EARLY AND LATE CHANGES IN ENDOTHELIAL DEPENDENT VASCULAR RELAXATION AND CONTRACTION RESPONSES IN THE MICROCIRCULATION OF DIABETIC RATS

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by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

AA	Arachidonic acid
AC	Adenylyl cyclase
ACh	Acetylcholine
BH_4	Tetrahydrobiopterin
BK_{Ca}	High conductance calcium activated potassium channel
Ca	Calcium
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
Ca_V	Voltage-dependent calcium channels
cGMP	Cyclic guanosine monophosphate
cGTP	Cyclic guanosine triphosphate
COX	Cyclooxygenase
CVD	Cardiovascular disease
DM	Diabetes mellitus
EDCF	Endothelium-derived contracting factor
EDH	Endothelium-dependent hyperpolarization
EDRF	Endothelium-derived relaxing factor
EET	Epoxyeicosatrienoic acid
eNOS	Endothelial nitric oxide synthase
FAD	Flavin adenine dinucleotide
FBG	Fasting blood glucose
FMN	Flavin mononucleotide
GC	Guanylyl cyclase
H_2O_2	Hydrogen peroxide
HbA_{1C}	Glycated haemoglobin
HRP	Horseradish peroxidase
IK_{Ca}	Intermediate conductance calcium activated potassium channel
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
IP	Prostacyclin receptor
IP_3R_1	Inositol-1, 4, 5-trophosphate receptor type-1
IRAG	IP3 receptor associated cGMP kinase substrate
K^+	Potassium ion
Kca	Calcium activated potassium channel
KCl	Potassium chloride
K_{IR}	Inward- rectifier potassium channel
LPC	Lysophosphatidylcholine
M	Muscarinic receptor
MEGJ	Myo-endothelial gap junction
MLC	Myosin light chain
MLCK	Myosin light chain kinase
Na^+/K^+-ATPase	
NADPH	Nicotinamide adenine dinucleotide phosphate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NS398	N-[2-(Cyclohexyloxy)-4-nitrophenyl]methanesulfonamide
PAD	Peripheral arterial disease

PBS	Phosphate buffer saline
PDE5	Phosphodiesterase 5
PE	Phenylephrine
PGI_2	Prostacyclin
PGIS	Prostacyclin synthase
PKA	Protein kinase A
PKG	Protein kinase G
PL	Phospholipase
PLA_2	Phospholipase A_2
PSS	Physiological saline solution
PVDF	Polyvidinylidene fluoride membrane
SBP	Systolic blood pressure
SD	Sprague Dawley
SDS	Sodium dodecyl sulfate
SERCA	Sarco/endoplasmic reticulum Ca^{2+} ATPase
SK_{Ca}	Small conductance calcium activated potassium channel
STZ	Streptozotocin
S18886	(3-[(6-amino-(4-chlorobenzensulphonyl)-2-methyl-5,6,7,8-tetrahydronapht]-1-yl)propionic acid
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline Tween-20
TXA_2	Thromboxane
TRAM 34	1-[(2-Chlorophenyl) diphenylmethyl]-1*H*-pyrazole
TRP	Transient receptor potential
TRPV4	TRP vanilloid type 4
UCL 1684	6,10-diaza-3(1,3)8,(1,4)-dibenzena-1,5(1,4)-diquinolinacyclodecaphane
VSMC	Vascular smooth muscle
WHO	World Health Organization

PERUBAHAN AWAL DAN LEWAT DALAM TINDAK BALAS PENGENDURAN DAN PENGECUTAN BERPERANTARA-ENDOTELIUM DI DALAM SALUR DARAH KECIL PADA TIKUS DIABETES

ABSTRAK

Diabetes berhubungkait dengan komplikasi salur darah kecil dan besar yang menyumbang kepada disfungsi lapisan endotelium dan membawa kepada penyakit kardiovaskular. Disfungsi endotelium (ED) dicirikan oleh gangguan pengenduran berperantara-endotelium dan peningkatan pengecutan-berperantara-endotelium. Sel endotelium menghasilkan faktor-faktor yang menyebabkan pengenduran dan pengecutan endotelium. Peranan faktor-faktor ini di dalam salur darah kecil tidak dicirikan dengan baik. Terdapat tiga objektif utama di dalam kajian ini; pertama adalah untuk mengetahui sumbangan nitrik oksida (NO), prostasiklin, hiperpolarisasi berperantara-endotelium (EDH) dan reseptor thromboxane (TXA_2) dalam pengenduran dan pengecutan berperantara-endotelium di dalam salur darah kecil tikus diabetes; kedua, untuk mengetahui ekspresi enzim dan reseptor yang terlibat dalam pengantaraan tindak balas endotelium dan akhirnya untuk mengetahui perubahan awal (2 minggu) dan lewat (10 minggu) pada tindak balas fungsi and molekular di dalam salur darah kecil tikus diabetes. Kajian in melibatkan empat kumpulan kajian: tikus normal 2-minggu, tikus diabetes 2-minggu, tikus normal 10-minggu dan tikus diabetes 10-minggu (n=15 ekor tikus bagi setiap kumpulan). Kajian ke atas fungsi salur darah ditentukan dengan menggunakan myograph dawai. Sumbangan setiap EDRF (NO, prostasiklin dan EDH) dan reseptor TXA_2 dalam pengenduran dan pengecutan berperantara-endotelium dinilai di dalam arteri ekor semua tikus kajian. Ekspresi dan taburan protein sintase nitrik

oksida endotelium (eNOS), siklooksigenase-1 (COX-1) dan siklooksigenase-2 (COX-2), sintase prostasiklin (PGIS), reseptor prostasiklin (IP), reseptor TXA_2 dan sintase TXA_2 ditentukan dengan pemblotan Western dan imunohistokimia. Pengenduran berperantara-endotelium berkurangan secara signifikan dalam tikus diabetes 2-minggu [R_{mak}; 73.49 (11.04)% vs 89.32 (10.03)%, p=0.002] dan tikus diabetes 10-minggu [R_{mak}; 58.84 (18.79)% vs 89.32 (10.03)%, p<0.01] masing-masing berbanding dengan tikus normal 2-minggu. Pengenduran berperantara–NO berkurangan dalam tikus diabetes 10-minggu berbanding dengan tikus normal 2-minggu (p<0.001) dan tikus normal 10-minggu (p<0.001). Pengenduran berperantara-EDH berkurangan dalam tikus diabetes 10-minggu berbanding dengan tikus normal 2-minggu (p=0.012) dan tikus normal 10-minggu (p=0.017). Kemorosatan pengenduran kepada prostasiklin dilihat dalam tikus diabetes 10-minggu berbanding tikus normal 2-minggu (p<0.001) dan tikus diabetes 2-minggu (p=0.033). Pemblotan Western dan imunohistokimia menunjukkan diabetes mengurangkan ekpresi protein eNOS, reseptor IP dan PGIS di dalam arteri ekor tikus. Dalam pengecutan berperantara-endotelium, peningkatan secara signifikan dilihat dalam tikus diabetes 10-minggu [$E_{mak;}$ 113.73 (51.32)% vs 34.80 (21.00)%, p<0.001], trend peningkatan dilihat dalam tikus diabetes 2-minggu [$E_{mak;}$ 71.80 (46.02)% vs 34.80 (21.00)%, p<0.058] berbanding dengan tikus normal 2-minggu. Peningkatan secara signifikan di dalam kedua-dua pengecutan berperantara-COX-1 dan COX-2, dan pengecutan berperantara-reseptor TXA_2 dilihat dalam tikus diabetes 10-minggu. Trend peningkatan pada ekspresi protein COX-2 dan reseptor TXA_2 diperhatikan dalam tikus diabetes 10-minggu, dan ini menyokong dapatan kajian fungsi. Ringkasannya, pengenduran dan pengecutan berperantara-endotelium dalam salur darah kecil didapati terjejas di dalam diabetes dan ini menjadi semakin

teruk dengan diabetes yang berpanjangan. Sehubungan itu, pencegahan awal diperlukan untuk menangani ED yang mungkin boleh pulih pada peringkat awal diabetes.

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ABSTRACT

Diabetes is associated with micro and macrovascular complications which contributes to endothelial dysfunction (ED) and leads to cardiovascular diseases. ED is characterized by impairment of endothelium-dependent relaxation and increases in endothelium-dependent contraction. The endothelial cells release factors that cause endothelial relaxation and contraction. The role of these factors in the microvasculature of diabetes is not well characterized. There were three main objectives of this study; firstly, to determine the contribution of nitric oxide (NO), prostacyclin, endothelium-dependent hyperpolarization (EDH) and thromboxane (TXA_2) receptor in endothelium-dependent relaxation and contraction in the microcirculation of diabetic rats; secondly, to determine the expression of enzymes and receptors involved in mediating endothelial responses and finally to determine the early (2 weeks) and late changes (10 weeks) in the functional and molecular responses in the microcirculation of diabetes rats. This study consisted of four experimental groups; normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats (n=15 rats per group). Vascular function studies were performed using wire myography. The contributions of individual EDRF (NO, prostacyclin and EDH) and TXA_2 receptor in mediating endothelium-dependent relaxations and contractions were evaluated in tail arteries of all the experimental groups. The expressions and distributions of endothelial nitric oxide synthase (eNOS), cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), prostacyclin

synthase (PGIS), prostacyclin (IP) receptor, TXA_2 receptor and TXA_2 synthase proteins were determined by Western blotting and immunohistochemistry. Endothelium-dependent relaxations were significantly decreased in diabetic 2-week [R_{max}; 73.49 (11.04) % vs 89.32 (10.03) %, p=0.002] and diabetic 10-week rats [R_{max}; 58.84 (18.79) % vs 89.32 (10.03) %, p<0.01], respectively compared with normal 2-week rats. NO-mediated relaxations were attenuated in diabetic 10-week rats compared with normal 2-week (p<0.001) and normal 10-week rats (p<0.001). EDH-mediated relaxations were lower in diabetic 10-week rats compared to normal 2-week rats (p=0.012) and normal 10-week rats (p=0.017). Diminished relaxations to prostacyclin were seen in diabetic 10-week rats compared to normal 2-week (p<0.001) and diabetic 2-week rats (p=0.033). Western blotting and immunostaining showed that diabetes reduced expression of eNOS, IP receptor and PGIS proteins in rat tail arteries. For endothelium-mediated contractions, significant increase in endothelium-dependent contractions were seen in diabetic 10-week rats [$E_{max;}$ 113.73 (51.32) % vs 34.80 (21.00) %, p<0.001], with a trend of increase in diabetic 2-week rats [$E_{max;}$ 71.80 (46.02) % vs 34.80 (21.00) %, p<0.058] compared to normal 2-week rats. Significant increases in both COX-1 and COX-2 mediated contractions, and TXA_2 receptor mediated contractions were seen in diabetic 10-week rats. Trend of increased expression of COX-2 and TXA_2 receptor proteins were observed in diabetic 10-week rats supporting the findings of functional studies. In conclusion, impairment in microvascular endothelium-dependent relaxations and increased endothelium-dependent contractions were observed in diabetic rats, and this worsened by prolonged diabetes. Therefore, early prevention is necessary to manage ED which may be reversible at an early stage of diabetes.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Vasculature

1.1.1 Introduction to vasculature and anatomy of the blood vessel wall

The human circulatory system is a huge network of organs and vessels which is responsible for the distribution of blood, nutrients, hormones, oxygen and other gases to cells in the body. It is involved in the removal of waste products such as carbon dioxide from the cells. It also plays a prominent role in stabilizing body temperature and pH, and maintains homeostasis. The circulatory system is made up of three main independent systems that work together: the heart (cardiac); lungs (pulmonary); and vascular system consisting of arteries, veins, coronary and portal blood vessels.

There are three major forms of blood vessels: artery, vein and capillary which differ in sizes and wall thickness. The arteries transport blood away from the heart while the capillaries facilitate the exchange of nutrients and gas between the blood and tissues. The blood is later transported back to the heart by the veins.

Both arteries and veins exhibit similar structure except for the wall thickness: arterial wall is thicker, as they carry blood under a higher pressure. Capillaries are made up of a layer of endothelium and occasional connective tissue. The walls of arteries and veins are made up of three distinctive layers known as tunica intima (inner layer), tunica media (middle layer) and tunica adventitia (outer layer) [Figure 1.1]. Tunica intima is lined by a mono layer of endothelial cells that surrounds the vascular lumen and comes in contact with blood flow. This layer is separated from the surrounding outer layers by the basal lamina. The middle layer or tunica media is

made up by vascular smooth muscle cells (VSMC) and circularly arranged elastic fibre. This layer is separated from the outer layer by a thick elastic tissue called the external elastic lamina. The tunica adventitia, the outer layer is completely composed of connective tissue and is interlaced with nerve fibres that supply the blood vessel (nervi vasorum) and nutrient capillaries (vasa vasorum) in the larger blood vessels (Figure 1.2).

As mentioned above, the core purpose of arteries is to supply body organs with blood. Due to the high pressure in the arteries, their walls are generally thicker than vein and as the blood flows to the organs, the arteries branches to smaller forms (Sandoo *et al.*, 2010). Arteries can be classified into conducting arteries, conduit arteries and resistance arteries based on their location in the arterial tree (Figure 1.3). Conducting arteries are the largest arteries (aorta, pulmonary and carotid artery) (McEniery *et al.*, 2007) in the body and have an enormous amount of elastic tissue that permits the vessel to expand and contract accordingly in response to ventricular contractions. These conducting arteries bifurcate into conduit arteries such as the brachial, radial and femoral arteries to direct blood to specific regions of the body (Pugsley and Tabrizchi, 2000). These conduit arteries further split to resistance arteries which are responsible for adequate perfusion of organ tissues and later distally form the microcirculation (Sandoo *et al.*, 2010).

The endothelial cell lining is present in all blood vessels, although the amount of connective tissue and smooth muscle vary according to the vessel's diameter and function. Therefore, endothelial cells are fundamental components that line the entire vascular system, from the heart to the smallest capillary, and play an important role in regulating the passage of materials (nutrients, gases, white blood cells) into and out of the bloodstream (Alberts *et al.*,2002)

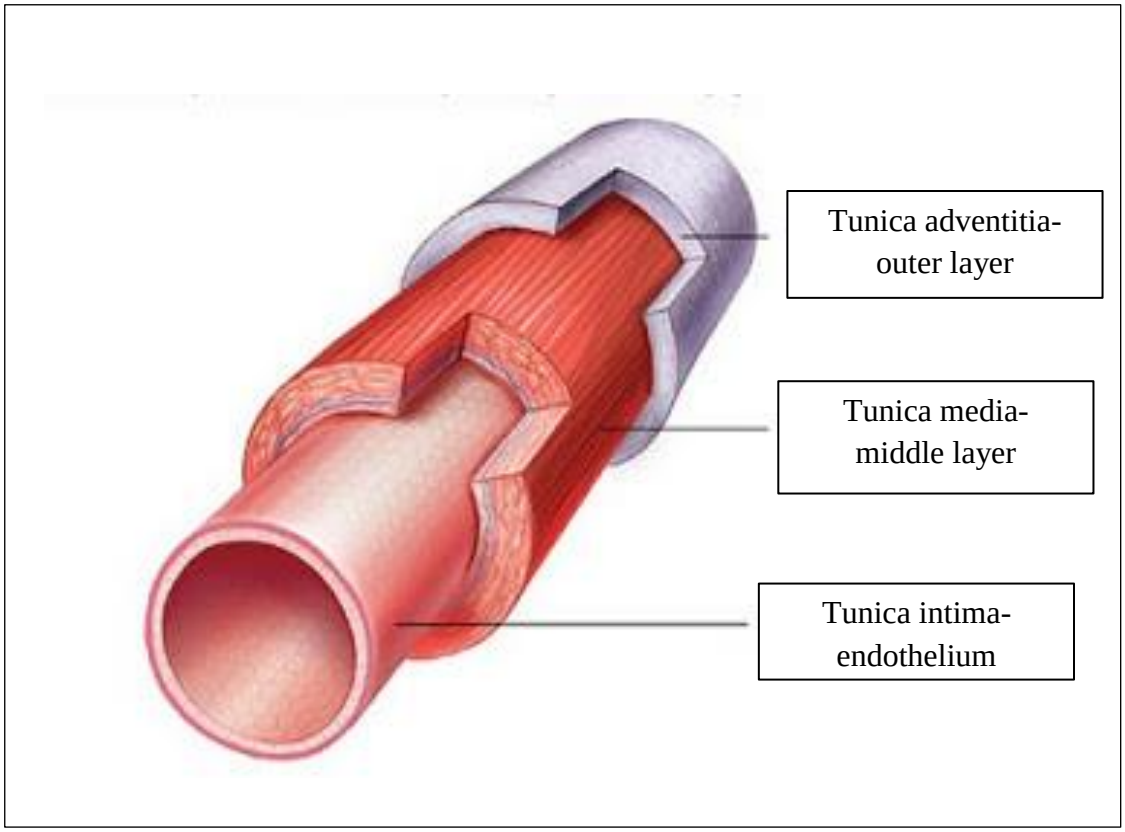


Figure 1.1 Three major layers of the human blood vessel
Modified from http://fibromusculardysplasia.blogspot.my/2011/10/classifying-fibromuscular-dyplasia.html

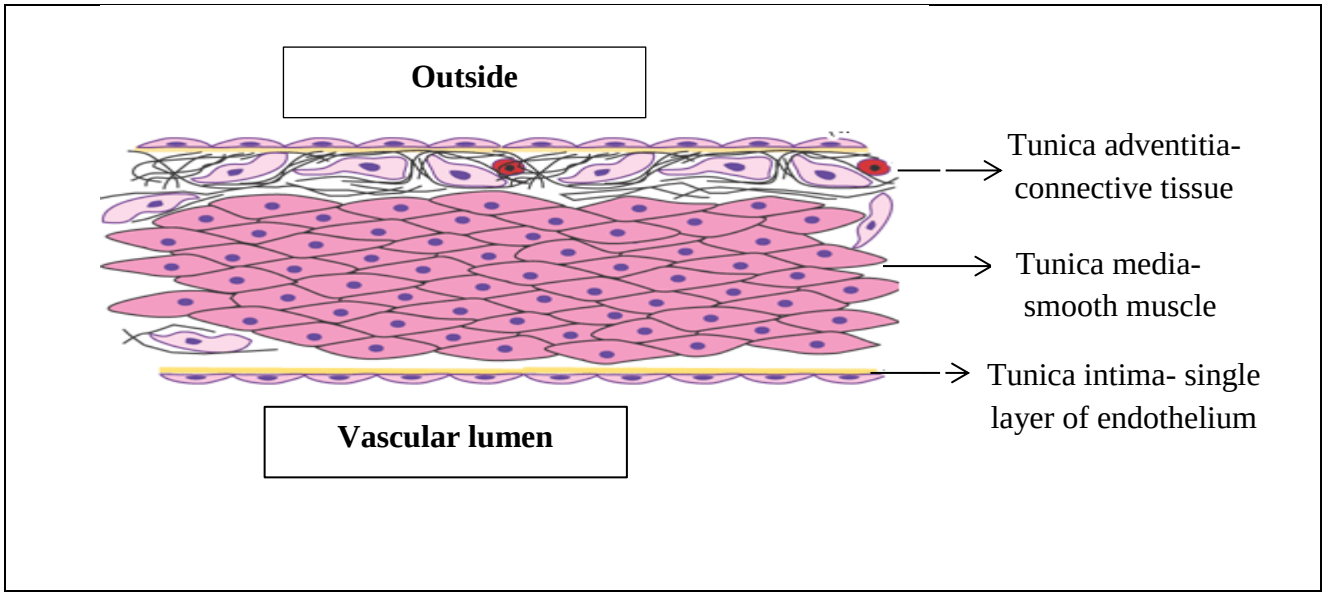


Figure 1.2 Histology of the blood vessels.
Modified from http://www.histology.leeds.ac.uk/circulatory/circ_common_str.php

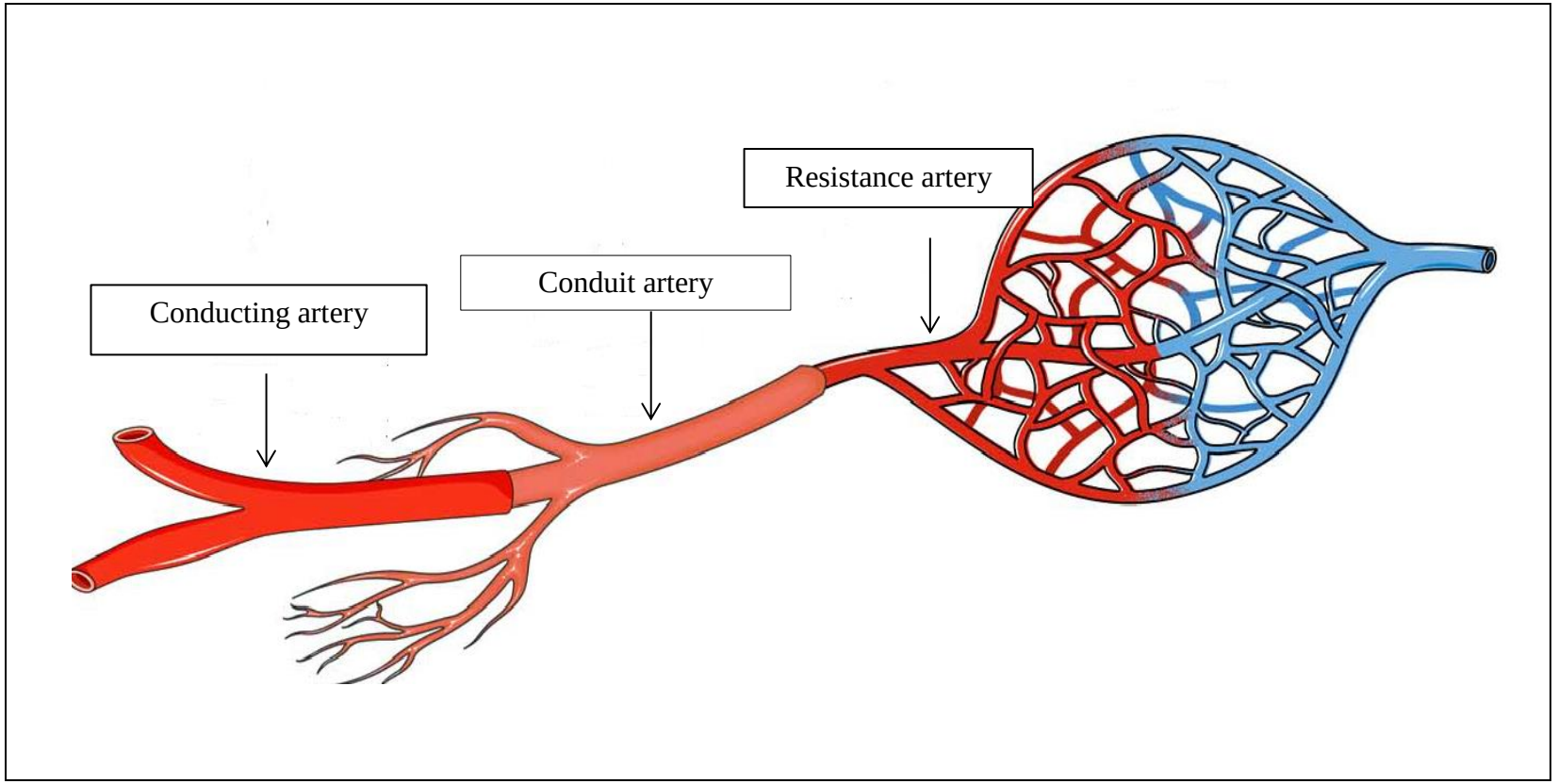


Figure 1.3 Illustration on the type of arteries in the human body. Modified from Sandoo et al 2010

1.1.2 Microcirculation

The microcirculation is described as small blood vessels of the vasculature that are embedded within the organs. These small vessels include small arteries, arterioles, capillaries, and venules (Levy *et al.*, 2001; Levy *et al.*, 2008). According to Mulvany *et al.*, 1990, small arteries with lumen diameter less than 500 μm are considered as microcirculation (Mulvany *et al.*, 1990).

The main function of the microcirculation is to optimize nutrient and oxygen supply within the tissue where adequate perfusion is crucial for the integrity of tissue and organ function (Levy *et al.*, 2001). Furthermore, it is noted that at the level of the microcirculation, substantial drop in hydrostatic pressure occurs in the blood vessel. These arteries are known as resistance arteries and can easily adjust to the changes (vasodilation or vasoconstriction) in compliance to the metabolic needs of the tissues. Due to their ability in adapting to changes in local blood regulations, small arteries are extremely important in the regulation of overall peripheral resistance and responsible in controlling the systemic blood pressure (Christensen and Mulvany, 2001).

Microvascular endothelial cells are one of the main targets for hyperglycemic damage, since the endothelial cells are unable to downregulate glucose transporter rate when the glucose concentration is high, thus causing intracellular hyperglycemia (Brownlee, 2005; Stehouwer, 2018). This causes microvascular endothelial dysfunction which is characterized by a decrease in nitric oxide (NO) availability, increased vascular permeability, increased leukocyte adhesion, and increased procoagulant activity (Brownlee, 2005; Stehouwer, 2018). Microvascular endothelial dysfunction can cause structural and functional alterations which contribute to the

risk of developing cardiovascular complications such as coronary heart disease, cardiomyopathy, congestive heart failure; cerebrovascular and peripheral arterial disease (Rizzoni *et al.*, 2003; Kalani, 2008; Lockhart *et al.*, 2009).

Studying the microvasculature bed has become an area of interest ever since the microcirculation is reported to be the initial site for early manifestation of vasculopathy (Levy *et al.,* 2001). Microvascular dysfunction takes place in multiple tissue beds long before the onset of atherosclerotic symptoms (Abularrage *et al.*, 2005). Impairment in the microvascular function is seen in patients with obesity (Al-Tahami *et al.*, 2011), diabetes mellitus (DM) (Caballero *et al.*, 1999), hypertension (Levy *et al.*, 2008) and renal diseases (Ramunni *et al.*, 2009).

1.1.3 The endothelium

Endothelium is a simple squamous cell lining the entire circulatory system, from the heart to the smallest capillaries (Rajendran *et al.*, 2013). Endothelial cells serve multiple purposes, though initially endothelium was considered to function as a barrier between the blood vessels and blood only (Galley and Webster, 2004; Rajendran *et al.*, 2013).

