyperlipidemia increases reactive oxygen species (ROS) production, resulting in oxidation and peroxidation of lipids, protein, and lipoprotein (Abbasnezhad *et al*., 2019). Low-density lipoprotein cholesterol (LDL-C) and it's oxidized form (oxLDL-C) play a major role in ED and atherogenesis. Oxidized LDL-C affects eNOS protein expression by increasing the synthesis of caveolin-1 (Davignon and Ganz, 2004), thus reducing NO production resulting in impairment of NO-mediated vasodilatory response (Blair *et al*., 1999; Wang *et al*., 2011).

Inflammatory markers are associated with risk factors for the development of T2DM and macrovascular complications. Inflammation is a contributor to ED and

plays a pivotal role in the development of atherosclerosis. Inflammation can modify the synthesis and degradation of vasodilators and vasoconstrictors. At the intima layer of an arterial cell wall, monocytes release a number of inflammatory cytokines such as interleukins-1 (IL-1), IL-1β, IL-6, and tumor necrosis factor-alpha (TNF- α); all of which contribute to vascular endothelium injury and induce vascular wall plaque formation (Sun *et al*., 2018). TNF-α activates NADPH oxidase resulting in increased superoxide production, leading to a reduction in NO bioavailability (Picchi *et al*., 2006). TNF-α also reduces the expression of eNOS via augmented superoxide generation and affecting the half-life of eNOS mRNA, thus further reducing NO bioavailability (Zhang *et al*., 2002; Bitar *et al*., 2005). NO protects blood vessels from endogenous injury by mediating molecular signals that inhibit leukocyte and platelet interaction with the vascular wall and prevent vascular smooth muscle cells (VSMC) proliferation and migration (Kubes *et al*., 1991; Sarkar *et al*., 1996). A reduction in endothelium-derived NO increases the activity of the pro-inflammatory transcription factor-like nuclear factor-kappa B (NF-κB), resulting in over-expression of leukocyte adhesion molecules and an increase in the production of inflammatory cytokines particularly TNF-α. These activities promote VSMC and monocyte migration into the intima and the formation of foam cells, characterizing the initial morphological changes of atherosclerosis (Creager *et al*., 2003).

Clinacanthus nutans (*C. nutans*) (Burm. f.) Lindau, commonly called Sabah Snake Grass, or "*Belalai Gajah*" in layman's terms, is widely used in Malaysia, Thailand, and Indonesia as traditional medicine. *C. nutans* extracts have been reported to have the following properties: antioxidant, hypoglycemic, hypolipidemic,

and anti-inflammatory. A previous study had demonstrated high radical scavenging activity of *C. nutans* extracts in high fat and high cholesterol fed rat model treated with *C. nutans* (Sarega *et al*., 2016). Two *in vitro* studies have shown that *C. nutans* extract inhibits α-glucosidase activity (Wong *et al*., 2014; Alam *et al*., 2017). α-glucosides ezyme is located at mucosal brush border of the small intestine; it functions to increase carbohydrate metabolism by increasing polysaccharide cleavage to glucose (Assefa *et al.,* 2020). Thus, inhibition of this enzyme reduces carbohydrate metabolism, reducing glucose absorption into the blood from the small intestine, thus reducing blood glucose levels. Observation from these *in vitro* studies has been supported by a recent study in type 2 diabetes (T2DM) rat model treated with *C. nutans* extract (Umar Imam *et al*., 2019).

C. nutans has also been reported to have hypolipidemic property as demonstrated in two previous studies (Sarega *et al*., 2016; Umar Imam *et al*., 2019). Both these studies demonstrated low serum total cholesterol (TC), triglycerides (TG), and LDL-C levels in hyperlipidemic (Sarega *et al*., 2016) and T2DM rats (Umar Imam *et al*., 2019) treated with *C. nutans*. In terms of inflammation, an *in vitro* study has reported that *C. nutans* reduced inflammatory cytokines, which include TNF-α, IL-1β and IL-6 in murine macrophages RAW 264.7 cells (Mai *et al*., 2016).