The discovery by Furchgott and Zawadzki in 1980 regarding the ability of endothelial to control vascular tone was a pioneering finding that leads to better understanding of endothelial functions beyond the barrier roles (Furchgott and Zawadzki, 1980). Apart from its role as a regulator of vascular tone, the endothelium is also involved in multiple roles such as in modulation of platelet activation, leukocyte adhesion, and thrombosis. It also functions in balancing the counter regulatory pathways which includes the control of vasomotion, cell proliferation, thrombosis, inflammation, and oxidation (Kuvin and Karas, 2003).

Any disruptions to endothelial function lead to endothelial dysfunction (ED) which has been linked with cardiovascular disease progression. Exposure to cardiovascular risk factors such as tobacco use, physical inactivity, and obesity, increasing age, hypertension, hyperlipidemia and insulin resistance / hyperglycaemia can result in reversible or irreversible endothelial damage (Celermajer, 1997; Obrenovic-Kircanski, 2007). These harmful risk factors compromised the endothelial defence mechanism causing endothelial cell activation and dysfunction.

Modifications in vasculature due to ED are linked to the pathogenesis of numerous diseases. One of the early signs of atherosclerosis is ED that occurs before morphological changes are visible in the vessel wall (Davignon and Ganz, 2004). ED plays a central role in the early pathophysiology of atherosclerosis in diabetes, hypertension and hyperlipidemia which are risk factors for cardiovascular disease (Park *et al.*, 2001; Low Wang *et al.*, 2016).

1.1.3(a) Endothelium as a vascular tone regulator

One of the most important roles of endothelium is to synthesize and secrete vasoactive substances that regulate vascular tone which contribute to vascular homeostasis. Vasoactive substances released by endothelial cells can be divided into two types: endothelium-derived relaxing factors (EDRFs) and endothelium-derived contracting factors (EDCFs) (Furchgott and Vanhoutte, 1989). EDRFs such as NO, prostacyclin (PGI_2) and endothelium derived hyperpolarizing factor (EDH) cause blood vessels relaxation whereas EDCF such as endothelin I, angiotensin II and thromboxane A_2 cause blood vessels contraction.

Generally, a healthy and intact endothelium maintains the vessel environment in a relatively neutral condition favouring dilatation over constriction. However, any

damage to endothelial cells leads to disturbance in vascular homeostasis. Damage to the endothelium disrupts the balance between vasodilators and vasoconstrictor and initiates the process of atherosclerosis that eventually leads to cardiovascular events (Davignon and Ganz, 2004).

1.1.3(b) Endothelium-derived relaxing factors (EDRF)

1.1.3(b)(i) Nitric oxide (NO)

One of the most important vasodilator produced by the endothelium is NO (Palmer *et al.*, 1988; Furchgott and Vanhoutte, 1989). It mediates endothelium-dependent vasodilation by opposing the effects of endothelial contracting factors.

NO is not only responsible for the vasodilatation which helps in blood pressure regulation (Moncada and Higgs, 1993), but also acts as anti-atherogenic substance. It impedes atherogenesis by inhibiting leukocyte and platelet activation, smooth muscle cell proliferation, oxidation of low density lipoproteins, synthesis of cytokines and platelet aggregation (Radomski *et al.*, 1987; Hogg *et al.*, 1995; Donald M. Lloyd-Jones and Kenneth D. Bloch, 1996; Rubbo *et al.*, 2002; Levine *et al.*, 2012)

Since NO plays a major role in vasodilation, diminished vasodilation is often related to the defect in NO production or activity. The reduced production of NO is mainly because of injury or damage to endothelial cells. The impairment in NO production promotes atherosclerosis by causing vasoconstriction, platelet aggregation, smooth muscle cell proliferation and migration, leukocyte adhesion, and oxidative stress (Davignon and Ganz, 2004).

NO is a soluble gas that is continuously synthesized by endothelial cells due to its naturally short half-life; ~6– 30 seconds (Palmer *et al.*, 1988; Dimitris *et al.*,

2012). It is synthesized from the amino acid L-arginine by the enzymatic action of nitric oxide synthase (NOS) (Dimitris *et al.*, 2012). Three different isoforms of NOs exist, that are endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) nitric oxide synthase, with each varying in structure and function. eNOS causes endothelial vasodilatation as it catalyses the oxidation of L-arginine resulting in the production of NO mainly, and L-citrulline as a by-product (Moncada and Higgs, 1993;Donald M. Lloyd-Jones and Kenneth D. Bloch, 1996). nNOS isoform acts as modulator of neuronal function which regulates synaptic neurotransmitter discharge (Prast and Philippu, 2001). Another isoform, iNOS is only expressed in cells that have been exposed to inflammatory conditions which activate the macrophages (Michel and Feron, 1997).

The ability of blood vessels to relax depends on the activity of eNOS. eNOS is present in two forms: constitutive NOS (cNOS; type III) and inducible NOS (iNOS; type II). Under basal conditions, nitric oxide is primarily synthesized by cNOS which plays a major role in blood pressure regulation (Rees *et al.*, 1989), whereas the production of NO from iNOS is very low. However during inflammation, the amount of NO produced by iNOS is greater than the amount produced by cNOS. The activity of iNOS is induced by immunological stimuli [bacterial endotoxins (e.g., lipopolysaccharide) and cytokines eg., tumor necrosis factor (TNF) and interleukins] and is Ca^{2+} independent (Schulz *et al.*, 1992)

Multiple cofactors such as reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavine adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin are also needed for NO production

The NO production in the intact blood vessel is mostly obtained from the activity of endothelial cNOS (Forstermann *et al.*, 1991). cNOS activity can be

triggered to chemical stimuli (acetylcholine, bradykinin, adenosine triphosphate, thrombin, serotonin, histamine, insulin and substance P) or physical stimuli (shear stress, pulsatile stretching of the vascular wall and hypoxia) (Schmidt et al., 1992; (Donald M. Lloyd-Jones and Kenneth D. Bloch, 1996), where both works by increasing the calcium level. Other vasoactive substances such as bradykinin can also trigger NO and the release other vasodilators (such as prostacyclin and EDH) resulting in the inhibition of platelet aggregation and blood vessels relaxation (Groves *et al.*, 1995; Drexler, 1998).

The three NOS isoforms depend on the enzyme's binding of the calcium regulatory protein calmodulin [CaM]. Both eNOS and nNOS are calcium dependent whereas iNOS is calcium independent and capable to bind with calmodulin even at low calcium level (Nathan and Xie, 1994; Michel and Feron, 1997).

Inactive eNOS is bound to the protein caveolin which is located in small invaginations in the cell membrane called caveolae (Bucci *et al.*, 2000; Sandoo *et al.*, 2010). The stimulation from agonist or other stimuli invokes a local release of calcium which in turn causes eNOS to detach from caveolin (Bucci *et al.*, 2000; Sandoo *et al.*, 2010). The intracellular calcium attaches to the CaM in the cytoplasm of the cell, causes structural changes that allows it to bind to eNOS to get activated (Fleming and Busse, 1999; Sandoo *et al.*, 2010). eNOS then converts L-arginine into NO (Palmer *et al.*, 1988; Sandoo *et al.*, 2010)

The mechanism of NO production is entirely reliant on the levels of intracellular of Ca^{2+} in the endoplasmic reticulum and extracellular stores which diffuses into the cell. A reduction in Ca^{2+} causes the calcium-calmodulin complex to dissociate from eNOS, which in turn binds with caveolin and becomes inactivated (Fleming and Busse, 1999)

As soon as NO is formed, it diffuses across the endothelial cell into the neighbouring smooth muscle where it binds to and activates the enzyme guanylyl cyclase (GC) (Ignarro *et al.*, 1986). The GC mediates the conversion of guanosine triphosphate (GTP) to cGMP, which in turn induces smooth muscle relaxation via various mechanisms. cGMP is later hydrolysed back to GMP by a family of phosphodiesterase 5 (PDE5).

cGMP stimulates protein kinase G (PKG) in order to activate myosin light chain phosphatase, the enzyme that dephosphorylates myosin light chains (MLC), preventing the MLC to bind to actin, thus preventing vasoconstriction and therefore promoting VSMC relaxation (Lee *et al.*, 1997; Jones *et al.*, 1999) .

Activated PKG also triggers activation of large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels which are present in all tissues especially at the smooth muscle (Nelson and Quayle, 1995). Opening of BK_{Ca} channels hyperpolarizes the membrane and closes voltage-dependent Ca^{2+} channels, reduces Ca^{2+} influx, and decreases intracellular Ca^{2+} concentration which leads to VSMC relaxation (Fukao *et al.*, 1999).

Besides, PKG also can reduce the release of intracellular Ca^{2+} in smooth muscle through the phosphorylation of both the IP3 receptor (IP3R) and the IP3R-associated PKG-I substrate (IRAG). Phosphorylation of IRAG and IP3 receptor inhibits the release of calcium from the endoplasmic reticulum and thereby promotes vasorelaxation (Komalavilas and Lincoln, 1996; Münzel *et al.*, 2003)

Most of NO-mediated relaxation results from cGMP-dependent protein kinase activation, however NO can also directly activate SERCA and cause the removal of Ca^{2+} from smooth muscle cytosol into the sarcoplasmic reticulum store (Figure 1.4).

cGMP also reduces Ca^{2+} release from the sarcoplasmic reticulum in the smooth muscle cell (Collins *et al.*, 1986) and also helps to restore Ca^{2+} to the sarcoplasmic reticulum (Cornwell *et al.*, 1991).

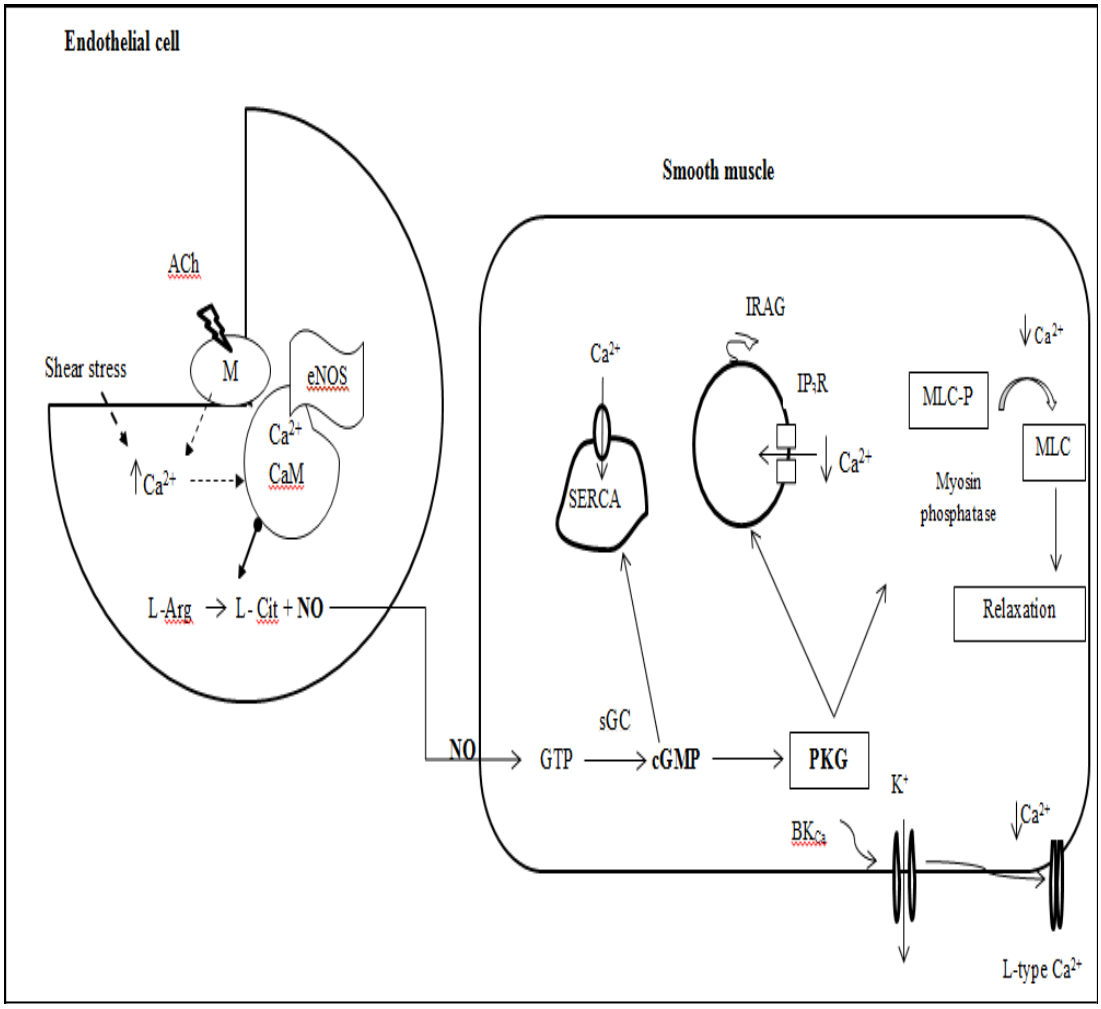


Figure 1.4 Schematic illustration representing possible mechanisms of NO-mediated relaxation in the blood vessel. Endothelial activation initiated by shear stress force or ACh lead to sudden rise in intracellular Ca^{2+} concentration causing NO generation. NO is made from L-arginine; this action is mediated by the endothelial nitric oxide synthase (eNOS). NO later diffuses into VSMC triggering the activation of sGC, causing production of cGMP and PKG. PKG can causes relaxation via: a) the activation of myosin phosphatase which prevents the binding of myosin to actin, thereby inhibits the constriction process; b) phosphorylation of BK_{Ca} channels that hyperpolarize smooth muscle cells and cause the closure of L-type Ca^{2+} channels, thus decreasing Ca^{2+} influx into VSMC; and c) phosphorylation of IRAG which reduced the release of Ca^{2+} from SERCA into VSMC cytosol. NO can also directly act on SERCA and assist the removal of Ca^{2+} from smooth muscle cytosol into the SERCA.

BK_{Ca}, high conductance calcium activated potassium channel, CaM, calmodulin; IRAG, IP3R receptor associated PKG-I substrate, IP3R1, Inositol-1,4,5-trophosphate receptor type-1; M, muscarinic receptor, MLC, myosin light chain kinase; MLC-P, myosin light chain phosphatase; PKG, protein kinase G; SERCA, sarco/endoplasmic reticulum Ca^{2+}ATPase.

1.1.3(b)(ii) Prostacyclin

Another agent that contributes to vasorelaxation of blood vessels is prostacyclin (Moncada *et al.*, 1976a). Both prostacyclin and NO are released by endothelial cells and their functions are relatively similar where they mainly prevent platelet aggregation and also cause vasodilatation (de Nucci *et al.*, 1988; Mitchell *et al.*, 2008)

Prostacyclin (also termed prostaglandin I_2 or PGI_2) is a member of the eicosanoid family of lipid molecules. Prostaglandin is produced via cyclooxygenase pathway which is also responsible for the production of thromboxane. These prostanoids, prostacyclin (PGI_2) and thromboxane (TXA_2) works antagonistically to maintain the homeostasis of vascular function (Vanhoutte and Tang, 2008). In contrast to prostacyclin, TXA_2 promotes vasoconstriction and causes platelet aggregation. Under normal circumstances, prostacyclin opposes TXA_2's biological effects, however when the blood vessel is severely damaged, prostacyclin may reverse its action and cause vasoconstriction (Vanhoutte and Tang, 2008; Félétou *et al.*, 2009).

Prostacyclin is released from endothelial cells when triggered by hormonal stimuli. It is formed from the reaction of an intermediate arachidonic acid which is made from endothelial phospholipid or diaglycerol and catalysed by phospholipase A2 which is calcium-dependent (Mitchell and Warner, 1999). This arachidonic acid can either enter the cyclooxygenase (COX) pathway or the lipoxygenase pathway to produce prostacyclin, TXA_2 or leukotriene respectively.

There are two types of COX enzymes (COX-1 and COX-2) (Mitchell and Warner, 1999; Mitchell and Warner, 2006) that lead to production of prostacyclin

and TXA_2. Both isoforms are expressed differently in endothelial cells: COX-1 is typically expressed in normal endothelial cells while COX-2 is mainly expressed when damage occurs to endothelial cells; or when endothelial cells are exposed to inflammatory cytokines (Mitchell and Warner, 1999; Mitchell *et al.*, 2006; Mitchell and Warner, 2006)

As mentioned above, prostacyclin is derived from arachidonic acid when catalysed by COX enzyme to form prostaglandin H_2. Prostaglandin H_2 acts as a precursor to produce other prostaglandins such as PGI_2, PGD_2, PGE_2, PGF_2 and TXA_2 when they are catalysed by their respective enzyme synthases. Prostacyclin is produced from the action of prostacyclin synthase (Figure 1.5)

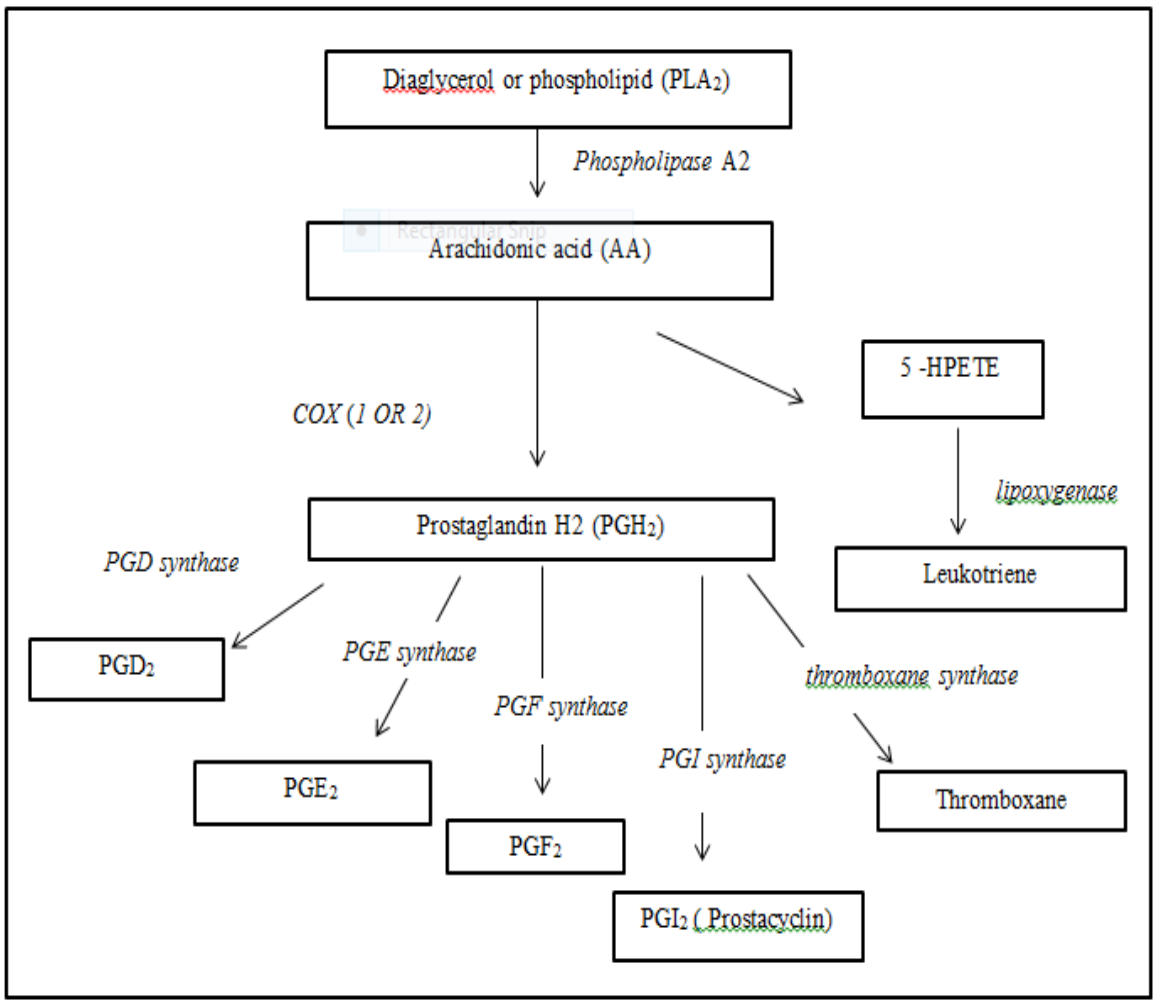


Figure 1.5 Production of prostaglandins in endothelial cells. Modified from Awtry and Loscalzo, 2000.
5-HPETE,5-hydroperoxyeicosatetraenoic acid; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; PGF_2; prostaglandin $F_{2;}$ PGI_2 , Prostacyclin; ;PGD synthase, prostaglandin D synthase; PGE, prostaglandin E synthase; PGF, prostaglandin F synthase PGF, prostaglandin F synthase; PGI synthase , Prostacyclin synthase; $PLA_{2,}$ phospholipid

As soon as prostacyclin is produced from the endothelium, it diffuses to VSMC to either cause vasodilatation or to nearby platelets to prevent platelet aggregation. In VSMC, prostacyclin binds to prostacyclin (IP) receptors on the cell surface, thereby initiating several processes that subsequently lead to vasodilation. Activation of IP receptors causes adenylyl cyclase to yield cyclic adenosine monophosphate which in turn activates protein kinase A (PKA). PKA causes the phosphorylation of the myosin light chain kinase (MLCK) to its inactive state and therefore prevents the binding of myosin to actin. PKA also decreases intracellular Ca^{2+} level which eventually leads to smooth muscle relaxation and vasodilation (Figure 1.6). Prostacyclin is a weak vasodilator and is rapidly broken down to 6-keto-PGF1 and has half-life of approximately 42 seconds (Gryglewski, 2008; Mitchell *et al.*, 2008; Majed and Khalil, 2012; Yu *et al.*, 2016)

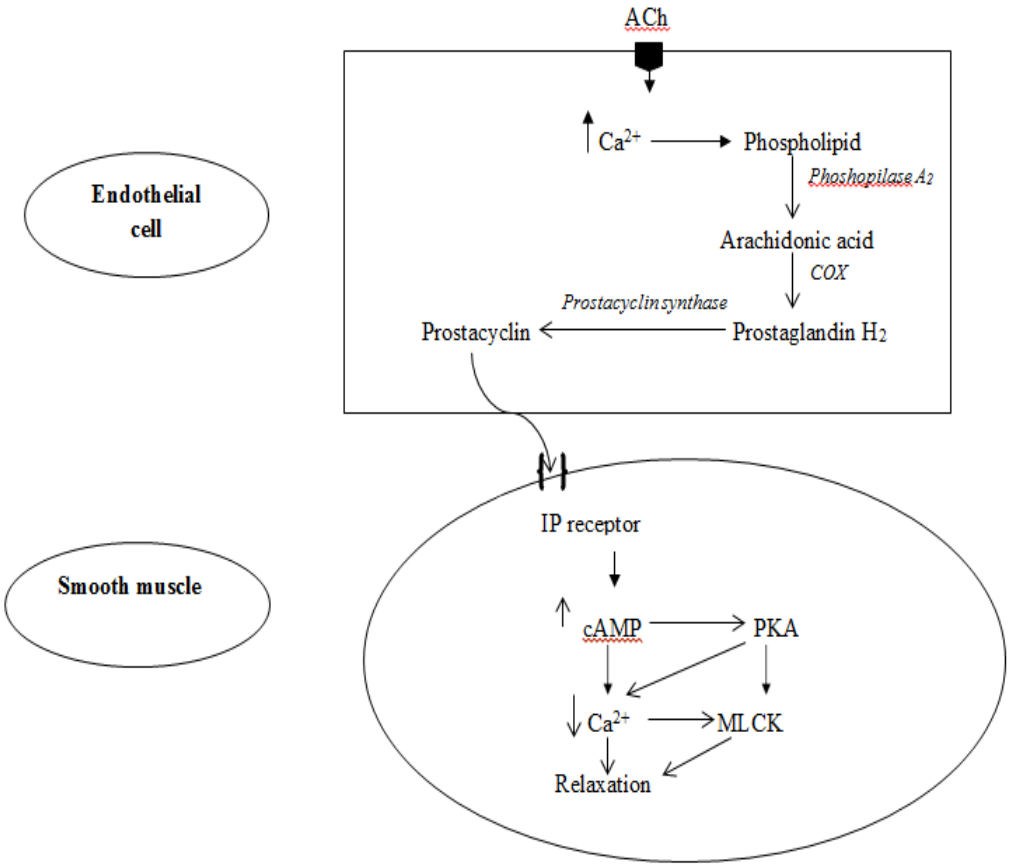


Figure 1.6 Prostacyclin productions by endothelial cells and vasodilation in vascular smooth muscle. Binding of ACh to muscarinic receptor causes sudden rise in intracellular Ca^{2+} concentration. This causes membrane phospholipid to form arachidonic acid which is then further catalysed to form prostaglandin H2 and prostacyclin. The released prostacyclin diffuses across the vascular smooth and binds to IP receptor (prostacyclin receptor) which in turn activates adenylyl cyclase to produce cAMP followed by activation of protein kinase A (PKA). PKA causes reduction in calcium level and phosphorylation of the myosin light chain kinase (MLCK), which thwarts the binding of myosin to actin and therefore inhibits vasoconstriction leading to smooth muscle relaxation and vasodilation. It can be noted that PGI_2 and TXA_2 work as physiological antagonists. Modified from (Mokhtar and Rasool, 2015)

AA, arachidonic acid; ACh, acetylcholine; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; EC, endothelial cells; IP, prostacyclin receptor; M, muscarinic receptor; MLCK, myosin light chain kinase; PGI2, prostacyclin; PGIS, prostacyclin synthase; PKA, protein kinase A; PL, phospholipids; PLA_2, phospholipase A_2 enzyme; VSMC, vascular smooth muscle cells.