1.2. Study Rationale

Currently, there are five major classes of therapeutic drugs that are used to treat diabetes mellitus: thiazolidinediones, sulfonylureas, biguanides, meglitinide,

and alpha-glucosidase inhibitors. Despite their effectiveness, most of them cause certain unwanted side effects and potential adverse effects. Thiazolidinediones have been reported previously to cause weight gain, fluid retention, and heart failure (Juurlink *et al*., 2009). A study by Nissen and Wolski (2007) has shown that treatment with thiazolidinediones, particularly rosiglitazone increased the risk of myocardial infarction in diabetic patients (Nissen and Wolski, 2007). Sulfonylureas may cause hypoglycemia, increase body weight, gastrointestinal disturbances, and headache (Schier *et al*., 2001). Biguanides, particularly metformin, could cause abdominal pain, diarrhea, nausea, and lactic acidosis to diabetic patients (Nzerue *et al*., 2003). Although there are many anti-diabetic drugs available, the number of T2DM patients are still increasing, many of them not properly controlled. This may be due to anti-diabetic drug adverse effects and perhaps the limitation of action of each drug. Furthermore, despite taking these drugs, vascular complications of diabetes such as atherosclerosis and subsequent cardiovascular events such as coronary artery and cerebrovascular events are still highly prevalent in diabetic patients.

Certain medicinal plants and natural products may have hypoglycemic effect with fewer side effects, easier to source locally, and at lower cost compared to current conventional drugs. They may be further investigated and developed as drugs, or used as adjunct to current drugs. It is possible that some of these natural products may be able to reduce diabetic vascular complications. Previous studies on *C. nutans* have shown that the leaves of this plant comprised of antioxidant, hypoglycemic, hypolipidemic, and anti-inflammatory effects. The antioxidant and

hypoglycemic effects of *C. nutans* might reduce the production of reactive oxygen species (ROS) particularly superoxide anion. Reduced superoxide anion ameliorates NO bioavailability in the endothelium, thus attenuate ED in diabetes. Besides that, the hypolipidemic property of *C. nutans* can reduce lipid levels in diabetes mellitus, particularly LDL-C, which may reduce LDL-C accumulation in the vascular wall. In addition, the anti-inflammatory property of *C. nutans* can reduce inflammatory cytokines production, particularly TNF-α. Reduced LDL-C and TNF-α levels attenuate the formation of foam cells and atherosclerotic plaque. Thus, these pharmacological properties of *C. nutans* have the potential to delay or prevent atherosclerosis in diabetes mellitus.

To date, there are no studies on the effects of *C. nutans* on vascular ED (an early precursor to atherosclerosis), vascular oxidative stress and inflammation status in T2DM. Thus, the present study aimed to determine the effects of methanolic leaves extract of *C. nutans* on these atherogenic risk markers in high-fat diet and low-dose streptozotocin (STZ)-induced T2DM animal model.

1.3 Hypothesis of the Study

1. T2DM rats induced by high-fat diet and low-dose STZ showed increased atherogenic risk.

2. Methanolic extract of *Clinacanthus nutans* (CNME) attenuates atherogenic risk markers in T2DM rats.

1.4 Objectives of the Study

1.4.1 General Objective

To determine the atherogenic risk of T2DM rats induced by high-fat diet and low-dose STZ, and investigate the effects of treatment with the methanolic extract of *Clinacanthus nutans* (CNME) leaves on atherogenic risk markers in T2DM rats.

1.4.2 Specific Objectives

1. To determine endothelium-dependent and -independent relaxation and contraction in the aorta of non-diabetic and T2DM rats, and evaluate the effects of CNME treatment on these parameters.
2. To determine the expression of eNOS protein in the aorta of non-diabetic and T2DM rats, and evaluate the effect of CNME treatment on eNOS protein expression.
3. To assess oxidative stress markers in the aorta of non-diabetic and T2DM rats by measuring SOD activity and MDA level, and evaluate the effects of CNME treatment on these markers.
4. To determine the level of inflammation (by measuring tumor necrosis factor-alpha, TNF-α) in the aorta of non-diabetic and T2DM rats, and evaluate the effect of CNME treatment on this marker.
5. To assess early vascular structural changes (intima-media thickness and presence of foam cells) in the aorta of non-diabetic and T2DM rats, and evaluate the effects of CNME treatment on these parameters.

CHAPTER 2

LITERATURE REVIEW

2.1 Vasculature

2.1.1 Structure and Function of Blood Vessel Wall

The blood vessel contributes to homeostasis by providing blood flow to and from the heart to organs and tissues, allowing for the exchange of nutrients and waste in tissues. They also play an important role in adjusting the velocity and volume of blood flow. There are three main types of blood vessels, which include arteries, capillaries and veins. Although blood vessel may vary in size, their general structure remains similar except at the level of the capillaries. Starting from the external surface, the blood vessel wall consists of the tunica adventitia, which is mainly made up of collagen and connective tissues (Figure 2.1). The vasa vasorum form a network of microvessels that lie in the adventitia. The middle layer is tunica media with the vascular smooth muscle cells (VSMC) and finally the tunica intima with the endothelium that surrounds the lumen where the blood flows. The endothelium is a thin layer of flattened cells that lines the inner surface of the entire cardiovascular system (Tortora and Derrickson, 2009).