1.1.3(b)(iii) Endothelium-dependent hyperpolarization factor

Besides NO and prostacyclin, another candidate that cause vasorelaxation is identified as endothelial dependent hyperpolarization (EDH). EDH was initially described in the late 1970s, where the researchers suggested that this hyperpolarization might be due to NO. However, their suggestions went wrong where in 1988, a group of researcher proved that the endothelium-dependent vasorelaxation is not only contributed by NO and prostacyclin but also by EDH (Chen *et al.*, 1988; Busse *et al.*, 2002; Félétou and Vanhoutte Paul, 2006; Mokhtar and Rasool, 2015)

There are two types of pathways which lead to EDH-mediated relaxation. One of the pathways is known as the classical EDH pathway. In this pathway, activation of endothelial cells is a prerequisite in order to begin the EDH response. Activation of endothelial cells by shear stress or agonist increases the intracellular calcium concentration where this event yields endothelial hyperpolarization opening of the small conductance calcium-activated potassium channels (SK_{Ca}) and intermediate conductance calcium-activated potassium channels (IK_{Ca}) (Félétou, 2016; Garland and Dora, 2017; Goto *et al.*, 2018; Murphy and Sandow, 2019). The importance of both potassium channels in mediating EDH response can be seen with the use of combined inhibitors such as apamin and charybdotoxin (or UCL 1684 and TRAM-34; block SK_{Ca} and IK_{Ca} respectively) which inhibit this response (Zygmunt and Hogestatt, 1996; Andersson *et al.*, 2000; Hinton and Langton, 2003). The use of combined blockers to SKCa and IK_{Ca} showed that both channels work via different pathways. The SK_{Ca} channel activates the inwardly-rectifying potassium channels (K_{IR}) on the VSMC whereas the IK_{Ca} channel stimulates the sodium/potassium pump (Na^+/K^+-ATPase) on VSMC (Jackson 2005; Dora Kim *et*

al., 2008). The opening of both channels causes outflow of K^+ ions from the endothelial intracellular compartment which causes rapid increase of K^+ in the myoendothelial spaces. Accumulation of extracellular K^+ concentration causes hyperpolarization which subsequently leads to vascular smooth muscle relaxation (Dora Kim *et al.*, 2008). Other than that, depending on the arteries and species studied, it is reported that EDH-mediated responses can be transferred via myo-endothelial gap junctions (MEGJ). Electrical coupling that happened between endothelial cells and vascular smooth cells is directly transmitted where this response is mediated by MEGJ. This MEGJ protein functions as communication channels between endothelial cells and surrounding VSMC (Sandow and Hill, 2000; Heberlein *et al.*, 2009; Félétou, 2016; Garland and Dora, 2017; Goto and Kitazono, 2019).

The intracellular Ca^{2+} released from the endoplasmic reticulum (ER) is needed to initiate the EDH response, however recent studies revealed that the Ca^{2+} influx through activation of transient receptor potential channels after ER calcium depletion also aids in the production of EDH via the downstream activation of SK_{Ca} and IK_{Ca} channels (Earley and Brayden, 2015; Garland and Dora, 2017; Grayson *et al.*, 2017; Behringer and Hakim, 2019; Goto and Kitazono, 2019; Murphy and Sandow, 2019).

Another type of pathway is known as the non-classical EDH pathway. In this pathway, hyperpolarization of vascular smooth muscle happens independently without the help of endothelial K_{Ca} channels. When then endothelial cells are triggered by ACh, diffusible factors such as NO, epoxyeicosatrienoic acids (EETs) and hydrogen peroxide (H_2O_2) are produced and transferred via internal elastic lamina to the underlying VSMC (Chen *et al.*, 1988; Campbell William *et al.*, 1996;

Miura *et al.*, 1999; Campbell and Fleming, 2010; Edwards *et al.*, 2010). This will lead to stimulation of different families of K^+ channels causing VSMC hyperpolarization. The hyperpolarization of VSMC reduce Ca^{2+} influx either by decreasing the opening probability of voltage-dependent calcium channels (Ca_V) or the Ca_V channel-dependent activation of the sarcoplasmic reticulum which will subsequently leads to VSMC relaxation (Figure 1.7) (Edwards *et al.*, 2010)

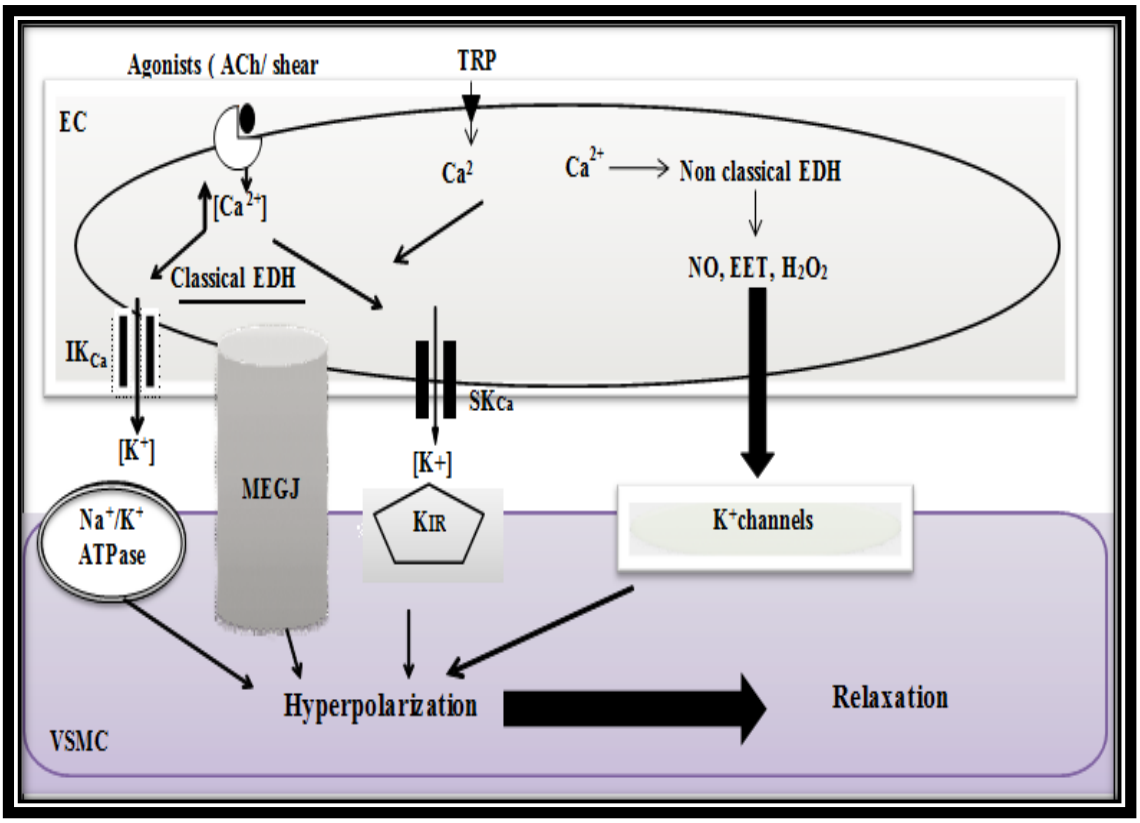


Figure 1.7 Endothelium-dependent hyperpolarization in the blood vessel. In classical EDH-type responses, endothelial cells are triggered with agonist or shear stress which increases the intracellular calcium concentration due to calcium release from endoplasmic reticulum and calcium influx through the endothelial nonselective cation channels of the transient receptor potential (TRP) family. This in turn activate SK_{Ca} and IK_{Ca} channels which causes vascular hyperpolarization either through activation of the Na^+/K^+- ATPase and K_{IR} channels respectively, or by means of direct transfer of electrical coupling through MEGJ. In non-classical EDH-type responses, presence of endothelium-derived diffusible factors such as NO, EETs and H_2O_2 leads to hyperpolarization of VSMC through activation of potassium channels. Adapted from Edwards *et al.*, 2010.

ACh, acetylcholine; IK_{Ca}, intermediate conductance calcium-activated potassium channels; K_{IR}, inwardly-rectifying potassium channels; M, muscarinic receptor; MEGJ, myo-endothelial gap junction; Na^+/K^+-ATPase, sodium/potassium pump; SK_{Ca}, small conductance calcium-activated potassium channels; TRP, transient receptor potential family

1.1.3(c) Endothelium contracting factors

Shortly after the discovery of NO and other members of endothelial relaxing factor, researchers explored the role of contracting factors in endothelial cells as it became obvious that despite the vasorelaxation role, under certain environments, endothelial cells cause contractions of the underlying vascular smooth muscle (Furchgott and Vanhoutte, 1989)

There are many factors contributing to endothelium-dependent contraction. For instance, reduced production of NO or other vasodilators leads to prominent decrease in overall endothelial-dependent relaxation activity. Moreover, the production of vasoconstrictor products such as angiotensin II, endothelin-1 and products of arachidonic acid metabolism (endoperoxides, TXA_2, and possibly isoprostanes) further augment contractions. These vasoconstrictors are also called endothelium derived contracting factors (EDCF) as they are directly involved in contractile activity of the VSMC. In addition, the sudden increase in oxygen-derived free radicals (superoxide anions) also intensifies endothelium-dependent contractions. The productions of EDCF are found greater in large vessels such as cerebral arteries and are further amplified with aging, spontaneous hypertension (Gluais *et al.*, 2005), and diabetes (Shi *et al.*, 2007).

1.1.3(c)(i) Thromboxane (TXA_2) as a vasoconstrictor

The involvement of thromboxane (TXA_2) as a vasoconstrictor was initially seen while studying the heterogeneity in endothelium-dependent responsiveness in canine veins. In canine veins, it was found that the mediators such as arachidonic acid and thrombin, which acts as endothelium relaxants in isolated arteries showed augmented contractions (De Mey and Vanhoutte, 1982). Similar observations were also obtained in other studies involving basilar artery of canine (Katusic *et al.*, 1987; Katusic *et al.*, 1988). Meanwhile, a study conducted in the aorta of spontaneously hypertensive rat (SHR) also reported that endothelium-dependent contraction is mediated by the product of COX (Luscher and Vanhoutte, 1986). Subsequent findings revealed that endothelium-dependent contractions are suppressed with the use of COX inhibitors proposing that down-stream products of cyclooxygenase enzyme (prostanoids) are responsible for endothelial contractions activity (Miller and Vanhoutte, 1985; Luscher and Vanhoutte, 1986; Yang *et al.*, 2002; Yang *et al.*, 2003; Wong and Vanhoutte, 2010). Most animal studies showed that endothelial contractions mediated by COX are augmented with aging (Koga *et al.*, 1989; Mombouli and Vanhoutte, 1993; Shi *et al.*, 2008), hypertension (Luscher and Vanhoutte, 1986; Auch-Schwelk *et al.*, 1989; Koga *et al.*, 1989; Yang *et al.*, 2002; Tang *et al.*, 2005), vasospasm (Dhein *et al.*, 1989), and high glucose (Tesfamariam *et al.*, 1990). Endothelium-dependent contraction is also considered to occur in humans as it was reported that in patients with essential hypertension; indomethacin (non-selective inhibitor of cyclooxygenases) practically normalizes the blunted vasodilator response to acetylcholine (Taddei *et al.*, 1997).

As described previously, early manifestation of endothelial dysfunction is associated with the loss of endothelial function especially in producing endothelial

derived relaxing factors. This could be related to acute endothelial injury or due to the presence of diseases such as diabetes, hypertension and cardiovascular disease. Under these environments, when the endothelial cells are exposed to shear stress or agonists such as thrombin, acetyl choline (Boulanger *et al.*, 1994), adenosine nucleotides (adenosine diphosphate and adenosine triphopspate) (Koga *et al.*, 1989; Mombouli and Vanhoutte, 1993) or calcium ionophore (Katusic *et al.*, 1988; Gluais *et al.*, 2006b; Shi *et al.*, 2007; Tang *et al.*, 2007; Shi *et al.*, 2008), the endothelial cells will produce more endothelial contracting factors which are mainly mediated by COX.

Upon stimulation of endothelial cells, sudden calcium influx is seen. This rapid accumulation of calcium originated either from sarcoplasmic reticulum release or from the activation of phospholipase C. An increase in calcium concentration is essential in causing endothelial contractions. For instance, when the extracellular calcium level is lowered (Okon *et al.*, 2002), reduction in endothelial contraction is seen and is completely absent in another study (Wong Siu *et al.*, 2009), whereas restoration of calcium ions is seen to normalise the endothelium contractions (Wong Siu *et al.*, 2009). Many studies utilize calcium ionophore A23187 (calcium-increasing agent) since it directly permits entry of calcium ions across the membrane and therefore increases intracellular calcium levels and causes endothelial contractions (Okon *et al.*, 2002; Shi *et al.*, 2007; Tang *et al.*, 2007; Vanhoutte Paul, 2011).

As mentioned previously, the calcium levels are increased when agonists such as acetyl choline or adenosine di- and triphosphate bind to specific receptors on the endothelial cells and causes phospholipase activation. Phospholipid activation is a series of processes resulting in the formation of inositol triphosphate which in turn

triggers the release of calcium from intracellular stores. This causes the decline in calcium levels intracellularly which in turn provokes calcium independent phospholipase A_2 ($iPLA_2$) to create more lysophospholipids (Tang *et al.*, 2007; Wong *et al.*, 2008; Vanhoutte Paul, 2011). The formed lyphospholipids opens the store-operated calcium channels and causes the inflow of extracellular calcium into the endothelial cells (Trepakova *et al.*, 2001; Smani *et al.*, 2004). This sudden outburst of cytosolic calcium ions will induce the calcium-dependent phospholipase A_2 ($cPLA_2$) to convert more membrane phospholipids into arachidonic acid (AA) (Wong and Vanhoutte, 2010; Vanhoutte Paul, 2011). AA can also be synthesized from DAG by diacylglycerol lipase (Hanna and Hafez, 2018). Depending on the need, the created AA will either enter COX or 5-lipoxygenase (5-LO) pathways to produce endoperoxides and leukocytes respectively.

In COX pathway, AA is converted to intermediate compounds, Prostaglandin G_2 (PGG_2) and then to Prostaglandin H_2 (PGH_2) which causes the production of five bioactive prostanoids (PGE_2, PGI_2, PGD_2, $PGF_{2\alpha}$) and TXA_2 by tissue-specific synthases (Breyer *et al.*, 2001; Funk, 2001). These prostanoids binds with their corresponding receptor located on smooth muscle (EP, FP, IP, TP, and DP) to produce biological actions. COX is seen as a rate-limiting enzyme and available in two isoforms, COX-1 and COX-2 (Garavito and DeWitt, 1999). These isoforms are important in the synthesis of prostaglandin, however their expression, availability and distribution within the cells depends on the species and the blood vessel studied and also the health conditions of the donor (Furchgott and Vanhoutte, 1989; Dalman and Porter, 1993; Tang and Vanhoutte, 2009; Vanhoutte, 2009; Vanhoutte *et al.*, 2009; Wong Siu *et al.*, 2009). Generally, COX-1 is highly expressed in normal arteries with low expression of COX-2 observed under same

vessels. However COX-2 is seen highly induced in the case of inflammation, aging, hypertension, diabetes and renal injury (Wong and Vanhoutte, 2010; Li *et al.*, 2018).

Among the product of cyclooxygenase, TXA_2 appears to evoke endothelial contractions. The formed TXA_2 readily diffuses to vascular smooth muscle and binds to TXA_2 receptor resulting in the increase of intracellular calcium level, activation of Rho kinase pathway and eventually contractions (Chan *et al.*, 2009; Wong and Vanhoutte, 2010). Binding to TXA_2 receptors triggers the calcium release from sarcoplasmic reticulum. The calcium increase enhances the binding of Ca^{2+} to calmodulin (CaM) which in turn activates myosin light chain kinase (MLCK) to phosphorylate the myosin light chain (MLC) of myosin II. The degree of MLC phosphorylation controls the extent to which VSMC contracts: MLC phosphorylation causes VSMC contraction while MLC dephosphorylation causes VSMC relaxation. The Rho-kinase pathway controls the degree of phosphorylation of the myosin light chain of myosin II, mostly via inhibition of myosin phosphatase, and subsequent Ca^{2+} sensitization (Fukata *et al.*, 2001). In many experimental studies, the release of TXA_2 is seen amplified where incubating the vessels with TXA_2 antagonist abolish the endothelial contractions (Auch-Schwelk *et al.*, 1990; Huang *et al.*, 2004; Vanhoutte Paul, 2011)

Other than TXA_2, PGH_2 which acts as an intermediate compound in TXA_2 production is also seen to exhibit contraction (Ge *et al.*, 1995; Gluais *et al.*, 2006a; Gluais *et al.*, 2007; Tang and Vanhoutte, 2008a; Wong and Vanhoutte, 2010) by activating TXA_2 receptors of vascular smooth muscle (Auch-Schwelk *et al.*, 1990; Kato *et al.*, 1990; Ge *et al.*, 1995; Wong and Vanhoutte, 2010). Moreover, PGH_2 together with prostacyclin become the main reservoir for EDCF. Prostacyclin plays a major role in causing endothelial contraction as prostacyclin does not cause

relaxation (Moncada *et al.*, 1976b; Zou *et al.*, 2002; Hink *et al.*, 2003; Gluais *et al.*, 2005). Instead of producing relaxation, prostacyclin appeared to cause endothelial contractions, though the expression of IP receptors is seen unaltered (Levy, 1980; Gluais *et al.*, 2005; Tang and Vanhoutte, 2008a; Liu *et al.*, 2012). This unresponsiveness of IP receptors might start a positive feedback on the endothelial cells, resulting in abundant overexpression of PGI_2 synthase with the major release of PGI_2 by endothelial cells provoked by acetylcholine or calcium ionophore A23187. Expression of PGI synthase is also seen greatly augmented with aging and spontaneous hypertension in aortic endothelial cells of the rat (Numaguchi *et al.*, 1999; Tang and Vanhoutte, 2008a; Vanhoutte Paul, 2011). Due to lack of responsiveness of IP receptors, prostacyclin binds to TXA_2 receptors and thus produces the contractions (Gluais *et al.*, 2005; Vanhoutte Paul, 2011).

In addition, TXA_2 receptor is also seen as a villain as it permits endoperoxides at higher concentrations to bind to it and thereby mediates vasocontraction (Feletou et al., 2010). Generally, endoperoxides are unstable however they are seen to activate thromboxane receptor (TXA_2 receptors) or known as TXA_2 receptor of VSMC (Ito et al., 1991; Asano et al., 1994; Ge et al., 1995). For example, greater releases of endoperoxides are seen in the aorta of the SHR than in that of normotensive rats (Ge et al., 1995; Ge et al., 1999; Vanhoutte Paul, 2011). The role of endoperoxides in mediating contraction become more significant when the activity of PGI_2 synthase is reduced due to the local production of peroxynitrite (Zou et al., 1999; Zou et al., 2002; Gluais et al., 2005) as this diverts arachidonic acid toward PGE_2 and PGF_2 synthases (Gryglewski *et al.*, 1986; Zou *et al.*, 1999; Zou *et al.*, 2002; Gluais *et al.*, 2005) to produce more of the endoperoxides and produce greater contractions (Figure 1.8).

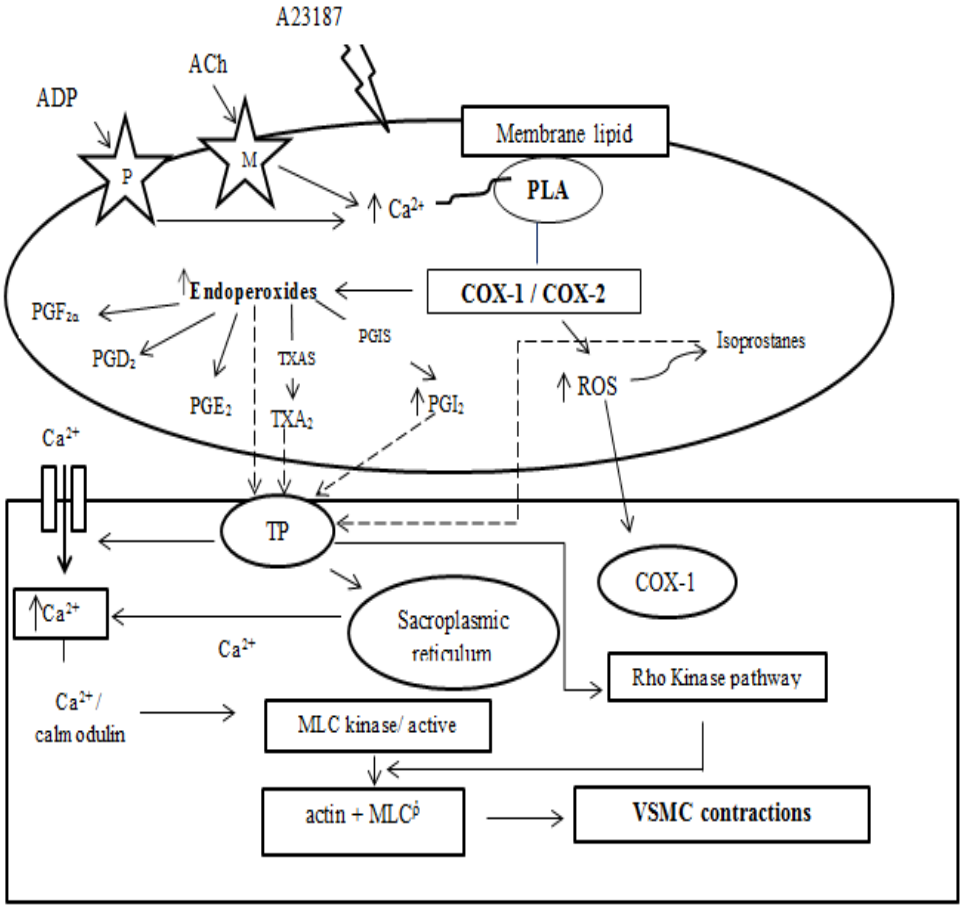


Figure 1.8 Endothelium-dependent contractions due to the production of prostanoids and ROS. Adapted from (Wong and Vanhoutte, 2010).

The endothelial contraction is initiated with sudden accumulation of intracellular Ca^{2+}. This increase is generated by receptor-dependent agonists, such as acetylcholine or ADP, or imitated with calcium increasing agents, such as the calcium ionophore A23187. This results in depletion of calcium which dislocates the inhibitory calmodulin (CaM) from $iPLA_2$. Triggered $iPLA_2$ produces lysophospholipids (LysoPL) which open store-operated calcium channels (SOCs) resulting entry of extracellular calcium into the endothelial cells. This sudden outburst of calcium ions activates $cPLA_2$, which causes the production of arachidonic acids (AA). The produced AA is then converted to prostanoids catalysed by cyclooxygenase-1 (COX-1). The produced prostanoids eventually binds to TP receptors which in turn increase Ca^{2+} levels. Binding to TP receptor activates increase in Ca^{2+} release from the sarcoplasmic reticulum. Intracellular increase in calcium levels enhances the binding of Ca^{2+} to calmodulin (CaM). This complex activates myosin light chain kinase (MLCK) to phosphorylate the myosin light chain (MLC) of myosin II. The Rho-kinase pathway modulates the level of phosphorylation of the myosin light chain of myosin II, mainly through inhibition of

myosin phosphatase, and contributes to agonist-induced Ca^{2+}-sensitization in smooth muscle contraction.

AA=arachidonic acid; ACh=acetycholine; ADP=adenosine diphosphate; $cPLA_2$, calcium dependent phospholipase A_2; COX, cyclooxygenase; $iPLA_2$, calcium independent phospholipase;m=muscarinic receptors; P=purinergic receptors; PGD_2=prostaglandin D_2; PGE_2=prostaglandin E_2; $PGF_{2\alpha}$=prostaglandin $F_{2\alpha}$; PGI_2=prostacyclin; PLA_2=phospholipase A_2; ROS=reactive oxygen species; TXA_2= thromboxane A_2, TP=thrombo-prostanoid receptor

1.2 Diabetes mellitus

1.2.1 Introduction to Diabetes Mellitus

Diabetes mellitus (DM) is one of the oldest diseases known to humankind as the early evidence of the symptoms had been documented on papyrus scrolls by ancient Egyptians about 3500 years ago (King and Rubin, 2003). This metabolic disease is characterized by the manifestation of high glucose level in blood, a condition called hyperglycemia. The presence of hyperglycemia results from defectiveness in insulin secretion, impaired insulin action, or both (American Diabetes Association, 2009).

1.2.2 Prevalence of diabetes

Diabetes mellitus becomes a major threat as the numbers of patients keep expanding yearly. Based from World Health Organization (WHO) report, it was estimated that 108 million adults were living with diabetes and this figure went up to 422 million adults in 2014. The prevalence among adults over 18 years of age has also increased from 4.7% in 1980 to 8.5% in 2014. WHO also reported that the largest numbers of people with diabetes were from South-East Asia and Western Pacific regions contributing nearly half diabetes cases in the world (WHO,2016). This figure is expected to double in the future as it is closely associated with our non- healthy life styles which include dietary habits and lack of physical activity.

Patients with diabetes have a shorter life expectancy. The average life span of a 50-year-old individual with diabetes is 6 years shorter than those without diabetes (Seshasai *et al.*, 2011). Reports by WHO showed that in 2012, diabetes alone has been the cause for 1.5 million deaths. It also contributed to an additional 2.2 million death, as diabetes is associated with cardiovascular risk and

other diseases. WHO also predicted that diabetes will be the seventh leading cause of death in 2030 (WHO, 2016).

According to the National Health and Morbidity Survey (NHMS) 2019 by the Ministry of Health (MOH), the prevalence rate of diabetes in adults has increased in Malaysia from 13.4 per cent in 2015 to 18.3 per cent in 2019, with diabetes defined as having sugar levels 7.0 mmol/L or above. The data also showed that an estimated 3.9 million adults in Malaysia aged 18 and above had diabetes as of last year, higher than 3.5 million in 2015. This rise is a concern as the National Health and Morbidity Survey in 2019 revealed that almost one in five Malaysian adults suffered diabetes. The NHMS 2019 survey revealed that 49 per cent of people with diabetes had never been examined or diagnosed with the chronic disease (National Health and Morbidity, 2019). These statistics revealed that females, older age group, Indians, and urban residents were at the highest risk of DM.

1.2.3 Aetiology of diabetes mellitus

Diabetes mellitus (DM) is a chronic disease that happens when our body failed to produce enough insulin or fail to use insulin. The presence of diabetes is characterised by high glucose level in the blood. Insulin, a hormone produced by beta cells of the pancreas normally regulates blood glucose level in the body. Insulin allows the transport of glucose from the bloodstream to the cells where it is consumed as a source of energy. This hormone along with glucagon help to keep the blood glucose level from getting too high (hyperglycemia) or too low (hypoglycaemia). The normal fasting glucose level in our body is between 3.9 to 5.5 mmol/L ((WHO, 2016).

There are three major forms of diabetes mellitus: diabetes mellitus type 1, diabetes mellitus type 2 and gestational diabetes mellitus (Triplitt *et al.,* 2015). Type 1 diabetes is autoimmune diseases where the insulin producing cells are destroyed by the body own immune system. Formerly this type of diabetes is known as insulin-dependent diabetes since the patients require daily administration of insulin due to the total insulin deficiency in the body. Type 1 diabetes is generally diagnosed during juvenile and adolescence which contributes for 5-10% of newly diagnosed diabetes. The cause of type 1 diabetes is unknown, however the researchers postulated that genetic and environmental factors played a role in the development of this disease (Aathira and Jain, 2014; Triplitt *et al.*, 2015). Type 1 diabetes is more severe compared to type 2 diabetes. Hypoglycaemia is reported to be a significant cause of morbidity and mortality in type 1 diabetes (Kyi *et al.*, 2015).

Type 2 diabetes contributes 85 to 95 percent of all diagnosed diabetes cases. The defect in insulin secretion or insulin resistance contributes to the development of type 2 diabetes. Decline in insulin secretion occurs when there is a gradual drop in pancreatic beta-cell function, and also reduced beta-cell mass, which is manifested before the onset of type 2 diabetes (Pratley and Weyer, 2001; Stumvoll *et al.*, 2005; Pratley, 2013; Triplitt *et al.*, 2015). Several data also suggested that by the time of diagnosis, a mere 20% of beta-cell function remains (DeFronzo *et al.*, 2010). The progress of chronic hyperglycemia additionally causes more damage to beta-cell function and insulin secretion (Pratley, 2013). Despite different aetiologies involved, all type 2 diabetes patients share abnormalities in carbohydrate, fat and protein metabolism (American Diabetes Association, 2009).

Gestational diabetes is first detected during pregnancy. It occurs in about 5% of pregnancies and is expected to increase as the prevalent of obesity continues

(Ben-Haroush *et al.*, 2004; Kampmann *et al.*, 2015). Gestational diabetes not only affects the mother but also the child. The mother is exposed to the risk of caesarean and operative vaginal delivery whereas in the new-born have increased risk for macrosomia, shoulder dystocia, neonatal hypoglycemia and hyperbilirubinemia (Catalano and Ehrenberg, 2006). Women with GDM are also at high risk of getting type 2 diabetes mellitus (T2DM) later in life; their children are also prone to develop diabetes type 2 and obesity early in life (HAPO Study , 2008).

1.2.4 Diabetes and microvascular complication

In the long run, the exposure to high blood glucose leads to other consequences such as microvascular and macrovascular complications. Microvascular complications include small blood vessel diseases such as diabetic retinopathy, diabetic neuropathy and diabetic nephropathy. Macrovascular complications occur in larger blood vessels such as in heart, brain and peripheral tissue which eventually causes coronary artery disease, stroke and peripheral vascular disease. These complications lead to early morbidity and mortality among diabetic patients (IDF, 2019). For example, diabetic retinopathy is one of the most frequent causes of impaired vision or blindness among adults aged 20-74 years (Fong *et al.*, 2003). Diabetes nephropathy causes chronic kidney disease and if left untreated, leads to end stage kidney disease which accounts for 20-40 % of all diabetic cases (Gheith *et al.*, 2016). Diabetic neuropathy, another type of microvascular complications involves both peripheral and autonomic nerves that predispose the risk for foot ulcers and limb amputation where it affects about 50% of diabetic patients (WHO, 2016). It is reported that diabetic patients are at the higher

risk of amputations, approximately 25 times greater compared to non-diabetics (Boulton *et al.*, 2005).

1.2.5 Diabetes and atherosclerosis

Diabetic patients with microvascular complications generally have higher tendency of accelerated atherosclerosis which can cause cerebrovascular and cardiovascular diseases and premature death (Kalofoutis *et al.*, 2007).

Atherosclerosis is referred as hardening of arteries due to plaque formation. This plaque formation consists of fatty substances, cholesterol, cellular waste products, calcium and other substances. The formation of plaque at specific sites will further cause intimal inflammation, necrosis, fibrosis, and calcification where overtime, the accumulation of plaques narrows the opening of lumen and thus reduces blood flow and oxygen supply which may abruptly cause macrovascular disease such as coronary heart disease, ischemic stroke, and peripheral vascular disease (Bentzon *et al.*, 2014).

1.3 RATIONALE OF THE STUDY

The number of diabetes cases in Malaysia is escalating with statistics in 2019 showing that 3.6 million Malaysians out of 32 million having diabetes. Out of the 3.6 million diabetic patients, 1.78 million were women. These figures are worrisome as this figure is expected to increase yearly together with many unreported diabetic cases. It was estimated that about 1.8 million Malaysians have diabetes, but is unaware of it as they do not go for the screening (National Health and Morbidity, 2019).

Diabetes leads to microvasculopathy which contributes to cardiovascular complications such as heart diseases, nephropathy, neuropathy and retinopathy. Endothelial dysfunction (ED), defined as impaired endothelial mediated relaxation is the initial pathology leading to atherosclerosis and vasculopathy in diabetes. Endothelial cells line the inner blood vessel and releases endothelial derived factors (EDF) such as endothelial derived relaxing factors (EDRF) that dilate blood vessels and endothelial contracting factors (EDCF) which constrict blood vessels. In larger vessels, imbalance in the availability of EDCF and EDRF has been reported to lead to ED. However, little is known in the diabetic microcirculation especially diabetics with peripheral artery disease (PAD). A few studies had previously reported on alterations in EDRF in the microcirculation of diabetic rats. However, no studies had addressed the roles and alterations caused by EDCF in the microcirculation of diabetic rats. No studies had reported on the effects of early and late diabetes on microvascular endothelial derived relaxing & contracting factors. In this study, the contribution of cyclooxygenase enzyme (which includes COX-1 and COX-2) and thromboxane (TXA_2) receptor in endothelium-mediated contraction were specifically evaluated. The effects of diabetes on EDRF [prostacyclin, nitric oxide

(NO), endothelium derived hyperpolarizing factors (EDH)] and thromboxane (TXA_2) receptor in early (2 weeks) and also late phases of diabetes (10 weeks) were also studied in streptozotocin induced diabetic rats.

This study methodology consists of two parts. The first part consists of *in-vitro* functional vascular response studies which assessed the contributions of each EDRF (NO, EDH and prostacyclin) to endothelium-mediated relaxation in the microcirculation of two and ten weeks diabetic and normal rats. The contribution of TXA_2 receptor to endothelium-dependent contraction and the enzymes responsible for TXA_2 production (COX-1 and COX-2) were also evaluated in normal and diabetic vessels. This method involved a series of experiments where microvessels were incubated with different pharmacological blockers that inhibit the synthesis or action of these factors.

The second part consists of molecular studies (western blot and immunohistochemistry) to determine presence and protein expression of the enzymes and receptors involved in the synthesis and action of these EDRF and TXA_2. Alterations in enzymes and receptors involved during the early (2 weeks diabetes) and late phases of diabetes (10 weeks diabetes) were studied.

In this study, tail artery of rats were studied as it was reported that the microcirculation of tail artery of the rats are similar to distal blood circulation of human limbs and digits in terms of the anatomy and reactivity profile (Wu *et al.*, 1995; Fang and Galiano, 2008; Spradley *et al.*, 2013). Distal blood circulation includes the microcirculation of the lower limbs. Besides, the tail artery of the rat has also been employed as an assay tissue to assess the impairment of vascular function that can be generalized to human peripheral vascular disease (Wakitani *et al.*, 1992).

Previous studies showed that diabetes patients with peripheral arterial disease (PAD) are at high risk of developing morbidity and mortality from cardiovascular diseases. PAD also is identified as a significant risk factor for diabetic foot diseases which contributes to foot amputation (American Diabetes Association, 2003, Prompers *et al.*, 2008; Chen *et al.*, 2015). Diabetic patients with PAD also demonstrated severe impairment in endothelial function (Marso and Hiatt, 2006; Kiani *et al.*, 2013). From this current study, we will have better insights on the mechanisms leading to the development of ED that might aid in the early management of diabetes patients particularly those with peripheral arterial disease. Findings from this study could be beneficial to improvise targeted therapeutic strategies and also introduce pharmacological intervention in the management of diabetic endothelial dysfunction in the lower extremities.

1.4 OBJECTIVES

The general objectives of this study are:

1. To determine the early and late changes in endothelium-dependent relaxation and contraction responses in the microcirculation of diabetic rats

The specific objectives of this study are:

1. To determine the contribution of NO, prostacyclin, EDH and TXA_2 receptor in endothelium-dependent relaxation and endothelium-dependent contraction in the microcirculation of diabetic rats
2. To determine the expression of enzymes (eNOS, PGIS, COX-1, COX-2 and thromboxane synthase) and receptors (IP receptor and thromboxane receptor) in mediating the endothelial relaxing and contracting responses in the microcirculation of diabetic rats.
3. To determine the early (2 weeks) and late (10 weeks) changes in the functional study evaluating endothelial dilators (NO, prostacyclin and EDH) and endothelial prostanoids (COX-1, COX-2 and TXA_2 receptor) together with molecular responses in the microcirculation of diabetic rats

CHAPTER 2

MATERIALS AND METHODS

2.1 Study Design

This study was conducted in 120 Sprague-Dawley rats with each body weight ranged from 250 to 350 grams and age between 10 to 12 weeks. In this study, endothelium-mediated contraction and relaxation of microcirculation was evaluated during early and late state of diabetes (two and ten weeks duration, respectively). The vascular responses in diabetic rats were compared with the responses in normal rats. In this study, rats were divided into 4 groups: (1) normal 2-weeks rats (2) diabetic 2-week rats (3) normal 10-week rats and (4) diabetic 10-week rats (Figure 2.0).

Previous studies reported that endothelial dysfunction started at to occur as early as one (Zhang *et al.*, 2012) or two weeks after diabetic induction (Rodríguez-Mañas *et al.*, 2003). However not all the studies agreed with that conclusion (Brands *et al.*, 1994; Pieper, 1999; Nacci *et al.*, 2009). Therefore in this study we choose duration of 2 weeks to evaluate microvascular endothelial function. Literatures also showed that diabetic complications such as neuropathy, nephropathy and retinopathy started to be observed after 8-14 weeks of diabetic induction (Suh, 1999; Parkar N and Addepalli V, 2014; Si *et al.*, 2014; Taskıran *et al.*, 2016; Lee et al., 2017; Peterson *et al.*, 2017). Generally microvascular complication due to diabetic manifested at the late stage of diabetes therefore the duration of 10 weeks chosen for this study.

2.1.1 Ethical approval

Animal study was approved by the Animal Ethics Committee of USM, Health Campus, Kubang Kerian, Kelantan (Appendix A).

2.1.2 Sample size calculation

The principal parameter of interest used in this study was percentage of maximum relaxation induced by acetylcholine (ACh). Two means equation with two-tailed alpha (α) set at 0.05 with power of 90% were applied. The mean difference of interest (14.24%) and standard deviation (11.78) from control rats were taken from previous findings Mokhtar *et al.*, 2016.

The calculation for the sample size is shown below:

Calculation of sample size.

$n = 2 (Z\alpha + Z\beta)^2 SD^2/d^2$

$n = 2 (1.96 + 1.28)^2 (11.78)^2/ (14.24)^2$

n = 15 (for each group)

Where n is defined as a sample size of each group

$Z\alpha = 1.96$ for α set at 0.05 (two-tailed)

$Z\beta = 1.28$ for β set at 90%

SD = standard deviation, 11.78

d = estimated smallest difference of significance, 14.24

Therefore, a minimum number of 15 rats required for each group to complete this study.

2.1.3 Animal handling and care

A total of one hundred and twenty healthy rats were received from the Animal Research and Service Centre (ARASC). The rats were allowed to

acclimatize to the surrounding condition a week before starting the study. Throughout the study period, the rats were caged in polypropylene cages in a well-ventilated room with room temperature of 25 ± 2°C and exposure to 12 hours light/ 12 hours of dark cycle. The rats had unrestricted access to tap water and rat pellets (unless they were fasted).

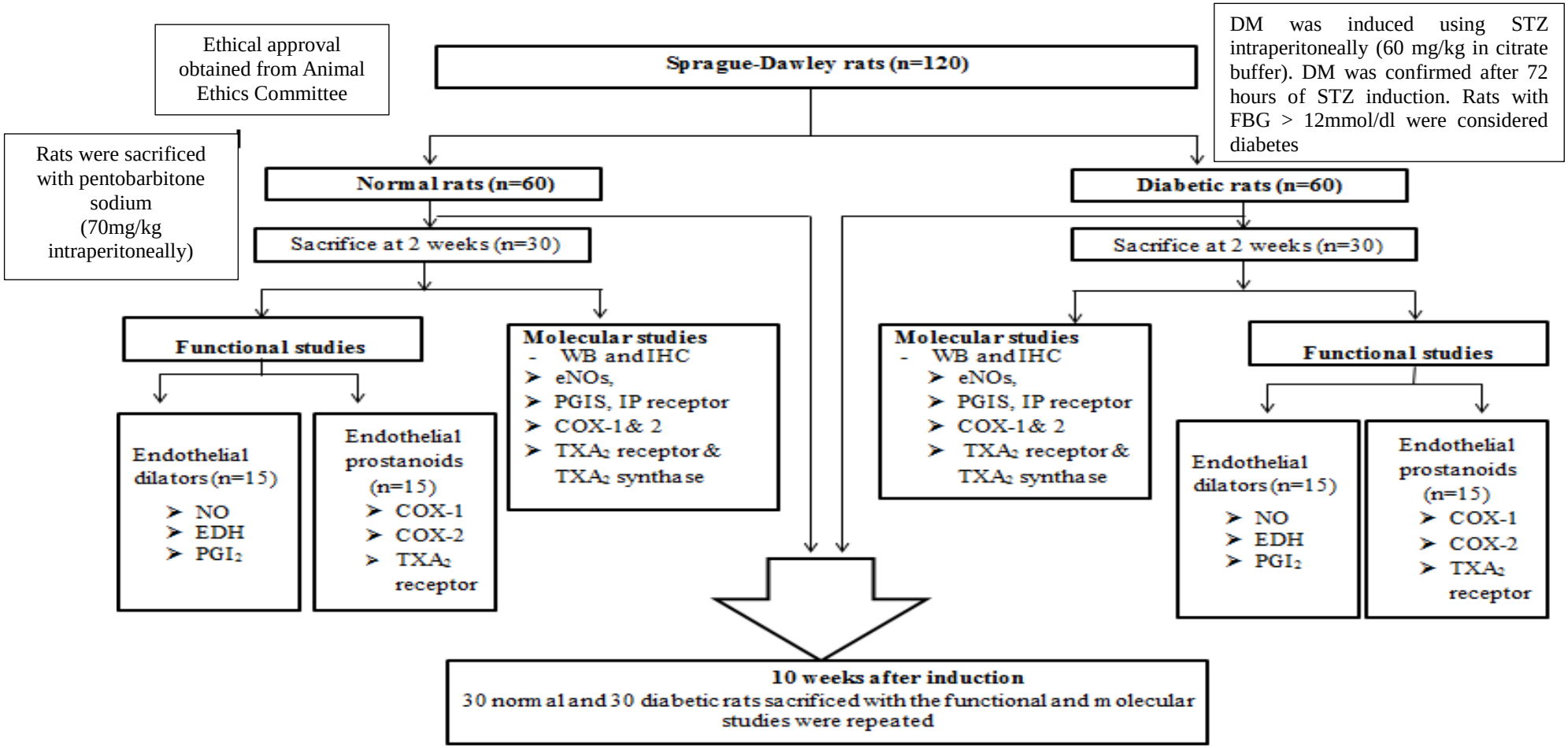


Figure 2.0 Study flow chart

2.1.4 Diabetes induction and confirmation

The rats in this study were divided into two groups; normal and diabetic group. Each group consisted of 60 rats. Normal rats were treated with sodium citrate buffer (0.1M) via intraperitoneal injection, while streptozotocin (STZ) was used to induce diabetes in the experimental rats. Prior to diabetes induction; the rats were fasted for 12-16 hours. The dosage of STZ used to induce diabetes in each rats were calculated based on their body weights. The fasted rats were later intraperitoneally injected with a single dose of STZ (60 mg/kg dissolved in sodium citrate buffer, 0.1 M, pH 4.5). Following the administration of STZ, the rats were monitored carefully for 72 hours. The presence of STZ causes the pancreas to lose its function that is by damaging Langerhans islet beta cells and thus induces experimental diabetes. To confirm the successful induction of diabetes in the experimental rats, blood from the tail tip was drawn after 72 hours of diabetes induction by tail pricking method. The blood glucose level was measured using the Accu-Check glucometer for continuous two days to ensure that the rats were diabetic. The rats with the fasting blood glucose readings higher than 12 mmol/dl were considered diabetic (Mokhtar *et al.*, 2016). Once the rats developed diabetes, random blood glucose levels were checked weekly until the end of study to ensure that rats were kept under diabetic condition. Other symptoms such as weight loss, increased in urination (polyuria), increased in appetite (polyphagia), thirsty (polydipsia) were also noted in diabetic rats.

2.1.4(a) Measurement of other parameters

Measurements of body weight for control and diabetic rats were also taken weekly starting from the baseline until the completion of the study. The intake of

water and food were also regularly monitored as the diabetic required more water and rat pellets.

2.1.4(b) Preparation of sodium citrate buffer (0.1 M, pH 4.5)

Streptozotocin which was measured according to rat's body weight was later diluted in sodium citrate buffer. To prepare 0.1 M sodium citrate buffer, 1.4705 g of tri-sodium citrate was dissolved in 50 ml double distilled water and the pH was adjusted to 4.5 with 2 M HCl.

2.1.4(c) Euthanization of rats

At week two, total numbers of 60 rats (30 rats from normal groups and 30 rats from diabetic groups) were sacrificed with pentobarbitone sodium (70 mg/kg, given intraperitoneally). The same procedure was repeated at week ten where rats from normal groups (n=30) and diabetic group (n=30) were sacrificed. The rat tail was dissected and kept in physiological saline solution (PSS) to be used in functional and molecular experimentation.

2.1.4(d) Tissue collection and dissection

The tail part of the rats was used in this study. The artery from the tail was preferred due to its subcutaneous localization and it reflects better view of peripheral arteries (Mokhtar *et al.*, 2016). The tail part can be divided into proximal, middle and distal segments with each part differing in diameters. The distal part of the artery (smallest in internal diameter, 286–315 μm) was selected for functional study while arteries from middle and proximal were used in Western blotting and immunohistochemistry studies (Mokhtar *et al.*, 2016) (Figure 2.1) .

In this study, to evaluate the endothelium-mediated contraction and relaxation responses, the experiments were done separately since the functional experiment required long hours and this causes the non-viability of the vessels.

After the confirmation of DM [72 hours after induction with STZ), the rats were killed at 2 weeks and 10 weeks. At week two, to study the role of endothelium-derived relaxing factors, thirty rats from normal groups (n=15) and diabetic groups (n=15) were sacrificed. Another thirty rats were also sacrificed at week two and used to evaluate endothelium-mediated contracting response. The proximal and middle part of the tail arteries were kept for molecular studies. The same procedure repeated at week 10 where another sixty rats were sacrificed to evaluate endothelium-mediated responses and to also to assess molecular studies.

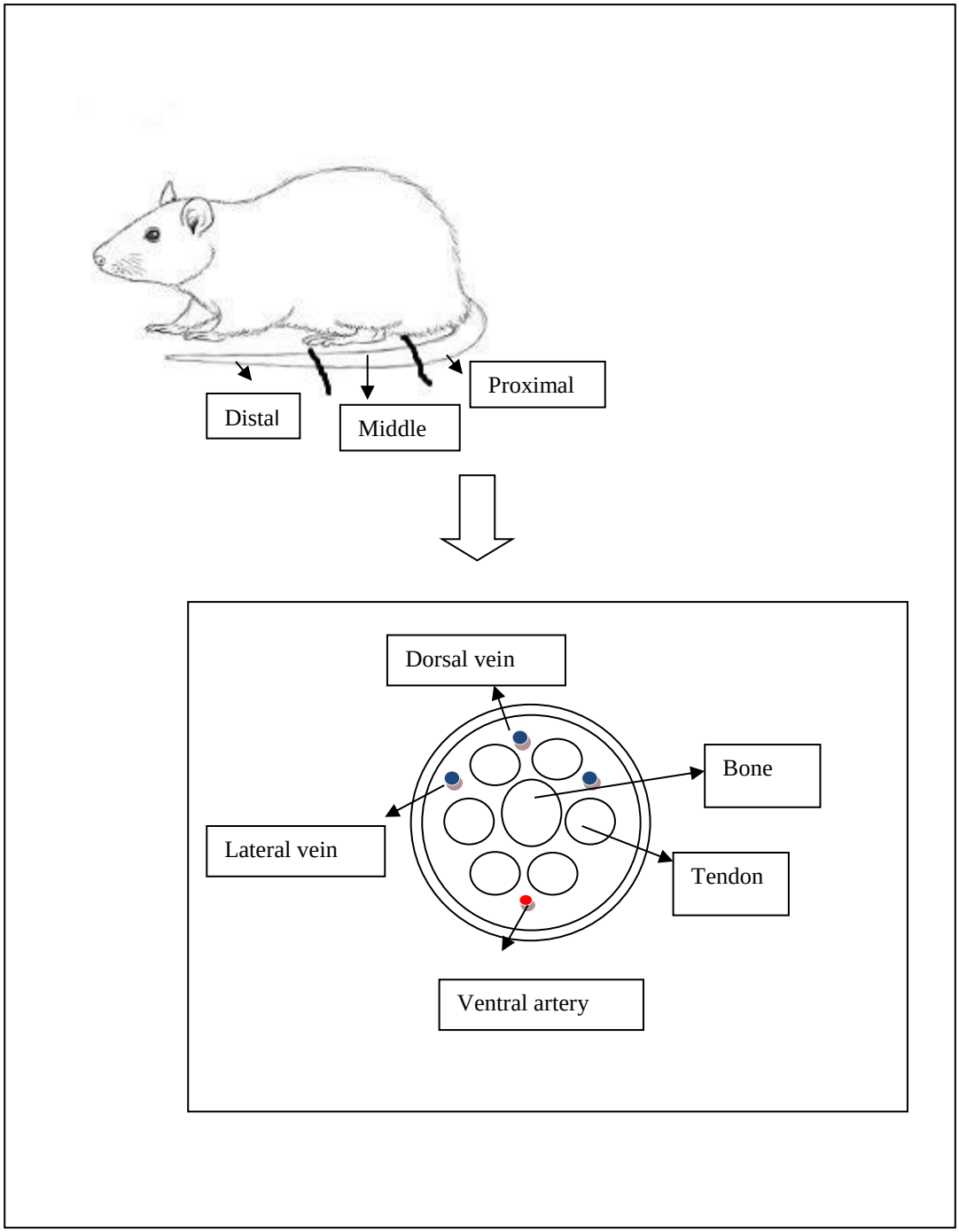


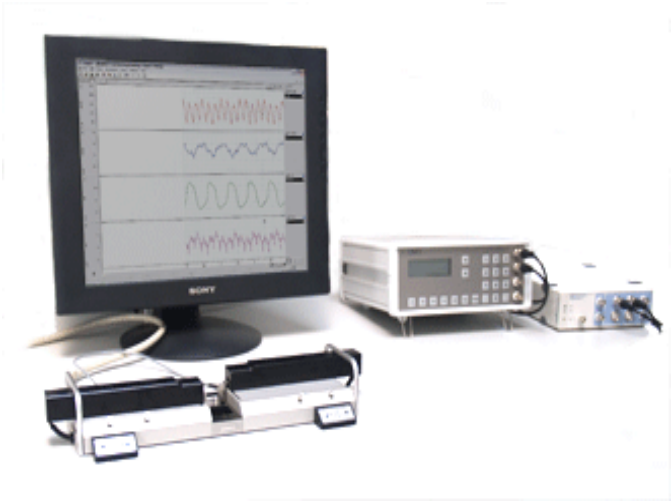
Figure 2.1 Diagram of transverse sections of rat tail pointing the dorsal vein, lateral vein and the ventral artery. Drawing adapted from Vanhoutte et al. 2002

2.2 Functional Study (Wire Myography)

In this *in vitro* procedure, the vascular reactivity at second (2^{nd}) and tenth (10^{th}) weeks of diabetes was evaluated. This experiment was carried out using dual wire myograph which was developed by Mulvany and Halpern to investigate active and passive properties of the vessels with the diameters between 100 and 1000 μm (Mulvany and Aalkjaer, 1990)

Functional study using wire myograph consist of few steps such as preparation of resistance arteries, mounting of the vessels, the normalization procedure, assessment of tissue viability, and the response of vascular function towards drugs with the presence of pharmacological inhibitors (Spiers and Padmanabhan, 2005).

In this study, we used dual wire myograph (410A), DanishMyo (DMT) Technology, Denmark which is designed to test responsiveness of two different small vessels (30 μm - 3 mm) simultaneously. This instrument also comes together with Powerlab 20, ADInstruments, Denmark where this instrument acquires tension data from the myograph in real time, which is displayed, recorded and analyzed using LabChart Pro software (Figure 2.2). It also consists of an internal heating system which keeps the chambers at a temperature of 37 °C.



Stereomicroscope

Dual wire Myograph

Figure 2.2: Equipment used in vascular functional study (Adapted from DMT manual)

2.2.1 Preparations of vessels for functional study

The procedure used in this study was done based on previous study (Mokhtar *et al.*, 2016). Once the vessels were isolated from the tail, the vessels were placed on petri dish filled with physiological saline solution. With the aids of micro dissecting scissors and forceps, the vessels were cleaned from the fats and connective tissues. The vessels were then cut into rings (2 mm in length). To study the endothelium-independent responses, the endothelial cell layers were removed by rubbing the lumen of the artery with human hair.

2.2.2 Preparation of buffers

(a) Physiological Saline Solution (PSS)

Physiological saline solution was prepared freshly before starting the experiment. This solution acts as internal buffer that maintains the same physiological conditions of the vessels as they exist in their normal body. To create similar environments, the physiological saline solution aerated with carbogen (95% oxygen and 5% carbon dioxide) for 20 minutes until the pH of the solution reached 7.4 and the experiment were conducted at 37 °C. To make physiological saline solution, all the chemicals listed below (Table 2.1) were weighed accordingly and dissolved in approximately 1000 ml deionized water.