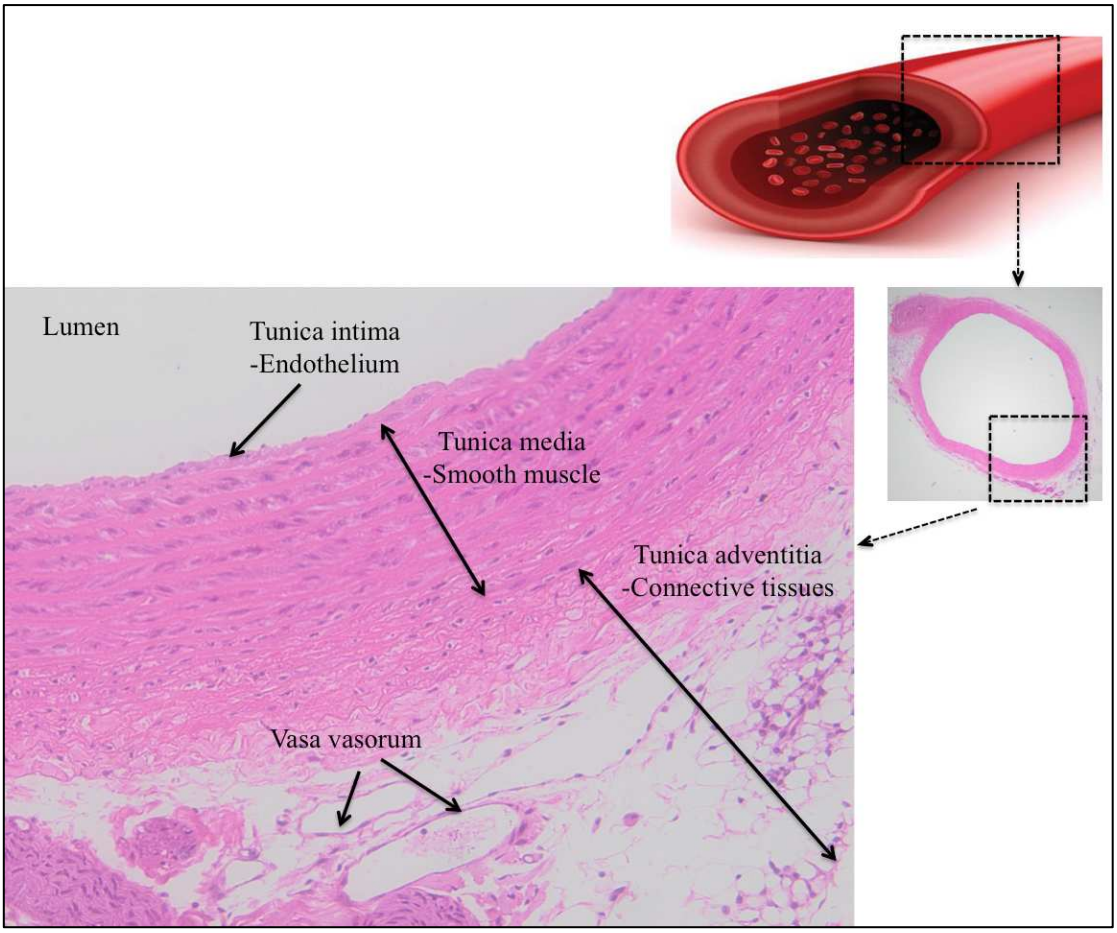

Figure 2.1 Anatomy of the blood vessel. Modified from https://image.shutterstock.com/image-illustration/blood-vessel-sliced.

2.1.2 Endothelium

The endothelium is a monolayer of endothelial cells that lines the interior surface of blood vessels and cardiac valves in the entire vascular system. This organ (endothelium) contains approximately 1×10^{13} cells and almost one-kilogram of weight. The shape of endothelial cells varies across the vascular tree, but they are generally thin and slightly elongated, with each endothelial cell being roughly 50-70 μm long, 10-30 μm wide and 0.1-10 um thick. Endothelial cells are metabolically active with important endocrine, paracrine, and autocrine functions. These functions

are indispensable for the maintenance of vascular homeostasis under physiological conditions (Bonetti *et al*., 2003; Sena *et al*., 2013).