Table 2.1. Recipe of the physiological saline solution

Chemicals	Molecular weight (g/mol)	Conc.(mmol/l)	Conc.(g/l)	Manufacturers
Sodium Chloride (NaCl)	58.44	118.99	6.954	Merck, Germany
Potassium Chloride (KCl)	74.56	4.69	0.35	Merck, Germany
Magnesium sulfate heptahydrate ($MgSO_4$-$7H_2O$)	246.48	1.17	0.29	Merck, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	136.09	1.18	0.16	Merck, Germany
Sodium hydrogen carbonate ($NaHCO_3$)	84.01	25.00	2.10	Merck, Germany
Glucose	198.77	5.50	1.09	Merck, Germany
Calcium chloride dehydrate ($CaCl_2$-$_2$H2O)	372.24	2.50	0.37	Merck, Germany

2.2.3 Mounting of the vessels

Before mounting the vessel in the myograph chamber, the chambers were filled with physiological saline solution with the heater (set to 37ºC) and gas (O_2) turned on. While waiting the temperature of the chamber to stabilize, the 2 mm of vessels placed in the petri dish was prepared to be cannulated under the microscope. By using the forceps, the vessel on the petri dish was gently threaded with first tungsten wire (40 μm diameter). The vessel was then moved to the chamber and placed at the myograph jaw which was attached to micrometer (Figure 2.3a). The wire was then placed in the center and was clamped by adjusting the micrometer (Figure 2.3b). Using the screwdriver, both end of the wires were wrapped under the screw of the jaw and tighten up (Figure 2.3c). Once the first wire fixed, the second wire was gently push into the lumen artery on top of the first wire (Figure 2.3d). The wire was introduced in one movement in order to avoid endothelial damage to the vessel. The jaws were moved gently to clamp the wire. The far end of the second wire was tied to the screw of the jaw which was attached to transducer (Figure 2.3e). Without damaging the mounted vessels, the jaws were opened slightly apart and the wires were adjusted parallel. The set-up of this experiment (two wires with one secured to micrometer and another to isometric force transducer) allows the wall forces produced by the vessel to be measured as it is held between the wires (Figure 2.4) (Bevan and Osher, 1972; Mulvany and Halpern, 1976; Mulvany and Aalkjaer, 1990; Schubert, 2005; Mokhtar *et al.*, 2016)

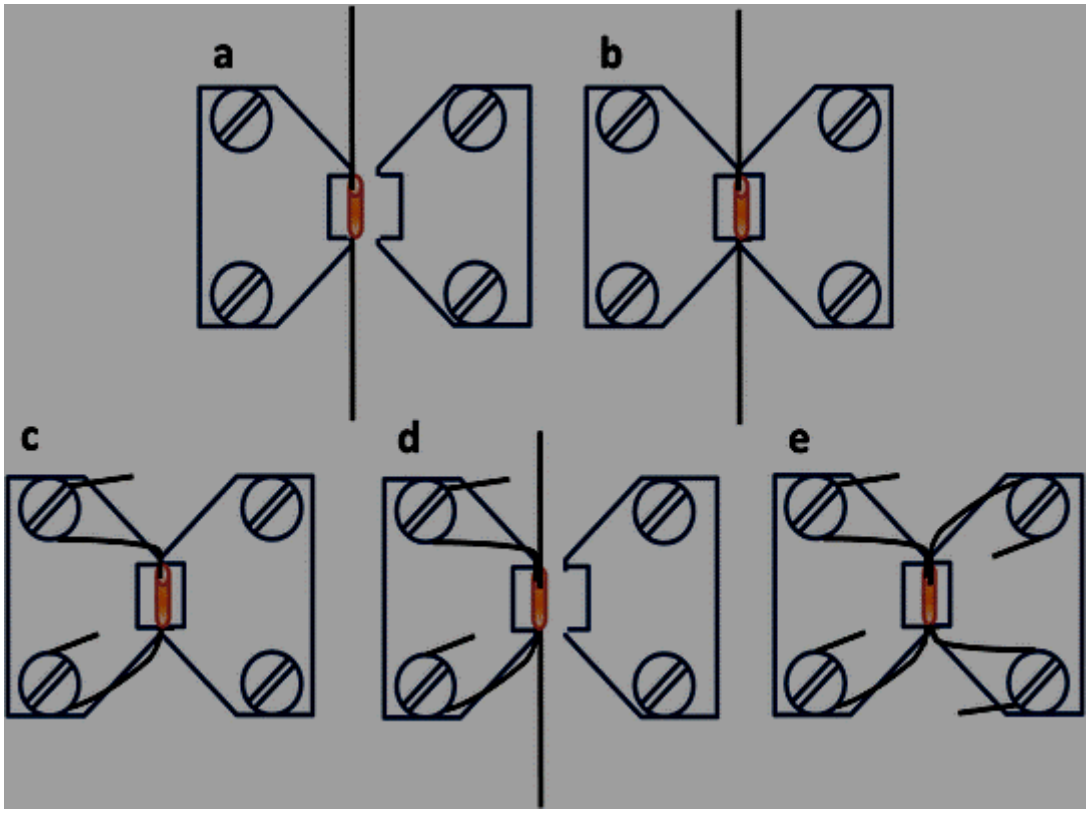


Figure 2.3 Mounting protocol of the artery; (a) the artery was placed in the middle of the jaw (b) the micrometre was adjusted to clamp the wire. (c) the end of the wires was secured tightly under the screw (d) the jaw was parted slightly before inserting the second wire on top of the first (e) the jaw was then closed before tying the wire underneath the screw.
Modified from https://musculoskeletalkey.com/wire-myography-to-study-vascular-tone-and-vascular-structure-of-isolated-mouse-arteries/

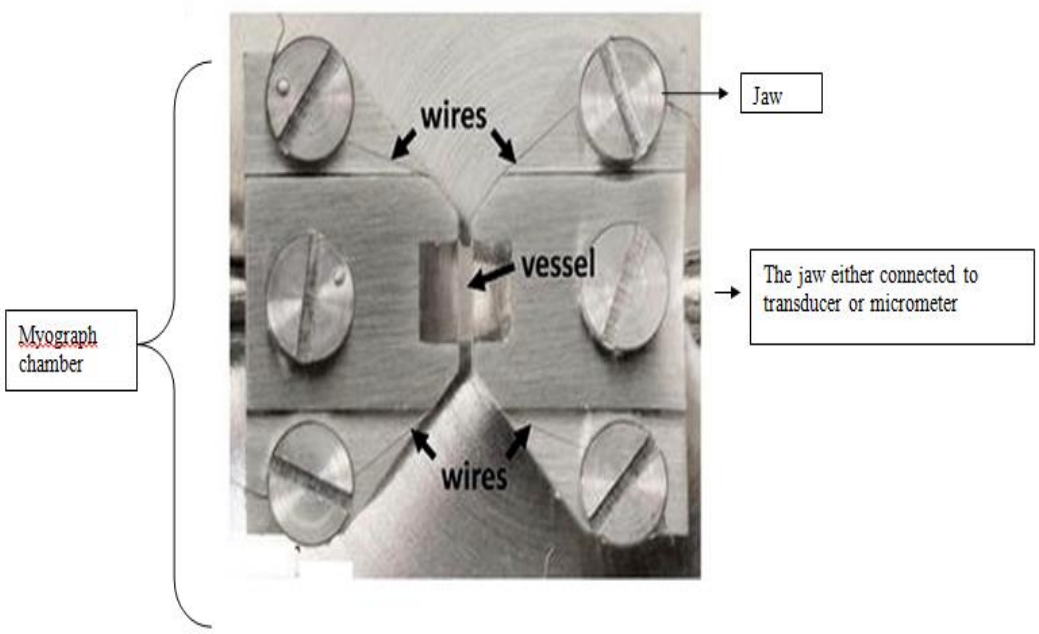


Figure 2.4 Vessel mounted on the jaws. Modified from Myograph manual book.

2.2.4 Normalization of vessel lumen

Before proceeding to the normalization, the mounted vessels were washed three times with PSS solution. The vessels were then allowed to equilibrate for thirty minutes.

The initial validation study on normalization procedure was conducted by (Mulvany and Halpern, 1977). From this study, it showed that the best experimental conditions were attained if one defines the vessel size as being the size when the vessel is fully relaxed and under a transmural pressure of 100 mmHg (internal circumference). Therefore in this study, the size of the vessel is first determined at a given transmural pressure (the pressure between the two sides of a vessel: internal – external wall pressure) and then set to the pressure at which contraction is optimal. According to Mulvany's group, the internal circumference of the vessel was determined at a standard target transmural pressure of 100 mmHg (IC_{100}=13.3 kPa), with the vessel relaxed. The size of the vessel that was optimal for contraction was at the IC_1=0.9XIC_{100}.

2.2.5 Wire Myograph protocol

2.2.5(a) Chemical used in smooth muscle viability and endothelial functions test

Table 2.2 showed the chemicals used in smooth muscle and endothelial function tests in wire myograph. Phenylephrine (PE) and acetylcholine (ACh) were freshly prepared each time the experiment conducted. Both drugs were diluted according to the desired concentrations with PSS (the final concentrations required for each drugs were determined in our preliminary study). Potassium chloride (KCl) were prepared and stored in room temperature. To check the smooth muscle function, 60 mM KCl (equivalent to 100 µl) was taken from the bottle and used. This concentration was determined from our previous study methodology (Mokhtar *et al.*, 2016).

Table 2.2 List of chemicals used in smooth muscle and endothelial function tests.

Chemicals	**Molecular Weight**	**Manufacturers**
Potassium Chloride (KCl)	74.56	Merck, Germany
Acetylcholine chloride (ACh)	181.66	Sigma Aldrich, USA.
Phenylephrine (PE)	203.67	Sigma Aldrich, Germany

2.2.5(b) Smooth muscle viability test (Contractile function)

After the normalization, the vessels were left to equilibrate for one hour. Then the vessels were treated with KCl. This causes vascular smooth muscle to depolarize, increase in inflow of extracellular Ca^{2+} and thus activates the contraction process. This step is conducted to confirm whether the function of VSMC remains intact as the vessels might be damage during the isolation or when the mounting task was carried out. KCl (60mM) was added into the chamber to observe for the contractile activity. The vessels were exposed to KCl for 10 minutes or until contractile activities became stable. The chamber was then washed with physiological saline solution until the vessels were allowed to relax to baseline. After 10 minutes, the vessels were then exposed to repeated dose of KCl. The vessels that failed to response to 60 mM of KCl were discarded.

2.2.5(c) Endothelium function test

This step is carried out to check the ability of the vessels to relax where endothelium can be easily damaged while mounting or dissecting. Besides that, this step is also needed to double check whether the endothelial cells were successfully removed in the case of studying endothelium-independent responses.

To check the endothelial viability function, the vessels was tested with a standard vasoconstrictor, phenylephrine (PE) and endothelium-dependent vasodilator, ACh. Phenylephrine causes contraction by acting on α1-adrenergic receptor of endothelial cells whereas ACh mediates endothelial relaxation by activation of muscarinic receptor on endothelial cells. ACh was used to stimulate the

release of nitric oxide or other relaxing factors from the endothelium and the ability of endothelium to relax

In this study, PE (10^{-4} M) was added to the chamber until contraction was stable. The concentration of PE was decided based from our initial observation where optimal contraction produced. From our initial observation when the wrong concentration of PE used, the arteries exhibited limited ability to maintain constriction. The mean tension to phenylephrine must reach 80% to that of KCl contraction. Once the PE contraction remained plateau, ACh (10^{-4} M) was introduced into the chamber so that vessels were able to relax. The vessels with intact endothelium were seen fully relaxed when introduced with ACh. The vessels in both chambers were washed and allowed to relax for 15 minutes before proceeding to further investigations.

2.2.6 Endothelium-dependent relaxation

2.2.6(a) Pharmacological inhibitors used for endothelium-dependent relaxation study

Table 2.3 listed type of inhibitors used in this protocol. Stock solutions were prepared in their respective solvents. The stock solutions were stored at -20°C freezer. They were freshly diluted to the appropriate concentrations with control solution before the experiment. The final concentration in the chamber for each drug was based on previous experience in the laboratory or studies reported in the literature (Table 2.3.1)

Table 2.3 List of pharmacological inhibitors used in endothelium-dependent relaxation

Chemical names	**Alternative name**	**Molecular weight**	**Manufacturer**
1-[(2-Chlorophenyl) diphenylmethyl]-1*H*-pyrazole	TRAM 34	344.84	Tocris Bioscience, UK
1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid	Indomethacin	357.8	Cayman, USA
6,10-diaza-3(1,3)8,(1,4)-dibenzena-1,5(1,4)-diquinolinacyclodecaphane	UCL 1684	654.44	Tocris Bioscience, UK
L-NG-Nitroarginine methyl ester hydrochloride	L-NAME	269.7	Cayman, USA
Potassium Chloride	KCl	74.56	Merck, Germany

Table 2.3.1 Inhibitors used in endothelium-dependent relaxation study

Inhibitors	Actions	Diluents	Final concentration in chamber	Reference
Indomethacin	• Non-selective inhibitor of cyclooxygenase (COX) • Thwarts prostacyclin production	DMSO	10^{-5} M (50µl)	(Luksha *et al.*, 2004; Mokhtar *et al.*, 2016)
L-NAME	• Inhibitor of NO synthase and hinders NO Productions	Distilled water	10^{-4} M (50µl)	(Georgescu *et al.*, 2011; Mokhtar *et al.*, 2016)
KCl	• Opening of K+ channels in the vascular smooth muscle cells abolished EDH activity.	Distilled water	30 mM (50µl)	(Shimokawa *et al.*, 1996)
TRAM 34	• Blocker of intermediate calcium-activated potassium channels (IKCa) inhibits EDH activity	DMSO	10^{-6} M (50µl)	(Leo *et al.*, 2011; Mokhtar *et al.*, 2014; Mokhtar *et al.*, 2016)
UCL 1684	• Blocker of small calcium-activated potassium channels (SKCa) and causes inhibition of EDH activity	DMSO	10^{-6} M (50µl)	(Leo *et al.*, 2011 (Mokhtar *et al.*, 2014; Mokhtar *et al.*, 2016)

2.2.6(b) Protocol of endothelium-dependent relaxation

Endothelium-dependent relaxations were investigated in pre-contracted artery. The purpose of this protocol was to find out the contribution of NO, prostacyclin and EDH in microvasculature of diabetic and normal rats at two and ten of the experiments.

Four vessels from four groups (diabetic 2-week rats, normal 2-week rats, diabetic 10-week and normal 10-week rats) that passed the viability and presence of endothelium tests were selected and treated under the following conditions:

a) Control solution: The vessels were not incubated with any inhibitors, and pre-contracted with PE (10^{-4} M) and exposed to cumulative concentration of ACh (10^{-9} to 10^{-4} M)

b) NO-mediated relaxation: The vessels were incubated for 30 minutes with the combination of indomethacin (10^{-5} M, non-selective inhibitor of COX that inhibits prostacyclin production) together with TRAM 34 (10^{-6} M), UCL 1684 (10^{-6} M) and KCl 30 mM (to inhibit EDH activity). After the incubation, the vessels were pre-constricted with PE (10^{-4} M) and exposed to cumulative concentration of ACh (10^{-9} to 10^{-4} M ACh)

c) EDH-mediated relaxation: The vessels were incubated with L-NAME [10^{-4} M, inhibitor of eNOS] and indomethacin (10^{-5} M) for 30 minutes. The vessels were then pre-contracted with PE (10^{-4} M) and exposed to cumulative concentration of ACh (10^{-9} to 10^{-4} M ACh)

d) Prostacyclin-mediated relaxation: The vessels were incubated with L-NAME [10^{-4}], TRAM 34 (10^{-6} M), UCL1684 (10^{-6} M) and 30 mM KCl for 30

minutes. The vessels were then pre-contracted with PE (10^{-4} M) and exposed to cumulative concentration of ACh (10^{-9} to 10^{-4} M ACh)

Table 2.3.2 showed the procedure involved in endothelium relaxation study in rat arteries. Figure 2.5 showed illustration of myograph tracing and formula to calculate the percentage of relaxation due to ACh.

Table 2.3.2 Procedures of endothelium-dependent relaxation study in rat arteries

Vessels	Controls	NO- mediated relaxation	EDH- mediated Relaxation	Prostacyclin- mediated relaxation
Standard procedures	**Normalization (**pre-stretching of an intact vessel) **Smooth muscle viability test (Contractile function)** - 2X exposure to KCl 60 mM **Endothelial function test** - Pre contracted with phenylephrine (10^{-4} M) - ACh (1 x 10^{-4} M)			
Incubation with blockers (30 minutes)	• No blockers	• Indomethacin (10^{-5} M) • TRAM34(10^{-6} M) • UCL1684(10^{-6}M) • KCl 30 mM	• LNAME (10^{-4} M) • Indomethacin(10^{-5}M)	• LNAME(10^{-4} M) • TRAM34(10^{-6} M) • UCL1684(10^{-6} M) • KCl 30mM
Pre-constriction	Phenylephrine(10^{-4} M)			
Cumulative concentration response curves	ACh (10^{-9} to 10^{-4} M)			

ACh, acetylcholine; EDH, endothelium-dependent hyperpolarization; KCl, potassium chloride; NO, nitric oxide.

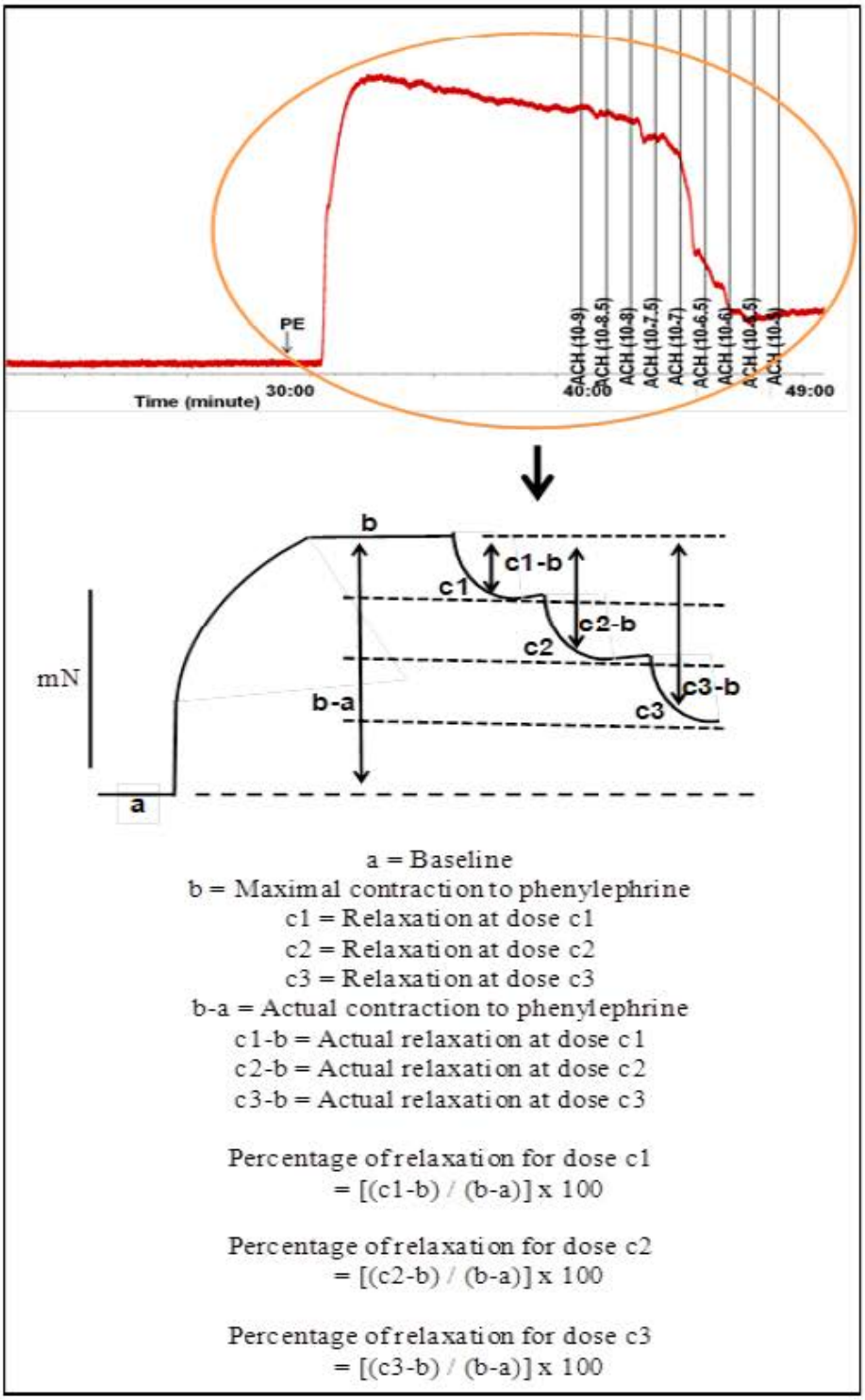


Figure 2.5 : Diagram of myograph tracing and calculation procedures for the percentage of relaxation.

2.2.7 Endothelium-independent relaxations

2.2.7(a) Chemical used to study the endothelium-independent relaxations

Table 2.4 listed the drugs used to study the endothelium-independent relaxations. The phenylephrine (PE) and sodium nitroprusside dehydrate (SNP) were freshly prepared and diluted with physiological saline solution (PSS) before the study. All the drugs were diluted to the appropriate concentrations with PSS solution before the experiment. The final concentration needed for the study was taken according to the previous literature (Table 2.4.1).

Table 2.4 List of chemicals used in endothelium-independent relaxation.

Chemicals	**Molecular Weight**	**Manufacturers**
Phenylephrine (PE)	203.67	Sigma Aldrich, Germany
Sodium Nitroprusside dehydrate	297.95	Sigma Aldrich, Poland

Table 2.4.1: Characteristic of the drugs used in endothelium-independent relaxation.

Drugs	**Actions**	**Diluent**	**Final concentration in chamber**	**Reference**
PE	α-1 adrenoreceptor agonist	Distilled water	10-$^{-4}$ M	Preliminaries study
SNP	NO donor	Distilled water	10^{-8} to 10^{-4} M	(Mokhtar *et al.*, 2016)

2.2.7(b) Protocol of endothelium-independent relaxation

To determine the endothelium-independent relaxations, the endothelium needs to be removed first. Therefore the purpose of this experiment is to check the relaxation of vascular smooth muscle without the involvement of endothelium. The endothelial cells were removed by rubbing the intimal surface of the lumen with a human hair (Kirkby *et al.*, 2012). Prior to conducting endothelium-independent tests, the absence of endothelial cells was confirmed by lack of relaxation caused by ACh agonist. The vessels with relaxation less than 30% were selected for endothelium-independent relaxation test. The selected vessels without endothelium were pre-contracted with PE and their responds towards cumulative concentration of SNP were tested.

Table 2.4.2 showed the procedure involved in endothelium- independent relaxation study in rat arteries. The calculation for endothelium-independent test was similar to calculation of endothelium-dependent relaxation (Please refer Figure 2.5).

Table 2.4.2 Preparation of endothelium-independent relaxation study in rat artery

Vessels	**SNP**
Standard protocol	**Normalization (**pre-stretching of an intact vessel) **Smooth muscle viability test (Contractile function)** - 2X exposure to KCl 60 mM **Endothelium function test** - Pre contracted with phenylephrine (10^{-4} M) ACh (10^{-4} M)
Pre-constriction	Phenylephrine (10^{-4} M)
Cumulative concentration response curves	SNP (10^{-8} to 10^{-4} M)

2.2.8 Endothelium-dependent contractions

2.2.8(a) Pharmacological inhibitors used in endothelium-dependent contractions study

Table 2.5 listed the type of inhibitors used in this protocol. Stock solutions were prepared in their respective solvents. The stock solutions were stored in a -20°C. They were freshly diluted to the appropriate concentrations with control solution before the experiment. The final concentration in the chamber for each drug was based on previous experience in the laboratory or studies reported in the literature (Table 2.5.1).

Table 2.5: List of drugs used in endothelium-dependent contractions.

Chemicals name	**Alternative name**	**Molecular Weight**	**Manufacturers**
1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid	Indomethacin	357.8	Cayman, USA
2-Valeryloxybenzoic acid	Valeryl salicyclate	222.24	Sigma Aldrich, USA
(3-[(6-amino-(4-chlorobenzensulphonyl)-2-methyl-5,6,7,8-tetrahydronapht]-1-yl)propionic acid)	S18886/ terutroban	407.91	Gift from University of Hong Kong, Hong Kong
Calimycin	Calcium ionophore A23187	523.62	Sigma Aldrich, USA
L-NG-Nitroarginine methyl ester hydrochloride	L-NAME	269.7	Cayman, USA
N-[2-(Cyclohexyloxy)-4-nitrophenyl]methanesulfonamide	NS398	314.36	Sigma Aldrich, USA

Table 2.5.1 Inhibitors used in endothelium-dependent contractions study

Drugs	Actions	Diluents	Final concentration in chamber	Reference
Calcium ionophore (A23187)	• Highly selective for Ca^{2+} (increase intracellular Ca^{2+} levels)	DMSO	10^{-9}-$10^{-4.5}$M	
Indomethacin	• Non-selective inhibitor of cyclooxygenase (COX) • Thwarts prostacyclin production	DMSO	10^{-5} M	(Okon *et al.*, 2002; de Sotomayor *et al.*, 2007; Tang and Vanhoutte, 2008b)
L-NAME	• Optimize EDCF- mediated response • Eradicate the effect of NO-induced relaxation.	Distilled water	10^{-4} M	(Tang *et al.*, 2005) (Okon *et al.*, 2002) (Qu *et al.*, 2010)

NS398	• Preferential blocker of COX-2	DMSO	10^{-6} M	(Zhang *et al.*, 2004; Gluais *et al.*, 2005; de Sotomayor *et al.*, 2007; Shi *et al.*, 2008)
S18886	• Potent thromboxane A2 (TXA_2) inhibitor	DMSO	10^{-7} M	(Tang *et al.*, 2005) (Gluais *et al.*, 2007; Tang and Vanhoutte, 2008b)
Valeryl salicyclate	• Preferential blocker of COX-1	DMSO	3×10^{-3} M	(Gluais *et al.*, 2005; Tang *et al.*, 2005; Vanhoutte and Tang, 2008)

2.2.8(b) Protocol of endothelium-dependent contractions

Endothelium-dependent contractions were studied in quiescent artery (artery with endothelium). The purpose of this protocol was to find out the roles of cyclooxygenase enzymes (COX-1 and COX-2), and thromboxane receptor (TXA_2 receptor) in endothelium-mediated contraction of the microvasculature in normal and diabetic rats at two and ten of the experiments.

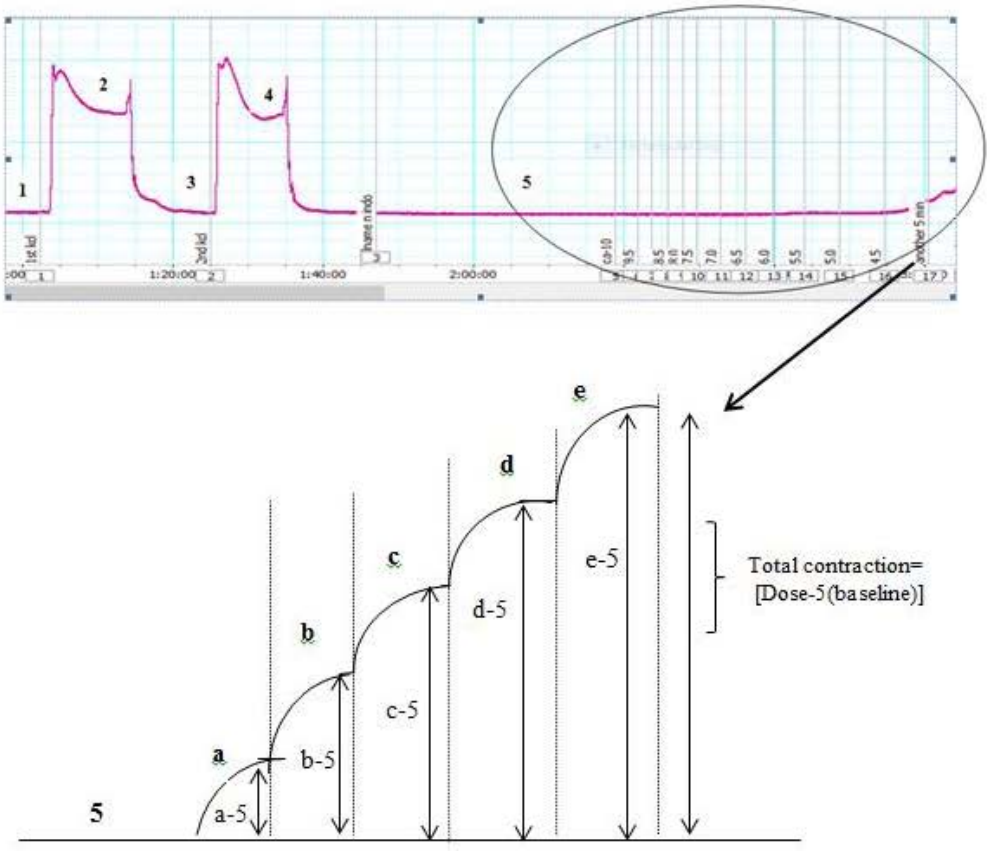
All the vessels were incubated with L-NAME (10^{-4}M), an NOS inhibitor, to optimize endothelium-dependent, COX-dependent contraction. Contractions to the calcium ionophore, A23187 (receptor independent activation) were studied under the following environments:

1. In control solution: the vessels were incubated with L-NAME for 30 minutes and were subjected to cumulative concentrations of calcium ionophore (10^{-8} M to $10^{-4.5}$ M). This step was to determine whether endothelium-dependent contractions occur in small arteries of the rat tail and were there alterations in the presence of diabetes.
2. In the presence of indomethacin: the vessels were incubated with L-NAME and indomethacin (10^{-5} M) for 30 minutes and then subjected to cumulative concentrations of calcium ionophore (10^{-8} M to $10^{-4.5}$ M). This step was to determine whether endothelium-dependent contraction was mediated by products of cyclooxygenase.
3. In the presence of valeryl salicylate: the vessels were incubated with L-NAME and valeryl salicyclate (3×10^{-3} M, an inhibitor of COX-1) for 30 minutes and then subjected to cumulative concentrations of calcium

ionophore (10^{-8} to $10^{-4.5}$M). This step was to determine if COX-1 plays a role in the contraction.

4. In the presence of NS398: The vessels were incubated with L-NAME and NS-398 (10^{-6} M, an inhibitor of COX-2) for 30 minute and then subjected to cumulative concentrations of calcium ionophore (10^{-8} to $10^{-4.5}$M). This step was to determine if COX-2 plays a role in the contraction.
5. In the presence of S18886: The vessels were incubated with the TXA_2 receptor antagonist, S18886 (10^{-7} M) and L-NAME for 30 minute and then subjected to cumulative concentrations of calcium ionophore (10^{-8} to $10^{-4.5}$ M). This step was to determine whether the endothelium-dependent contractions of small arteries of the rat tail involves activation of TXA_2 receptors on the vascular smooth muscle

Figure 2.6 showed illustration of myograph tracing and formula to calculate percentage of contraction induced by calcium ionophore. Increases in tension were expressed as percentage of the reference contraction to 60 mM KCl before drug incubation.



1= Baseline for 1st KCl
2= 1st maximal contraction to KCl
3= Baseline for 2nd KCl
4= 2nd maximal contraction to KCl
5= Baseline

1st KCl = 2-1

2nd KCl – 4-3

Average KCl contraction

a-5= Total Contraction at dose a
b-5=Total Contraction at dose b
c-5– Total Contraction at dose c
d-5= Total Contraction at dose d
e-5 =Total Contraction at dose e

Percentage of contraction at dose a : [a- 5/ (Average KCl contraction)] × 100 %
Percentage of contraction at dose b : [b- 5/ (Average KCl contraction)] × 100 %
Percentage of contraction at dose c : [c- 5/ (Average KCl contraction)] × 100 %
Percentage of contraction at dose d : [d- 5/ (Average KCl contraction)] × 100 %
Percentage of contraction at dose e : [e- 5/ (Average KCl contraction)] × 100 %

Figure 2.6 Diagram of myograph tracing and calculation procedures for the percentage of contraction.

2.3 Western blotting

2.3.1 Introduction to western blot

In this method, a mixture of proteins is parted according to their molecular weight, transferred to solid support or membrane and the target proteins were marked using primary and secondary antibody for the detection. We studied the expression of cyclooxygenase 1 (COX-1), cyclooxygenase 2 (COX-2), thromboxane receptor (TXA_2 receptor), thromboxane synthase (TXA_2 synthase), endothelial synthase (eNOs), prostacyclin receptor (IP receptor) and prostacyclin synthase (PGIS). The expressions of these proteins were then compared between normal and diabetic rats, and between 2 and 10 weeks diabetes.

2.3.2 Materials

Table 2.6, 2.6.1 and 2.6.2 listed the instruments, antibodies and chemicals and consumables used in Western blotting.

Table 2.6 List of instruments used in Western blotting

Instruments	Manufacturers
Ice-maker AF 200	Scotsman
Image analyser	Vilber Lourmat Fusion FX7-826 France
Mini Protean B cells	BioRad, USA
NanoDrop 1000 UV- Vis Spectrophotometer	Thermo Fisher Scientific, USA
PowerPac Basic	BioRad, USA
Refrigerated Centrifuge	Hettich Zentrifugen, Tokyo
Scanner	
Spectrophotometer	Thermoelectron Corporation, UK
Trans-blot SD Semidry transfer cell	BioRad, USA
Vortex mixer	IKA

Table 2.6.1 List of antibodies used in Western blotting

Type of antibodies	Manufacturers
Anti β-actin antibody	Abcam, UK
Anti-Cyclooxygenase 1 antibody	Abcam, UK
Anti-Cyclooxygenase 2 antibody	Abcam, UK
Anti- Prostaglandin I synthase antibody	Abcam, UK
Anti-Thromboxane A2 receptor antibody	Abcam, UK
Anti-Thromboxane A2 synthase antibody	Abcam, UK
Donkey anti-goat IgG (HRP)	GeneTex, Taiwan
Goat Anti- Rabbit IgG H&L (HRP)	Abcam, UK
IP Receptor (Prostaglandin II receptor)	Santa Cruz Biotechnology, INC
NOS3 antibody [endothelial nitric oxide synthase, eNOS]	Santa Cruz Biotechnology, INC

Table 2.6.2 List of chemicals and consumables used in Western blotting

Chemicals/Consumables	Molecular weight	Manufacturer
2-Mercaptoethanol	78.13	Merck, Germany
30% acrylamide/bis		Bio-Rad Laboratories. Inc, CA
Ammonium persulfate	228.20	Biorad Laboratory Inc., USA
Bovine serum albumin		Amresco,Ohio
Bromophenol blue	669.96	Sigma Aldrich, Austria
Chemiluminescent HRP substrate		Nacalai Tesque, Japan
Glycerol	92.1	Calbiochem, CA
Glycine	75.6	Merck, Germany
Immobilon-P, PVDF membrane	-	Millipore Corporation, USA
Methanol	32.04	Ajax Firechem, Australia
Phosphate buffer saline tablet		Sigma Aldrich, USA
Prestained broad range protein marker ladder (5 to 245 kDa)		SMOBIO, U.S.A
RIPA lysis buffer with protease inhibitor	-	Sigma Aldrich, USA
Skimmed milk powder	-	Anlene
Sodium Chloride	58.44	Merck, Germany
Sodium dodecyl sulfate (SDS)	288.38	Biorad Laboratory Inc., USA
TEMED	-	Sigma Aldrich, USA
Trizma Base	121.1	Sigma Chemical Co., USA
Trizma hydrochloride	157.6	Sigma Chemical Co., USA
Tween-20	-	Sigma Chemical Co., USA

2.3.3 Buffers for SDS PAGE electrophoresis and Western blotting

a) 0.5 M Tris- Base pH 6.8

To prepare Tris- base with the concentration of 0.5 Molar, 6.06 g of Tris- base were weighed and dissolved in 70 ml distilled water. The pH of the buffer was then adjusted to 6.8 with HCl. The solution was then refill up to 100 ml and was stored at 4°C.

b) 1.5 M Tris Base pH 8.8

To prepare 1.5 M Tris base, 90.86 gram of Tris- base was dissolved in 250 ml of double distilled water and the pH was adjusted to 8.8. The buffer was top up with double distilled water to reach final volume of 500 ml and was stored at room temperature

c) Ammonium persulfate (10%)

To make ammonium persulfate (10%) freshly, 0.1 g of ammonium persulfate was dissolved in one ml of distilled water.

d) Blocking buffer (5%)

Milk was used as blocking buffer: to prepare 5% of it, five gram of non-fat dry milk powder, Anlene was dissolved in 100 ml phosphate buffer saline (PBS). This buffer was made freshly each time before the use

e) Coomassie blue solution

To prepare Coomassie blue stock solutions, 0.8 g of Coomassie blue powder was dissolved in 100 mL of methanol which were then added with 260 ml of distilled water. Before the usage, the 90 mL of stock solution were added with 10 mL of acetic acid. Both solutions were stored in room temperature.

f) Destaining solution

The solution was prepared by mixing 400 mL of methanol with 100 mL of acetic acid and 500 mL of distilled water.

g) Phosphate buffered saline/Tween-20 (PBS-T)

PBS was bought readily from Sigma Aldrich, USA. To prepare one litre of PBS, about five tablets were dissolved in one litre of distilled water.

PBST was prepared by adding one ml of Tween-20 to 1000 ml of PBS. This PBST solution was then used as washing buffer and to dilute the antibody.

h) Ponceau S solution

The solution was prepared by dissolving 0.2 g of Ponceau red with 1 mL of acetic acid. The solution was then made up to 100 mL with the addition of double distilled water.

i) Reducing sample buffer/ Laemmli buffer

Sample buffer was prepared by adding 0.76 g of Tris-base, one g of SDS and 10 ml of glycerol in 30 ml double distilled water and the pH was adjusted to 6.8. Prior to use, 10% 2-mercaptoethanol and 0.05% bromophenol blue were added to the buffer.

j) Running buffer

Running buffer was prepared by dissolving three g of Tri-base, 14.4 g of glycine and one g of SDS in 800 ml double distilled water and the pH was adjusted to 8.3. The buffer was top up with double distilled water to reach final volume 1000 ml and was stored at 4 °C.

k) Sodium dodecyl sulfate (SDS)

10% of SDS was prepared by dissolving 10 g of SDS powder to 80 ml of distilled water. These mixtures were then made up to 100 ml by adding distilled water.

l) Stacking buffer

Stacking buffer was prepared by dissolving 6 g of Tri-base and 0.4 g of sodium dodecyl sulfate (SDS) in 70 ml double distilled water and the pH was adjusted to 6.8. The buffer was top up with double distilled water to reach final volume of 1000 ml and was stored at 4 °C.

m) Transfer buffer

Transfer buffer was prepared by dissolving 3 g of Tri-base, 14.4 g of glycine and 0.37 g of SDS in 800 ml double distilled water and followed by the addition of 200 ml methanol. The pH was adjusted to 8.3 and was stored at 4 °C.

2.3.4 Tissue lysate preparation

The proximal and distal part of tail arteries were harvested and placed in centrifuged tube containing one hundred microliter of ice-cold RIPA buffer. By using the micro scissors, the tissues were crushed into small pieces so that the tissues were completely lysed and released the protein of interest. The homogenized sample lysate was let to incubate for 10 minutes at 4 °C before the lysate was centrifuged at 12 000 rpm for 20 minutes at 4 °C. The supernatant of the lysate was collected and were stored in deep freezer (-80 °C). The remaining tissues were discarded.

2.3.5 Protein assay quantification

The prepared samples contain unknown protein concentration. The amount of the protein presence in the samples were determined by using NanoDrop 1000 UV- Vis Spectrophotometer (Thermo Fisher Scientific, USA) at 280nm absorbance . This step is to ensure equal loading of protein in gels electrophoresis. Two microliter of the sample was placed on top of the pedestal. The absorbance value of the protein was detected by the internal spectrometer of NanoDrop.

2.3.6 Preparation of gel for gel electrophoresis

The protein molecules were parted according to their molecular weight by SDS-PAGE. To begin the electrophoresis process, two separate gels (4% stacking gel and 8.0 % resolving gel) were prepared freshly. This method is known as Laemmli (Laemmli, 1970). The glass plates were washed and wiped with 70% ethanol before assembling it onto casting stand. The short plates were laid on top of bigger plates and the plates were clamped together. Distilled water was applied onto it to check whether it was tightly sealed and no leakage. The water was then discarded. The solutions for resolving gel was prepared according to recipes listed in the table

2.6.3. The resolving gel was poured into the plates until one particular level (briefly leaving behind 2 cm from the top so that the comb was inserted later). Distilled water was then added on top of resolving gel and the gel was let to polymerize. The water was then discarded once the gel was polymerized. Similarly, the stacking gel was prepared (recipe in the table 2.6.3) and was added on top of resolving gel and the comb were inserted. The gel was let to harden. After the gel was harden, the comb was carefully removed and the wells were cleaned with distilled to remove the acrylamide debris. The gel was then detached from the casting stand and transferred to the electrophoresis tank containing running buffer.

Table 2.6.3 Recipe of stacking and resolving gel preparations

Chemicals	**Volume**
Stacking gel (4%)	
30% acrylamide/bis	0.53 ml
0.5 M Tris Base	1ml
Double distilled water	2.4 ml
Ammonium persulfate	40 µl
SDS	40 µl
TEMED	4 µl
Resolving gel (8%)	
30% acrylamide/bis	2.67 ml
1.5 M Tris Base	2.5 ml
Double distilled water	4.6 ml
Ammonium persulfate	100 µl
SDS	100 µl
TEMED	10 µl

2.3.6(a) Sample preparation

Prior to loading the samples in the well of the gel, the samples needed to be boiled. We used 80 µg of samples where the volume of the samples to be added was calculated from the protein quantification method (2.3.5). The calculated samples

were pre-mixed with sample buffer in an equal ratio before boiling at 95˚C for 5 minutes. The volume of the sample to be added to the gel to get 100 µg of protein was determined from the protein assay quantification method.

2.3.6(b)SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

About five microliter of the protein ladder (5 kDA to 245 kDA) was loaded to first lane of the well. The boiled samples were added into other empty wells. The black lead of electrophoresis tank was connected to the negative terminal and the red lead to the positive terminal of power lab. The gel was run at 120 V until the dye reached the bottom of the gel. The gel was then subjected to protein blotting immediately.

2.3.7 Semi dry protein transfer

Before transferring the protein onto the PVDF membrane, the membrane was activated with methanol for few minutes before treating them in transfer buffer for 10-15 minutes. The gel was removed from the electrophoresis tank and also from the glass plates before washing it with distilled water. The gel was then equilibrated in transfer buffer for 10-15 minutes. The filter pads were also wetted with the transfer buffer. The filter pads were then assembled on top of transblot which served as an anode followed by the PVDF membrane, gel and finally the filter paper. Air bubbles were removed by rolling the falcon centrifuge tube on top of each layer. This sandwich type arrangement was then covered with the cathode plate and the transfer was performed at 18 V for 2 hours 30 minutes. The membrane was stained with Ponceau S solution to ensure the efficiency of the transfer. The gel was also treated with Coomasie Blue solution to detect the presence of protein in gel.

2.3.8 Antibody incubation and detections

The membrane was blocked for one hour at room temperature in blocking PBS buffer containing 5% non-fat dry milk and 0.1% Tween 20. Membrane was then incubated with the corresponding primary antibodies in blocking buffer. The detail of dilution factor and duration of antibodies incubation were listed in the Table 2.6.4. After being washed three times with washing PBS buffer containing 0.1% Tween 20 (PBS-T) for 5 minutes, the membrane was incubated for one hour with polyclonal secondary antibody which was horse-radish peroxidase (HRP)-conjugated in blocking buffer. Membrane was then washed three times prior to detection by Chemi-Lumi One Super. Image analysis was performed using Image Analyser (Vilber Lourmat Fusion FX7-826 France). The intensity of protein bands representing the amount of proteins was measured with Image J software (http://rsb.info.nih.gov/ij/). The relative protein presence of eNOS, COX-1, COX-2, PGIS or IP receptors was expressed as the total amount of protein (indicated by the intensity of protein band for β-actin) in the rat sample and the intensity of protein bands.

Table 2.6.4 Dilution and duration of incubation for antibodies used in Western blotting

Antibody	Western blotting	
	Dilution	**Incubation Duration**
Anti β-actin antibody	1:5000	Overnight
Anti-Cyclooxygenase 1 antibody	1:2 000	2 hours
Anti-Cyclooxygenase 2 antibody	1:2 000	2 hours
Anti-Prostaglandin I synthase antibody	1:2 000	2 hours
Anti-Thromboxane A2 receptor antibody	1:2000	2 hours
Anti-Thromboxane A2 synthase antibody	1:2000	2 hours
Donkey anti-goat IgG (HRP)	1:2000	1 hours
Goat polyclonal secondary antibody to rabbit IgG-H&L (HRP)	1:2000	1 hours
IP Receptor (Prostaglandin II receptor)	1:2000	Overnight
NOS3 Antibody (eNOS)	1:1000	Overnight

2.4. Immunohistochemistry

In this study, immunohistochemistry (IHC) methods were applied to detect the presence and locations of selected proteins in tissue sections. The distribution and locations of COX-1, COX-2, TXA_2 receptor, TXA_2 synthase, eNOs, PGIS and IP receptor were performed in four different groups of rats [normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats] .

2.4.1. Materials

Table 2.7, 2.7.1 and 2.7.2 refer to the instruments, chemicals and consumables used during immunohistochemistry.

Table 2.7 List of instruments used in IHC

Instruments	**Manufacturers**
Cold plate	Thermoelectron Corporation, USA
Hot plate	Thermoelectron Corporation, USA
Light microscope with image analyser	Olympus Corp., Japan
Microtome, Shandon 325	Thermoelectron Corporation, USA
Tissue embedding centre	Thermoelectron Corporation, USA
Tissue floatation bath, HI 1210	Leica, Solms, Germany
Tissue processor, TP 1020	Leica Solms, Germany

Table 2.7.1 List of antibodies used in IHC

Type of antibodies	Manufacturers
Anti-Cyclooxygenase 1 antibody	Abcam, UK
Anti-Cyclooxygenase 2 antibody	Abcam, UK
Anti- Prostaglandin I synthase antibody	Abcam, UK
Anti-Thromboxane A2 receptor antibody	Abcam, UK
Anti-Thromboxane A2 synthase antibody	Abcam, UK
Donkey anti-goat IgG (HRP)	GeneTex, Taiwan
Goat Anti- Rabbit IgG H&L (HRP)	Abcam, UK
IP Receptor (Prostaglandin II receptor)	Santa Cruz Biotechnology, INC
NOS3 antibody[endothelial nitric oxide synthase, eNOS]	Santa Cruz Biotechnology, INC

Table 2.7.2 List of chemicals and consumables used in IHC

Chemicals/ Reagents	Molecular weight	Manufacturers
Acetic acid	60.05	J.T. Baker, PA,USA
Ammonia solution 25%	17.03	Merck
Ethanol absolute	46.07	HmbG Chemicals, Germany
Formaldehyde	30.03	Mallinckrodt chemicals St.Louis, MO, USA
Hydrogen peroxide (30%)	34.01	J.T. Baker
Mounting DPX		Invitrogen, MA, USA
Sodium Chloride	58.44	Merck
Stable Diaminobenzidine (DAB)		Invitrogen, USA
Trisodium citrate dehydrate	294.10	
Trizma hydrochloride	157.6	Sigma Aldrich
Tween-20	1228	Sigma Aldrich
Xylene	106.17	Sigma Aldrich

2.4.2 Recipe of the buffers

a. Preparations of blocking solutions (5%)

5% of blocking buffer was prepared by dissolving five gram of milk powder in 100 ml of TBST. This solution was freshly prepared each time before the experiment.

b. Preparations of ethanol (95%)

The solution was prepared by adding 950 ml of pure ethanol (100%) into 50 ml of double distilled water.

c. Preparations of ethanol (80%)

Eighty percent of pure ethanol was prepared by mixing 800 ml of pure ethanol (100%) with 200 ml of double distilled water.

d. Preparations of Ethanol (70%)

Seventy percent of pure ethanol was prepared by adding 700 ml of pure ethanol (100%) with 300 ml of double distilled water.

e. Preparations of Ethanol (50%)

Fifty percent of pure ethanol was prepared by equally adding 500 ml of pure ethanol (100%) with 500 ml of double distilled water.

f. Preparations of Hydrogen peroxide, 3%

Ten millilitre of 30% hydrogen peroxide solution was diluted in 90 ml double distilled water to obtain 100 ml of 3% hydrogen peroxide

g. 10 mM Sodium citrate buffer, 0.05% Tween-20, pH 6.0

10 mM sodium citrate buffer was prepared by dissolving 2.94g of tri- sodium citrate dehydrate in 1000ml of distilled water. The pH was then adjusted to 6.0 with 1 molar of HCl. 0.5 ml of Tween-20 was added into the solution and was stored at room temperature

h. Tris Buffered Saline (TBS)

TBS was prepared by dissolving 2.43 of Tris-HCl and 8 g of sodium chloride in 1000 ml of double distilled water with the pH was adjusted to 7.6 by using sodium hydroxide.

i. Tris Buffered Saline/ Tween-20 (TBS-T)

In this study, TBST was used as washing buffer and antibody diluents. TBST was prepared by adding 1 ml of Tween-20 into 1000 ml of TBS prepared before and mixed well. The solution was then placed at room temperature.

2.4.3 Protocols involved in IHC

2.4.3(a) Tissue collection and slide preparation

The tail arteries were cleaned from fat and rinsed with Krebs solution to prevent the presence of hematologic antigens which intervene the detection of target antigens. The tail arteries were then cut into 4 to 5 mm in length before immersing them in formaldehyde. The purpose of the tissues immersed in 4% of formaldehyde (pH 7.4) for 18 to 24 hours to prevent autolysis and also to preserve the tissues. The preserved tissue were then wrapped in the gauze and placed in the cassettes before proceeding with tissue processing.

2.4.3(b) Tissue processing

The tissues were then placed in an automated closed tissue processor (Tissue processor, TP 1020 Leica) for overnight where the tissues undergo a series of steps including dehydration, clearing and infiltrations (Table 2.7.3). The purpose of tissue processing is to dehydrate the samples and to infiltrate the samples with paraffin wax.

Table 2.7.3 Steps involved in tissue processing

Steps	Reagent	Time (hour)	Process
1	Formalin	1	Fixation
2	70% Ethanol	1	Dehydration (removing all the water from tissue)
3	95% Ethanol	1	Dehydration
4	95% Ethanol	1	Dehydration
5	100% Ethanol (1)	1	Dehydration
6	100% Ethanol (2)	1	Dehydration
7	100% Ethanol (3)	1	Dehydration
8	Xylene (1)	1	Clearing (traces of alcohol were removed)
9	Xylene (2)	1	Clearing
10	Xylene (3)	1	Clearing
11	Paraffin wax (1)	1	Impregnation (to ensure infiltration of paraffin into tissues)
12	Paraffin wax (2)	1	Impregnation
13	Paraffin wax (3)	1	Impregnation
14	Paraffin wax (4)	1	Impregnation

2.4.3(c) Embedding, tissue sectioning and slide preparations

Following tissue processing, the paraffin-infiltrated tissues were embedded in the paraffin mould. The tissues were aligned in the paraffin mould carefully before filling them with paraffin wax. The paraffin wax inside the mould was allowed to cool on a cold plate until the wax easily detached from the mould. The sides of paraffin blocks were then trimmed to remove access paraffin so that it can fix the microtome. The prepared blocks were kept in refrigerator prior TO the sectioning. This step was to ensure that the blocks were cold enough so that the ribbons from the sectioning produced will be smooth and without damaged. Once the block was placed in the microtome, the block was trimmed at 5 μm thicknesses. The ribbons produced from the block were then floated in a 42°C water bath. The best ribbons of sectioned intact tissue with no wrinkles or splits were chosen before mounting it on

poly-L-lysine slides. The slides were then left to dry to remove water that may be trapped under the section. Before proceeding to the next step, the slides were heated on a hot plate at the temperature of 50°C for 60 minutes

2.4.3(d) Deparaffinization and rehydrating section

Prior to staining, the slides need to undergo deparaffinization and rehydrating (removing paraffin). Incomplete removal of paraffin can cause poor staining of the slides. This method involved several steps described as follows:

1. The slides were placed in the rack and transferred to xylene I to dewax the slides for five minutes in room temperature
2. The rack was later transferred to different container containing xylene II for another 5 minutes
3. The rack was later immersed in a container filled with 100% ethanol followed by 95%, 80%, 70% and 50% of ethanol respectively for 5 minutes in each solution.
4. The rack was then immersed in distilled water, TBS and distilled water again for 5 minutes in each solution.
5. Any excess reagents were drained by tapping the edge of the slide. The slides were then immediately subjected to peroxidase quenching

The slides were not allowed to dry throughout the experiment since drying out can causes non- specific antibody and lead to high back ground staining.