The functions of vascular endothelium include vascular growth and remodeling, regulation of vessel integrity, metabolism, cell adhesion, angiogenesis, vascular permeability, tissue growth, homeostasis and immune responses. In addition, the vascular endothelium plays a major role in the regulation of vascular tone, maintaining blood fluidity, inflammatory responses and controlling tissue blood flow (Félétou *et al*., 2011; Sena *et al*., 2013). As a main regulator of vascular homeostasis, the endothelium maintains the balance between vasodilation and vasoconstriction, inhibition and promotion of the migration and proliferation of smooth muscle cells, fibrinolysis and thrombogenesis (Félétou *et al*., 2011; Sena *et al*., 2013). Endothelial cells produced endothelium-derived relaxing factors (EDRF) and endothelium-derived contracting factors (EDCF) to regulate vasodilation and vasoconstriction respectively. Generally, EDRF consist of nitric oxide, prostacyclin and endothelium-derived hyperpolarizing factor. EDCF comprise of thromboxane A_2, endothelin-1 and angiotension II. Any disturbance in this tightly regulated equilibrium of vasodilation and vasoconstriction leads to endothelial dysfunction (ED).

2.1.3 Nitric Oxide

Nitric oxide (NO) is a structurally simple molecule that exerts effects on a wide variety of actions in the vasculature. NO is an endothelium-derived relaxing factor (EDRF) that was discovered in 1980 by Furchgott and Zawadzki (Furchgott

and Zawadzki, 1980); it has been recognized as a key determinant of vascular homeostasis, regulating several physiological properties of blood vessel, including vascular permeability, vasodilation and anti-thrombotic properties (Jin and Loscalzo, 2010). The bioavailability of NO represents a central feature of the normal vascular function and is required for maintaining vasodilator tone and inhibiting platelet activation, thereby preventing thrombosis and its clinical vascular consequences. Decreased production or increased metabolism of NO lead to NO insufficiency within the vasculature and its pathological consequences (Jin and Loscalzo, 2010).

2.1.3 (a) Synthesis of NO

NO synthesis occurs in a range of cell types within the vasculature, which include macrophages and the vascular endothelium (Jin and Loscalzo, 2010). NO is synthesized by a class of enzymes known as nitric oxide synthase (NOS), which include endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). The three NOS isoforms are characterized by their site of synthesis, pattern of expression, and calcium (Ca^{2+}) tendency: NOS I or nNOS is expressed primarily in neurons; NOS II or iNOS, is expressed in macrophages, neutrophils, platelets and VSMC, as wells as in other nonvascular cells; NOS III or eNOS is constitutively expressed in endothelial cells. nNOS and eNOS are more critical for normal physiology, whereas iNOS is associated with injury and inflammation (Kaszkin *et al*., 2004).

All of the enzymes catalyzed the five-electron oxidation of one of the terminal guanidine nitrogen atom of L-arginine to produce L-citruline and NO. Enzyme activity requires flavin mononucleotide (FMN), flavin adeninine dinucleotide (FAD), tetrahydrobiopterin (BH_4), Ca^{2+}-calmodulin, and heme, which serve as cofactors, along with nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen, which serve as co-substrates (Jin and Loscalzo, 2010) (Figure 2.2).

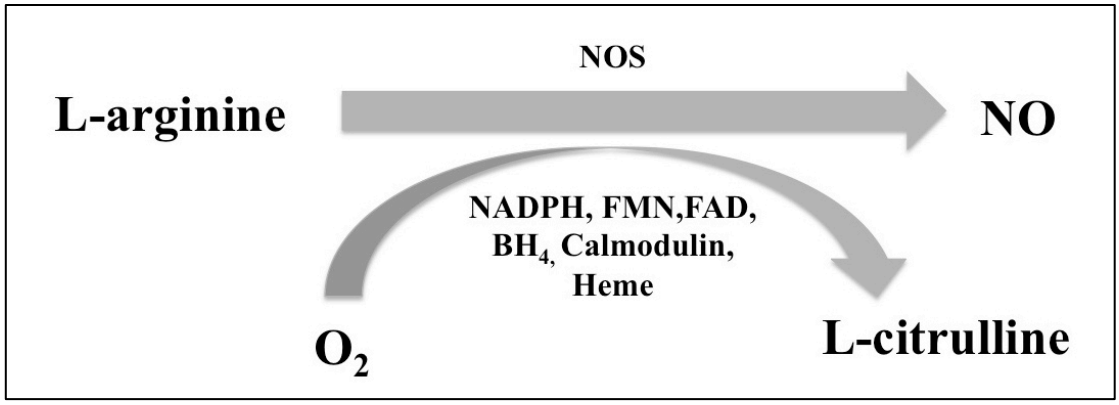

Figure 2.2 The synthesis of the nitric oxide. Figure modified from Jin and Loscalzo, (2010).

2.1.3 (b) Pathways of NO

Once synthesized, NO diffuses across the endothelial cell membrane and enters the VSMC where it stimulates soluble guanylyl cyclase (sGC) to induce formation of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). cGMP activates protein kinase G (PKG) which prevent Ca^{2+} influx from voltage-dependent Ca^{2+} channel (VDCC) and Ca^{2+} release mediated by inositol 1,4,5-

triphosphate receptor (IP_3R). PKG also act as sarco/endoplasmic reticulum calcium ATPase (SERCA) to promote the reuptake of cytosolic Ca^{2+} into the sarcoplasmic reticulum. It results in the reduction of intracellular Ca^{2+} concentration and inactivation of calmodulin, which is no longer able to activate myosin light chain kinase (MLCK). The depletion of intracellular Ca^{2+} also increases the activity of myosin light chain phosphatase (MLCP). The actin-myosin cross-bridge is broken and smooth muscle relaxation ensues (Figure 2.3)

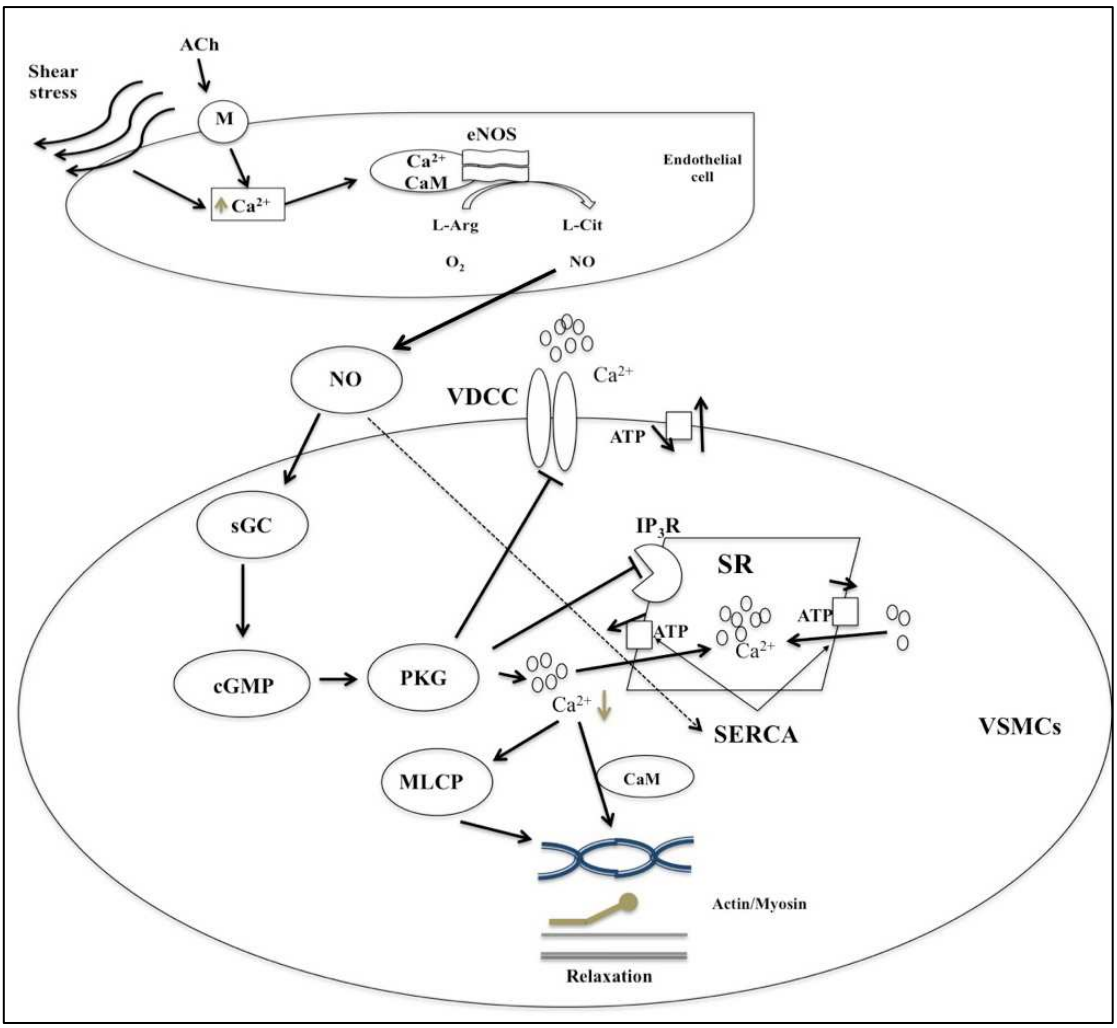

Figure 2.3 Schematic diagram of possible mechanism of NO-mediated response in the blood vessel. The activation of endothelial cells by ACh or shear stress leads to an increase in intracellular Ca^{2+} concentration and NO is produced. NO diffuse into VSMC and activates sGC, leading to production of cGMP. cGMP activates PKG. NO can also directly stimulate SERCA and facilitate the removal of Ca^{2+} from smooth muscle cytosol into the sarcoplasmic reticulum store. Figure modified from Zhao *et al*. (2015) (Zhao *et al*., 2015).

ATP, adenosine triphosphates; CaM, calmodulin; M, muscarinic receptor; MLCK, myosin light chain phosphatase; PKG, protein kinase G; SERCA, sarco/endoplamic reticulum Ca^{2+} ATPase; SR, sarcoplasmic reticulum

2.2 Diabetes Mellitus

2.2.1 Definition and Classification

Diabetes mellitus is group of metabolic disorders characterized by hyperglycemia resulting from defects in insulin secretion and/ or insulin action (American Diabetes Association, 2020). Diabetes can be classified into three categories depending on the underlying etiology.

Type 1 diabetes mellitus (T1DM), previously known as insulin-dependent diabetes, results from autoimmune destruction of β-cells of the pancreas. In this form of diabetes, the rate of β-cells destruction is quite variable, being rapid in some individuals (mainly infants and children) and slow in others (mainly adults). Some patients, particularly children and adolescent, may present with ketoacidosis as the first manifestation of the disease. Others have modest fasting hyperglycemia that can rapidly change to severe hyperglycemia and /or ketoacidosis in the presence of infection or other stress. At this latter stage of the disease, there is little or no insulin secretion (American Diabetes Association, 2020).