2.4.3(e) Peroxidase quenching

This step was conducted to eliminate endogenous peroxidase activity which may cause non-specific staining (i.e., a false positive). In this study, endogenous

peroxidase activity was blocked using 3% hydrogen peroxide in methanol for one minute. The slides were then washed 3 times with TBS for two minutes.

2.4.3(f) Antigen retrieval

Prior to antibody incubation, the step was carried out to reverse the loss of antigenicity that might occur during fixation. The formation of methylene bridges during fixation can crosslinks the proteins and prevent antibody binding by masking the antigenic site.

In this step, the slides were placed in a glass beaker containing citrate buffer at pH 6.0. The buffer was let to boil at 95 ° C for 30 minutes. The beaker was then removed and the slides were left to cool for 20 minutes at room temperature. The slides were then washed with TBS before continuing with antibody incubation.

2.4.3(g) Primary and secondary antibody incubation

Primary and secondary antibodies were diluted with TBS containing 5% non-fat dry milk and 0.1% Tween 20. The antibodies listed in Table 2.7.4 were used. The slides were initially incubated with primary antibodies for two hours with at room temperature. The slides were then washed with TBST-T for 5 minutes for three times before incubating the slides with secondary antibody. The slides incubated with secondary antibodies conjugated with HRP for one hour at room temperature. The slides were then washed again with TBS-T three times for 5 minutes.

Table 2.7.4 Dilution and duration of incubation for antibodies used in IHC

Antibodies	Dilution	Incubation Time
Anti-Cyclooxygenase 1 antibody	1:200	2 hours
Anti-Cyclooxygenase 2 antibody	1:200	2 hours
Anti- Prostaglandin I synthase antibody	1:300	2 hours
Anti-Thromboxane A2 receptor antibody	1:200	2 hours
Anti-Thromboxane A2 synthase antibody	1:200	2 hours
Donkey anti-goat IgG (HRP)	1:200	1 hour
Goat polyclonal secondary antibody to rabbit IgG H&L (HRP)	1:200	1 hour
IP Receptor (Prostaglandin II receptor)	1:200	2 hours
NOS3 Antibody (eNOS)	1:100	2 hours

2.4.3(h) Slide staining

The slides were incubated with stable diaminobenzidine (DAB) for 5 minutes and then rinsed with deionised water.

2.4.3(i) Dehydrating and stabilizing with mounting medium

The slides were placed in a rack and need to undergo dehydration process. This step was described as follows:

1. The rack was transferred to increasing ethanol concentration [70 %, 80%, 95%, and 100 % (2 times)] by immersing them for 2 minutes in each solution.
2. The rack was later transferred to xylene I and xylene II respectively and was left in each solution for 5 minutes.
3. The slides were taken from the racks and the sections were mounted with DPX.
4. Mounted sections were covered with coverslips.

2.4.3(j) Slide visualization

The slides were studied under the light microscope for presence of brown colour indicating the presence and localization of protein of interest. The colour intensity of the protein of interest was compared between the experimental groups by histopathologist in a blinded manner.

2.5 Statistical analysis

Statistical analyses were executed using Statistical Package for Social Science (SPSS) Software version 24.0. The normality of data was checked through several methods such as histogram with normal curve and Kolmogorov-Smirnov test. Normally distributed data were analysed by parametric tests while non-normally distributed data was analysed using non-parametric test.

In this study, parametric tests were used as the data were normally distributed. The one way ANOVA test followed by Bonferroni post hoc was used to evaluate the differences between four groups. Paired t-test, a parametric test was used to study the differences within the groups before and after treatment. The data was reported as means (SD).

In functional study, the concentration response curves were calculated and presented using the GraphPad Prism Version 5 for Windows (GraphPad Software, San Diego, CA, USA). The maximal relaxation (R_{max}) and maximal contraction (E_{max}) were chosen as main parameters investigated. The maximal relaxation (R_{max}) and maximal contraction (E_{max}) were defined as the utmost relaxation/ contraction to the agonist investigated respectively. The figures displayed were reported as means ± SEM. Statistical significance was sited at $p<0.05$.

CHAPTER 3 RESULTS

3.1 Characteristics of Sprague Dawley rats

3.1.1 Weight measurements

Table 3.0 showed the body weight of normal 2-week rats, diabetic-2 week rats, normal 10-week rats and diabetic 10-week rats. The mean average initial body weight of the rats was 307.75 g. No significant differences were observed between the groups. The final body weight measurement was also taken and significant differences were noted between the groups. Overall, the normal rats experienced weight gains whereas weight losses were noted in diabetic rats.

Figure 3.0 showed the difference in body weight before and after intervention. The normal 2-week rats exhibited a significant increase in body weight (+ 49.73 g, $p<0.001$) whereas in diabetic 2-week rats, there was a significant decrease in body weight (- 41.67 g, $p< 0.05$). The same pattern of body weight were also observed in normal 10-week rats (+ 143.80 g, $p< 0.001$) and in diabetes 10-week rats (-104.4 g, $p< 0.001$). Diabetic rats in both groups were also seen to show polyuria (frequent urination) compared to normal rats.

Table 3.0 Initial and final body weights of normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats

Groups	Initial weight (g)	F-statistics (df)	p-value	Final weight (g)	F- statistics (df)	p-value
Normal 2-week rats	303.27 (34.78)	1.82 (3, 56)	0.154	353.00 (35.47)[a]	62.49 (3, 56)	<0.001
Diabetic 2-week rats	309.33 (44.53)			267.67 (58.01)[a,b,c,]		
Normal 10-week rats	289.93(64.30)			433.73 (48.96)[a]		
Diabetic 10-week rats	328.47 (34.20)			224.07 (35.93)[a,b,c]		

Values were presented as mean (SD).
[a] indicates a statistically significant difference within the same group (Paired t-test, $p< 0.05$). [b] indicates significant differences vs. diabetic 2-week rats between groups (ANOVA, $p< 0.05$); [c] indicates significant differences vs. diabetic 10-week rats between groups (ANOVA, $p< 0.05$).

3.1.2 Fasting blood glucose (FBS)

Table 3.2 showed the FBS of the four groups after administration of STZ or sodium citrate vehicle. FBS in diabetic 2-week rats are higher compared to normal 2-week rats ($p<0.001$) and normal 10-week rats ($p<0.001$). Similar results were also seen in diabetic 10-week rats when compared to normal 2-week rats ($p<0.001$) and normal 10-week rats ($p<0.001$)

Table 3.2 FBS of normal 2-week rats, diabetic-2week rats, normal 10-week rats and diabetic 10-week rats after STZ or sodium citrate injection

Groups	FBS (mmol/L)	F-statistics (df)	p-value
Normal 2-week rats	6.15 (0.53)	1789.38(3, 56)	<0.001
Diabetic 2-week rats	31.78 (2.13) [a, b]		
Normal 10-week rats	6.11 (0.47)		
Diabetic 10-weeks rats	33.00 (1.60) [a, b]		

Values were presented as mean (SD). Data were analysed using ANOVA followed by Bonferroni post hoc test.
[a] indicates significant differences vs. normal 2-week rats; [b] indicates significant differences vs. normal 10-week rats

3.2 Contractile responses to KCl and PE

3.2.1 Contractions to KCl (60mM) in four different groups

Table 3.3.1 showed contractions to KCl in vessels with or without endothelium. No significant differences were seen in vessels with or without endothelium in all the groups

Table 3.3.1 Contractions to KCl in vessels with or without endothelium in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic-10 week rats

Type of vessels	KCl +EC (mN/mm)	F-statistics (df)	p-value	KCl –EC (mN/mm)	F- statistics (df)	p-value
Normal 2-week rats	6.85 (2.04)	2.26 (3, 56)	0.108	6.75 (3.18)	1.62 (3, 56)	0.193
Diabetic 2-week rats	6.05 (2.23)			6.67 (3.51)		
Normal 10-week rats	7.01 (1.35)			5.52 (1.42)		
Diabetic 10-week rats	5.53 (1.70)			5.07 (1.17)		

Values were presented as mean (SD). Data were analysed using one way ANOVA followed by Bonferroni post hoc test. KCl +EC; KCl with endothelium, KCl –EC; KCl without endothelium

3.2.2 Contractions to PE (10^{-4} M) in four different groups.

Table 3.3.2 showed the vascular responses to PE (10^{-4} M) in vessels with or without endothelium. No significant differences were seen between all the groups.

Table 3.3.2 Contractions to PE (10^{-4} M) in vessels with or without endothelium in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats

Type of vessels	PE +EC (log M/mN)	F-statistics (df)	p-value	PE –EC (log M/mN)	F- statistics (df)	p-value
Normal 2-week rats	7.51 (2.77)	1.78 (3, 56)	0.162	7.73 (2.95)	1.35(3,56)	0.268
Diabetic 2-week rats	6.64 (3.24)			5.95 (2.87)		
Normal 10-week rats	8.23 (2.15)			7.67 (3.64)		
Diabetic 10-week rats	6.25(2.08)			6.59(1.83)		

Values were presented as mean (SD). Data were analysed by using one way ANOVA followed by Bonferroni post hoc test.
PE +EC, PE with endothelium; PE-EC, PE without endothelium.

3.3 Endothelium-dependent relaxation within the groups

3.3.1 Relative contributions of NO, EDH and prostacyclin in endothelium-mediated relaxation in normal 2-week rats

Figures 3.0 showed the maximum relaxations to ACh which specifies the contribution of each vasodilator in causing relaxations in normal 2-week rats.

The overall net response of ACh-mediated relaxation in control solution was 89.32 (10.03) %. The maximal relaxation caused by NO (vessels incubated with indomethacin together with TRAM 34, UCL 1684 and KCl, 30 mM) was 63.87 (12.66) % which was highest relaxation when compared with two other vasodilators. Percentage relaxation contributed by prostacyclin (vessels incubated with with L-NAME, TRAM 34, UCL1684 and 30 mM KCl) was 41.26 (9.51) %, which was significantly lower compared to NO-mediated relaxation ($p<0.001$). The EDH-mediated relaxation was 37.22 (10.65) % and significantly lower compared to control solution ($p<0.001$]. The relaxation of EDH (vessels incubated with L-NAME and indomethacin) was significantly lower to NO ($p<0.001$), but was not significantly different compared with prostacyclin.

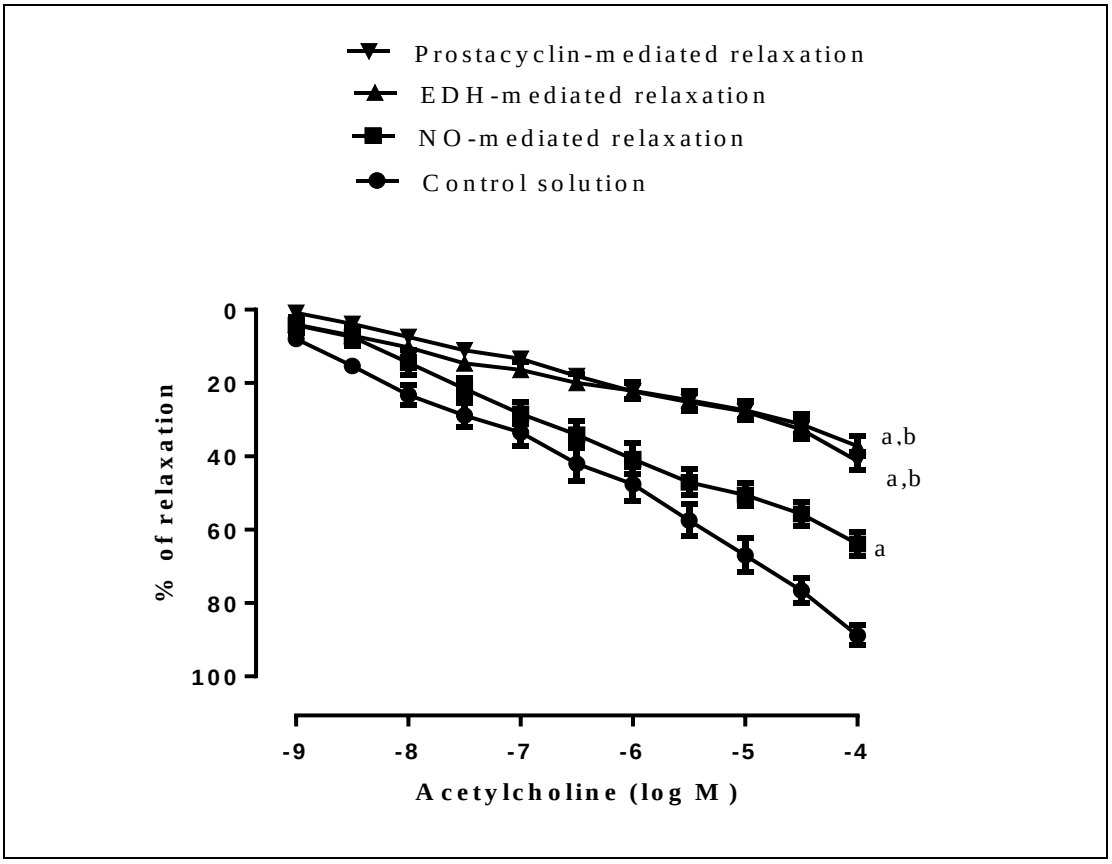


Figure 3.0 Concentration-response curves of ACh in normal 2-week rats. Arteries were incubated with the respective inhibitors initially before subjecting to cumulative ACh dose response in order to study the individual contribution of vasodilators. Relaxations are expressed as a percentage of the contraction induced by PE (10^{-4}M). [a] indicates significant difference vs. control solution; [b] indicates significant difference vs. NO-mediated relaxation using ANOVA followed by Bonferroni post hoc test

3.3.2 Relative contributions of NO, EDH and prostacyclin in endothelium-mediated relaxation in diabetic 2-week rats

Figures 3.1 showed the maximum relaxations to ACh of each vasodilator in diabetic 2-week rats

Overall, the net response of ACh-mediated relaxation in control solution was 73.49 (11.05) %. NO-mediated relaxation contributed greatest relaxation which was 53.67 (18.01) % when compared with two other vasodilators. The percentage relaxation mediated by prostacyclin was 35.10 (14.04) %, which was significantly lower than control solution ($p< 0.001$) and NO-mediated relaxation ($p=0.005$). The relaxation by EDH was 30.13 (13.80) %, which was lower than control solution ($p< 0.001$) and NO-mediated relaxation ($p < 0.001$).

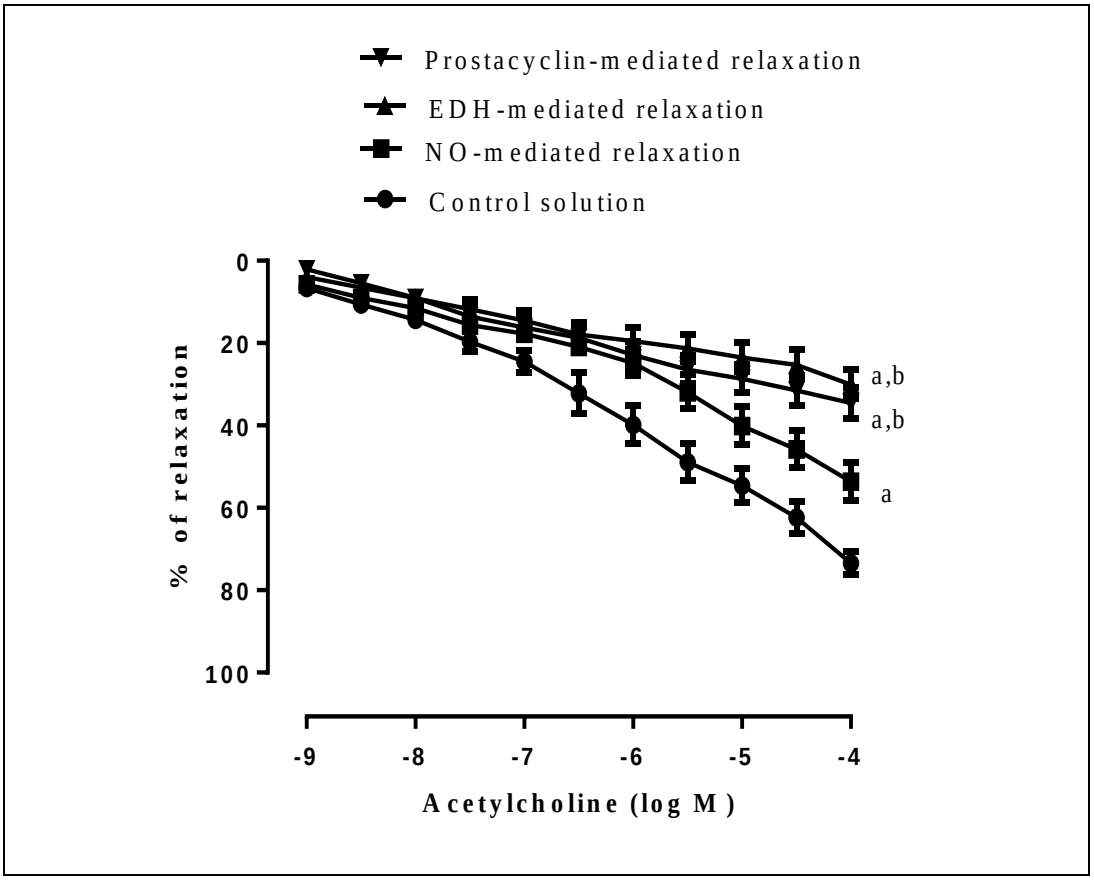


Figure 3.1 Concentration-response curves of ACh in diabetic 2-week rats. Arteries were incubated with the respective inhibitors initially before subjecting to cumulative ACh dose response in order to study the individual contribution of vasodilators. Relaxations are expressed as a percentage of the contraction induced by PE (10^{-4}M).

[a] indicates significant difference vs. control solution; [b] indicates significant difference vs. NO-mediated relaxation using ANOVA followed by Bonferroni post hoc test.

3.3.3 Relative contributions of NO, EDH and prostacyclin in endothelium-mediated relaxation in normal 10-week rats

Figures 3.2 showed the maximum relaxations to ACh identifying the contribution of each vasodilator in causing relaxations in normal 10-week rats.

The percentage of maximal ACh-mediated relaxation in control solution was 86.99 (8.82) %. Maximal relaxation by NO was 55.49 (15.18) % and it was significantly lower compared to control solution ($p < 0.001$). The maximal EDH-mediated relaxation was 36.53 (15.10) %, which was significantly lower than control solution ($p < 0.001$) and NO-mediated relaxation ($p = 0.002$). Relaxation contributed by prostacyclin was 33.70 (13.06) %, which was significantly lower than control solution ($p < 0.001$) and NO-mediated relaxation ($p < 0.001$).

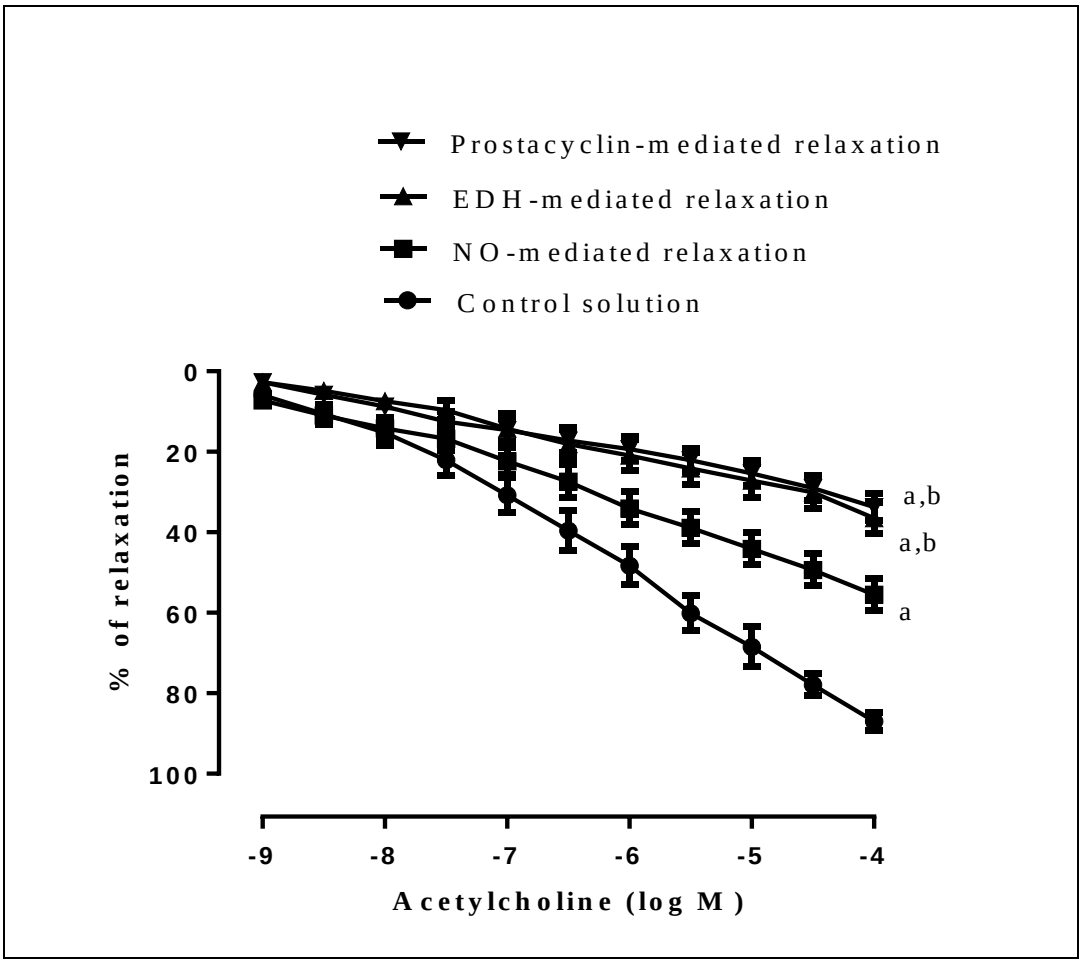


Figure 3.2 Concentration-response curves to ACh in normal 10-week rats. Arteries were incubated with the respective inhibitors initially before subjecting to cumulative ACh dose response in order to study the individual contribution of vasodilators. Relaxations are expressed as percentage of the contraction induced by PE (10^{-4} M).

[a] indicates significant difference vs. control solution; [b] indicates significant difference vs. NO-mediated relaxation using ANOVA followed by Bonferroni post hoc test

3.3.4 Relative contributions of NO, EDH and prostacyclin in endothelium-mediated relaxation in diabetic 10-week rats

Figures 3.3 represented the maximum relaxations of each vasodilator in causing ACh-mediated relaxations in diabetic 10-week rats.

The maximal percentage of ACh-mediated relaxation in control solution was 58.48 (18.79) %. Maximal NO-mediated relaxation being 31.31 (18.24) % and it was significantly lower than control (p <0.001). Maximal relaxation by prostacyclin was 20.38 (17.86) % and was reduced when compared to control solution (p< 0.001). Maximal relaxation contributed by EDH was 19.80 (18.28) %, which was lower than control solution (p <0.001).