Type 2 diabetes mellitus (T2DM) is associated with a relative insulin deficiency rather than absolute (American Diabetes Association, 2020). Most patients with T2DM have some degree of insulin resistance associated with obesity as one of the major risk factors. Obesity is defined as excessive fat accumulation in adipose tissue that extends to morbidity and mortality due to weight-related complications; to the extent that health is impaired. Insulin resistance occurs due to reduced insulin sensitivity such that the cells are unable to respond well to insulin,

thus there is impairment in glucose uptake by cells, thus inability of cells to use glucose for energy. Hence, more insulin needs to be produced by the pancreatic β-cells to compensate for insulin resistance. Progressively, the amount of insulin become deficient in meeting further cellular requirements, a condition known as relative insulin deficiency, predisposing to the onset of hyperglycemia. Chronic condition of hyperglycemia could further damage the pancreatic β-cell, resulting in absolute insulin deficiency (Pratley, 2013).

Gestational diabetes mellitus is a metabolic disorder associated with glucose intolerance, which was first identified during pregnancy (American Diabetes Association, 2020). It occurs when placenta produce insulin-blocking hormones that leads to high blood glucose level. Women affected with gestational diabetes have a seven-fold increased risk of developing T2DM in the future after initial diagnosis. Their children are also prone to develop T2DM early in life (Bellamy *et al*., 2009; Rayanagoudar *et al*., 2016).

2.2.2 Diagnosis, Symptoms and Medication

T2DM is most commonly diagnosed using the parameters glycated haemoglobin (HbA_{1c}) and fasting blood glucose (FBG). HbA_{1C} indicates the average blood glucose levels for the past three months while FBG is the blood glucose level after an overnight fast. Individuals with HbA_{1C} levels of 6.5% or higher and FBG levels of 7.0 mmol/L or more is diagnosed as having T2DM (American Diabetes Association, 2008). Symptoms of diabetes may include frequent urination, excessive

thirst, increase in appetite, unusual fatigue, sudden weight loss, nausea, dizziness and slow wound healing.

Pharmacological management of T2DM may require the use of oral hypoglycemic agents, especially when a strict diet and exercise plan have failed to reduce blood glucose level. The oral hypoglycemic agents are classified into five major classes, which include biguanides, meglitinides, sulfonylureas, α-glucosidase inhibitors and thiazolidinediones (TZD). The biguanide class has metformin as a member, which improves insulin sensitivity and increase glucose uptake. Meglitinides include repaglinide and nateglinide; while sulfonylureas include gliclazide, glibenclamide and glimepiride. Both meglitinides and sulfonylureas promote the release of insulin from pancreatic β-cells. α-glucosidase inhibitors include acarbose and miglitol; they, preventing the digestion of carbohydrate in the intestine. TZD include pioglitazone and rosiglitazone; these drugs reduce insulin resistance and makes body tissue more sensitive to the effects of insulin insulin's effects. Insulin therapy was considered if the situation of inadequate glycemic control on optimal dose and number of oral hypoglycemic agents (Ministry of Health Malaysia, 2015b).

In this study, metformin has been used as a positive control in the treatment of T2DM rat model. Metformin is the often the first line of drug used in the management of T2DM (Kinaan *et al*., 2015). It is decreasing insulin resistance thus increases glucose uptake and use by target tissues. This also reduces hyperglycemia. Other reported mechanisms of action of metformin in reducing blood glucose levels

include: i) reduced hepatic and renal gluconeogenesis; ii) slowing of glucose absorption from gastrointestinal tract and improve peripheral glucose uptake and utilization; iii) reduction of plasma glucagon levels.

2.2.3 Animal Models of Diabetes Mellitus

Animal models have been used extensively to investigate *in vivo* efficacy, mode of action and side effects of anti-diabetic drugs and plants. Due to the heterogeneity of diabetic conditions in man, no single animal model is entirely representative of a particular type of human diabetes. Thus, many different animal models have been used, each displaying a different selection of features seen in human diabetic states (Eddouks *et al*., 2012). The most widely used animal models are small rodents, which are less expensive to maintain than larger animals and generally show a more rapid onset of their diabetic condition consistent with their short lifespan.

Diabetes can be developed via surgical, pharmacologic, or genetic manipulations in several animal species. Streptozotocin (STZ) is the most frequently used drug to induce diabetes and this model has been useful for the study of multiple aspects of diabetes mellitus. STZ is often administered parentally via intraperitoneal, intravenous or subcutaneous routes (Eddouks *et al*., 2012). STZ enters the β-pancreatic cell via a glucose transporter 2 (GLUT2) and causes alkylation of DNA (Ishizaka *et al*., 2010; Eddouks *et al*., 2012). As a result of the DNA alkylation, pancreatic β-cell is destroyed by necrosis. The destruction of pancreatic β-cells

disrupts the ability of the pancreas to produce insulin, leading to a T1DM animal model.

A high-fat diet can be formulated by using a standard diet combined with animal fat (ghee) and sugar (to increase the caloric value). Feeding high-fat diet in developing a T2DM rat model has become increasingly popular in recent years. Feeding high-fat diet leads to the development of obesity and consequently insulin resistance. Subsequent administration of a low-dose STZ injection causes mild dysfunction in pancreatic β-cell without destroying all the pancreatic β-cells. This will compromise insulin secretion by the pancreas. Thus, feeding high-fat diet with administration of low-dose STZ closely mimics the natural development of T2DM (from insulin resistance to β-cell dysfunction) as well as its metabolic features (Guo *et al*., 2018). Studies have shown that mice fed with only high-fat diet develop symptoms related to T2DM such as hyperinsulinemia and insulin resistance but not hyperglycemia (Srinivasan *et al*., 2005; Magalhães *et al*., 2019). On the other hand, using a high-fat diet with a low-dose STZ has shown positive results in establishing obesity, insulin resistance, T2DM and hyperglycemia (Magalhães *et al*., 2019). However, currently there are no standardization on the development of T2DM animal model in terms of composition of the high-fat diet, dose of STZ to be administered, the species and the age of animals to be used (Atanasovska *et al*., 2014; Magalhães *et al*., 2019).

2.3 Diabetes and Vascular Complications

Cardiovascular complications are the primary cause of death in diabetic patients. Diabetes can cause microvascular and macrovascular complications. Microvascular complications include retinopathy (eye damage), neuropathy (neuron damage) and nephropathy (kidney damage). Macrovascular complications comprise of coronary artery disease, cerebrovascular disease and peripheral arterial disease (Forbes and Cooper, 2013). Diabetes affects the function of multiple cell types in the blood vessel, including the endothelium and VSMC.

2.3.1 Effect of Diabetes on the Endothelium

Diabetes is associated with endothelial dysfunction (ED). ED is an early step in the development of atherosclerosis. ED is characterized by impaired endothelium-mediated relaxation. It occurs due to the imbalance between vasodilation and vasoconstriction due to reduction in the bioavailability of vasodilators, particularly NO (Zhu *et al*., 2011). In diabetes, hyperglycemia increases reactive oxygen species (ROS) production via glucose autoxidation and advanced glycation end products (AGEs). Increase ROS production augmented its tendency to react with NO to form cytotoxic oxidant peroxynitrite. Peroxynitrite lead to degradation of eNOS cofactor tetrahyrobiopterin (BH_4), causing the uncoupling of eNOS, thus reducing NO bioavailability (Endemann and Schiffrin, 2004). In diabetes, ED leads to increased endothelial cell permeability, which increase LDL uptake into subendothelial space. LDL-C undergoes oxidation (oxLDL-C) due to increase ROS level; resulting from hyperglycemia in diabetes. oxLDL-C is taken up by macrophages and undergo

inflammatory process, thus lead to foam cell formation and subsequently atherosclerotic plaque. In addition, reduced NO bioavailability upregulated adhesion molecules such as intracellular adhesion molecule (ICAM), vascular adhesion molecule (VCAM) and E-selectin in endothelial cells via induction of nuclear factor kappa B (NF-κB) expression (Khan *et al*., 1996). Increases in adhesion molecules initiate the inflammatory process that lead to the atherosclerotic plaque formation.

2.3.1 (a) Endothelium-dependent Relaxation

Previous studies have shown that NO-mediated relaxations were reduced in the aorta of Goto Kakizaki (non-obese, spontaneous T2DM rat model) rats (Sena *et al*., 2011; Kobayashi *et al*., 2012) and high-fat diet fed with low-dose STZ-induced T2DM rats (Oztürk *et al*., 2015). It was shown that the impairment of ACh-induced endothelium-dependent relaxation was due to reduced bioavailability of NO, decreased expression of eNOS mRNA, and higher oxidative stress level in rat aorta of both diabetic rat models (Sena *et al*., 2011; Oztürk *et al*., 2015). In diabetes, hyperglycemia increases production free radical species particularly superoxide anion. High production of superoxide anion affects the activity of NO; it reacts with NO to form peroxynitrite, which reduced NO bioavailability resulting in the impairment of NO-mediated relaxation.

2.3.1 (b) Endothelium-dependent Contraction

A previous study by Tesfamariam *et al*. (1989) has shown that ACh-induced endothelium-dependent contraction was increased in aortic rings of diabetic rabbits

(Tesfamariam *et al*., 1989). Another study by Shi *et al*. (2007) reported that calcium ionophore-induced endothelium-dependent contraction was increased in the aorta of T1DM diabetic rats (Shi *et al*., 2007). It has been shown that the hypercontractility in T1DM diabetic arteries were associated with high oxidative stress level which increases the production of endothelium-derived contracting factors (EDCF) (Tang *et al*., 2007; Matsumoto *et al*., 2008;). Due to hyperglycemia, increased production of superoxide anion reduces the activity of the antioxidant enzyme SOD to counteract the superoxide anion, thus reducing NO bioavailability. Reduced NO leads to EDCF activation and production, thus resulting in augmented smooth muscle contraction.

2.3.2 Effect of Diabetes on Vascular Smooth Muscle

2.3.2 (a) Vascular Smooth Muscle Relaxation

Studies have shown that the response of VSMC to the NO donor, sodium nitroprusside was not affected in renal arteries and aorta of T2DM rats (Viswanad *et al*., 2006; Sena *et al*., 2011). However, a previous study has shown that sodium nitropruside-induced relaxation was attenuated in female pregnant T2DM rat model (Goulopoulou *et al*., 2014).

2.3.2 (b) Vascular Smooth Muscle Contraction

Vascular smooth muscle response to the endothelium-independent vasoconstrictor, phenylephrine was shown to be increased in small mesenteric and aorta of 12 weeks old T2DM *db/db* mice (Guo *et al*., 2005) and thoracic aorta of 24 weeks old T2DM *ob/ob* mice (Nevelsteen *et al*., 2013). It has been shown that