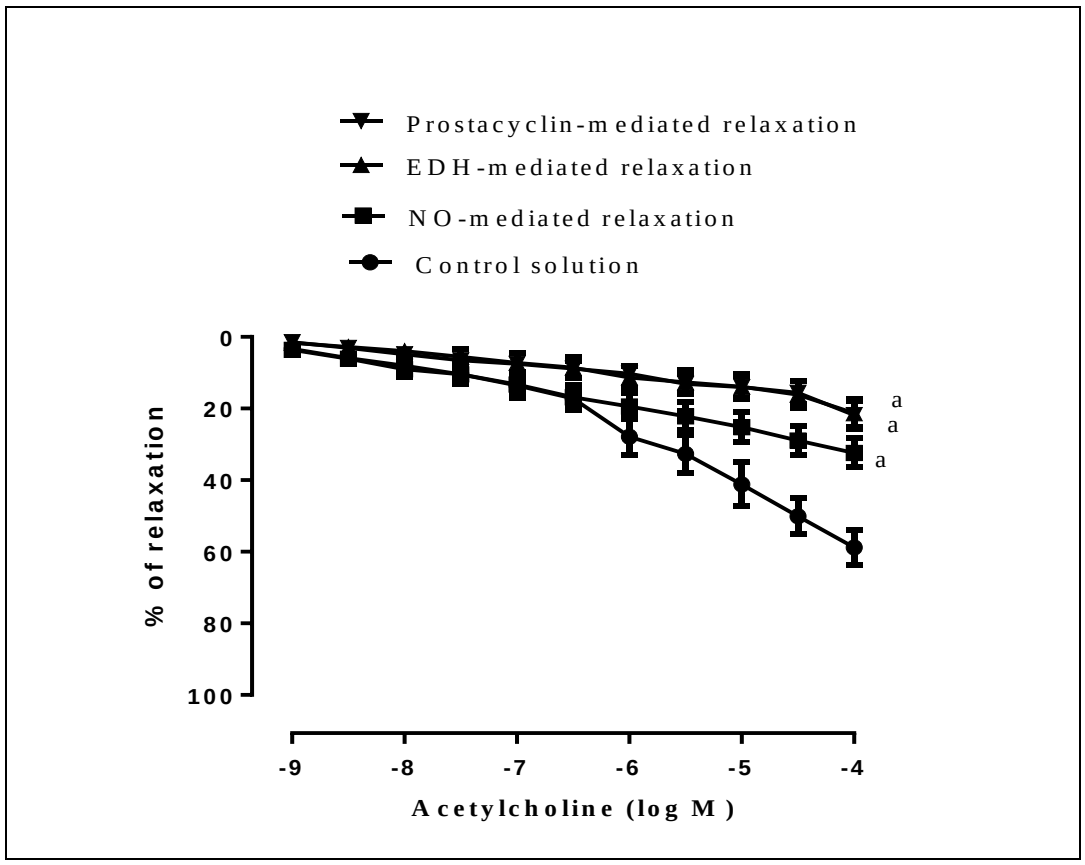


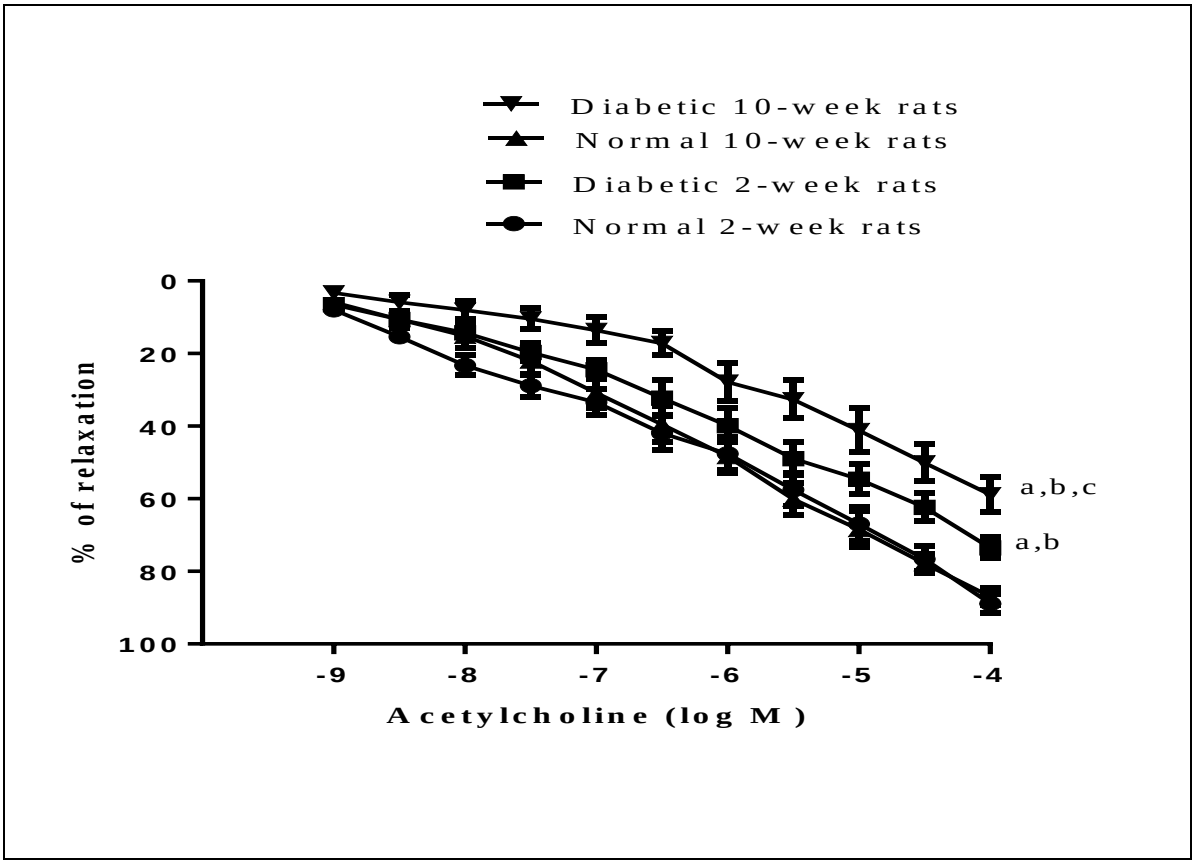
Figure 3.3 Concentration-response curves of ACh in diabetic 10-week rats. Arteries were incubated with the respective inhibitors initially before subjecting to cumulative ACh dose response in order to study the individual contribution of vasodilators. Relaxations are expressed as percentage of the contraction induced by phenylephrine (10^{-4} M).
[a] indicates significant difference vs. control solution using ANOVA followed by Bonferroni post hoc test.

3.4 Endothelium-dependent relaxation between the groups

3.4.1 Maximal ACh-mediated relaxation in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats

Figures 3.4 showed the maximal relaxation to ACh in the normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats.

The maximal relaxation to ACh in normal 2-week rats was 89.32 (10.03) %. In diabetic 2-week rats, the maximal relaxation to ACh was 73.84 (11.04) %, which was significantly lower compared with normal 2-week rats (p= 0.002) and normal 10-week rats (p= 0.006). The maximal relaxation to ACh in normal 10-week rats was 86.99 (8.82) %.The percentage of maximal relaxation in diabetic 10-week rats was 58.48 (18.79) % which were vastly reduced when compared with normal 10-week (p< 0.001) and normal 2-week rats (p <0.001). There was significant reduction in relaxation in diabetic 10-week rats when compared diabetic 2-week rats (p =0.016).



Figures 3.4 Concentration-response curves to ACh-mediated relaxation in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. Relaxations are expressed as percentage of the contraction induced by PE (10^{-4} M). [a] indicates significant difference vs. normal 2-week rats; [b] indicates significant difference vs. normal 10-week rats; [c] indicates significant difference vs. diabetic 2-week rats using ANOVA followed by Bonferroni post hoc test

3.4.2 Maximal NO-mediated relaxation in normal 2-week rats, diabetic-2 week rats, normal 10-week rats and diabetic 10-week rats

Figures 3.5 showed the maximal relaxation mediated by NO in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats.

The maximal relaxation contributed by NO in normal 2-week rats was 63.87 (12.66) %. In diabetic 2-week rats, the maximal relaxation caused by NO was 50.34 (18.01) %, which was significantly higher than diabetic 10-week rats [R_{max}: 50.34 (18.01) %. vs 31.31 (18.24) %, $p< 0.001$]. There was a trend of reduced relaxation in diabetic 2-week rats when compared to normal 2-week rats ($p=0.080$). The maximal relaxation of NO in normal 10-week rats was 55.49 (15.18) %. The maximal relaxation of NO in diabetic 10-week rats was 31.31 (18.24) % which were significantly higher lower than normal 2 weeks ($p< 0.001$) and normal 10 weeks ($p< 0.001$).

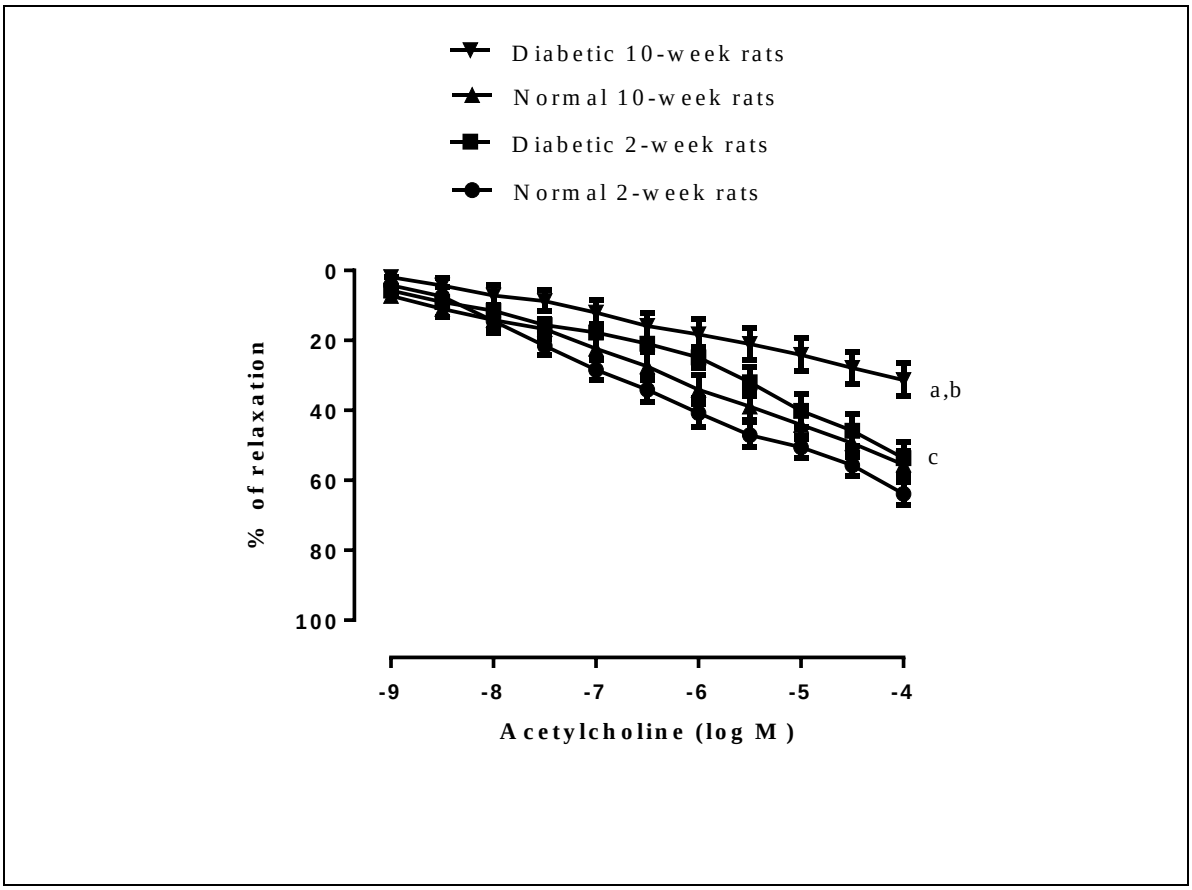


Figure 3.5 Concentration-response curves to NO-mediated relaxation in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. The vessels were incubated with indomethacin, TRAM 34 plus UCL 1684 and 30 mM KCl. Relaxations were expressed as a percentage of the contraction induced by PE (10^{-4}M).

[a] indicates significant difference vs normal 2-week rats, [b] indicates significant difference vs. normal 10-week rats, [c] indicates significant difference vs. diabetic 10-week rats using ANOVA followed by Bonferroni post hoc test. There was a trend of reduced relaxation in diabetic 2-week rats when compared to normal 2-week rats (p=0.080).

3.4.2(a) Endothelial nitric oxide synthase (eNOS) protein expression in rat tail arteries

Figures 3.5.1 and 3.5.2 showed the eNOS protein expression, obtained via Western blot in tail arteries of normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. Expression of eNOS in diabetic 10-week rats were significantly lower than normal 2-week rats ($p<0.001$), normal 10-week rats ($p<0.001$) and diabetic 2-week rats ($p=0.023$).

Plate 3.1 represents immunohistochemical staining highlighting the distributions of eNOS in the four groups: The intensity of eNOS protein was low in diabetic 10-week rats compared to other groups (normal 2-week rats, diabetic 2-week rats and normal 10-week rats).

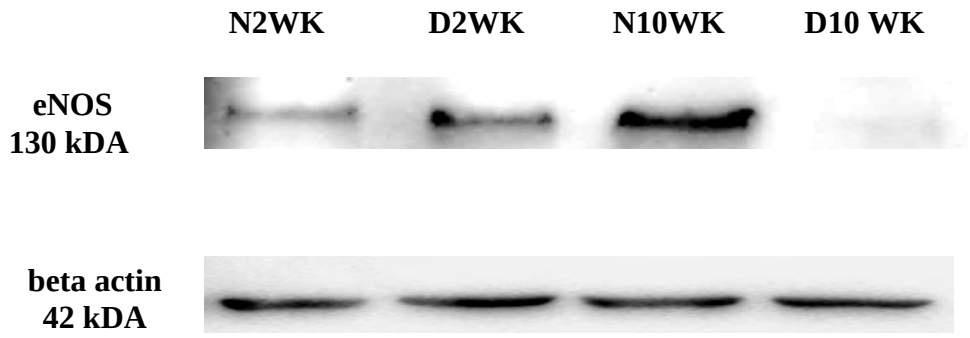


Figure 3.5.1 Representative Western blots illustrating eNOS protein expression in tail arteries of normal 2-week (N2WK), diabetic 2-week (D2WK), normal 10-week (N10WK) and diabetic 10-week (D10WK) rats. n = 10 rats for each group

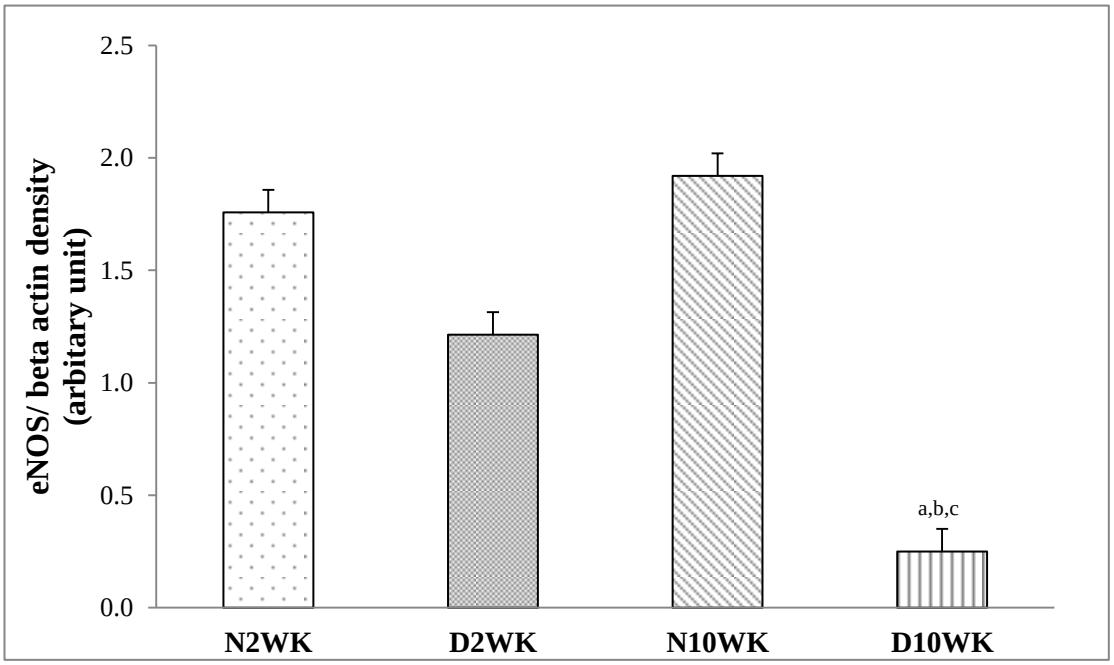
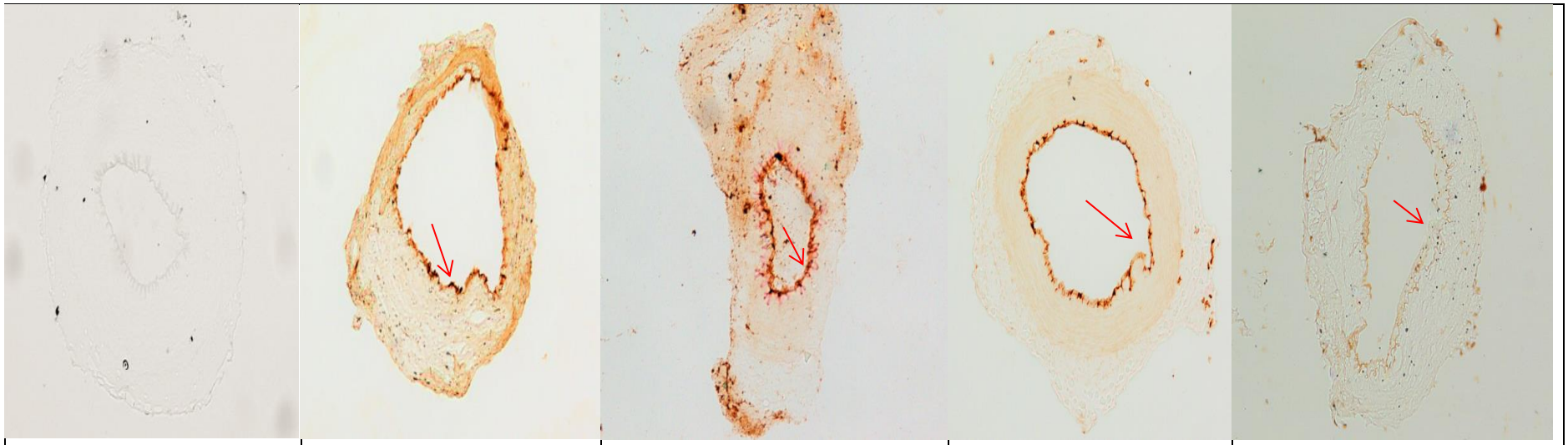


Figure 3.5.2: Graphical representation of the eNOS protein expression normalized to β-actin. [a] indicates significant difference vs. normal 2-week rats; [b] indicates significant difference vs. diabetic 2-week rats ; [c] indicates significant difference vs. normal 10-week rats using ANOVA followed by Bonferroni post hoc test.
N2WK: normal 2-week rats; D2WK: diabetic 2-week rats; N10WK: normal 10-week rats; D10WK: diabetic 10-week rats



Negative control	Normal 2-week rats	Diabetic 2-week rats	Normal 10-week rats	Diabetic 10-week rats

Plate 3.0 Immunohistochemical staining photos of the distributions of eNOS protein expression in the tail arteries of four experimental groups. Sections without primary antibodies were used as negative controls. The brown stain indicates expression of eNOS proteins in the vessels (red arrows). Magnifications 100x.

3.4.3 Maximal prostacyclin-mediated relaxation in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats

Figures 3.6 showed the maximal relaxation caused by prostacyclin in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. The maximal relaxation by prostacyclin in normal 2-week rats was 41.26 (9.51) %. Prostacyclin-mediated relaxation in diabetic 2-weeks rats was 35.10 (14.04) %.

Maximal relaxations by prostacyclin in normal 10-week rats was 33.70 (13.05) %. Trend of reduction was seen in diabetic 10-week rats (p=0.069) compared to normal 10-week rats with no other changes noted in other groups. Prostacyclin-mediated relaxation in diabetic 10-weeks rats was 20.38 (17.96) %, which were significantly lower compared to normal 2-week rats (p <0.001) and diabetic 2-week rats (p=0.033).

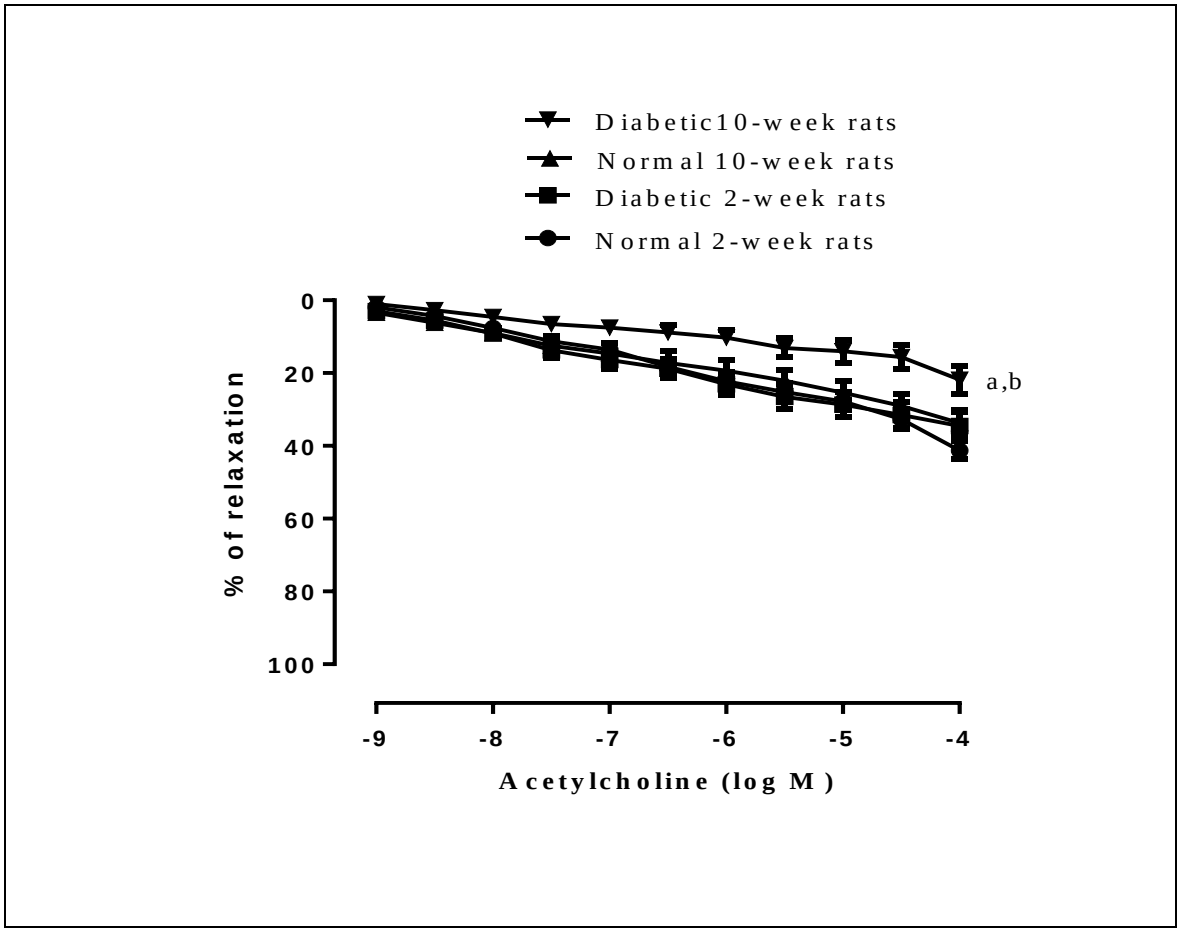


Figure 3.6 Concentration-response curves to prostacyclin-mediated relaxation in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. The vessels were incubated with L-NAME, TRAM 34 plus UCL 1684 and 30 mM KCl. Relaxations were expressed as a percentage of the contraction induced by phenylephrine. [a] indicates significant difference vs. normal 2-week rats ; [b] indicates significant difference vs diabetic 2-week rats.
There was a trend of reduction in diabetic 10-week rats when compared to normal 10-week (p= 0.069) using ANOVA followed by Bonferroni post hoc

3.4.3(a) IP receptor protein expression in rat tail arteries

Figures 3.6.2 and 3.6.3 showed the IP receptor protein expression, obtained via Western blot in the tail arteries of normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. Expression of IP receptor protein in diabetic 10-week rats were significantly lower than normal 2-week rats (p=0.45), normal 10-week rats (p=0.009) and diabetic 2-week rats (p=0.049).

Plate 3.1 represents immunohistochemical photos highlighting the distributions of IP receptor protein in tail arteries of four experimental groups: normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. The intensity of IP receptor protein was lower in diabetes 10-week rats compared to other groups.

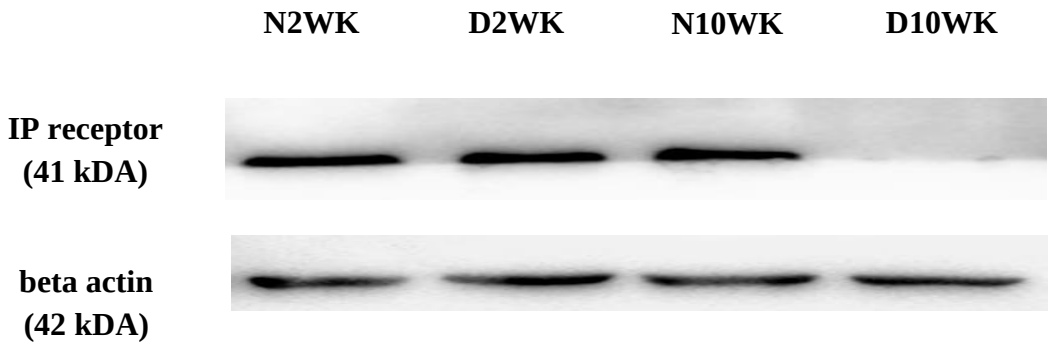


Figure 3.6.2 Representative Western blots illustrating IP receptor protein expression in tail arteries of normal 2-week rats (N2WK), diabetic 2-week rats (D2WK), normal 10-week rats (N10WK) and diabetic 10-week rats (D10WK). n =10 rats

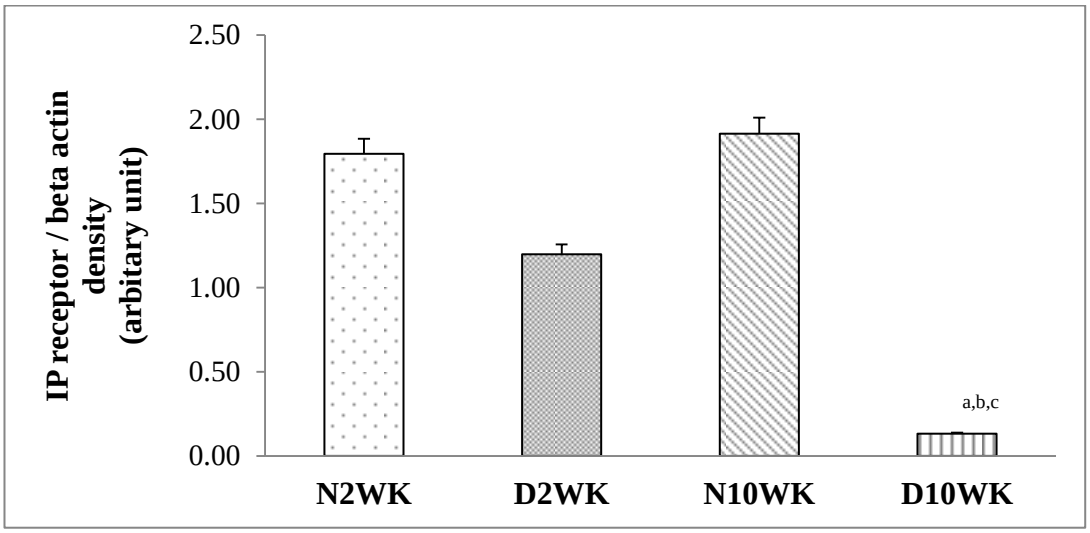
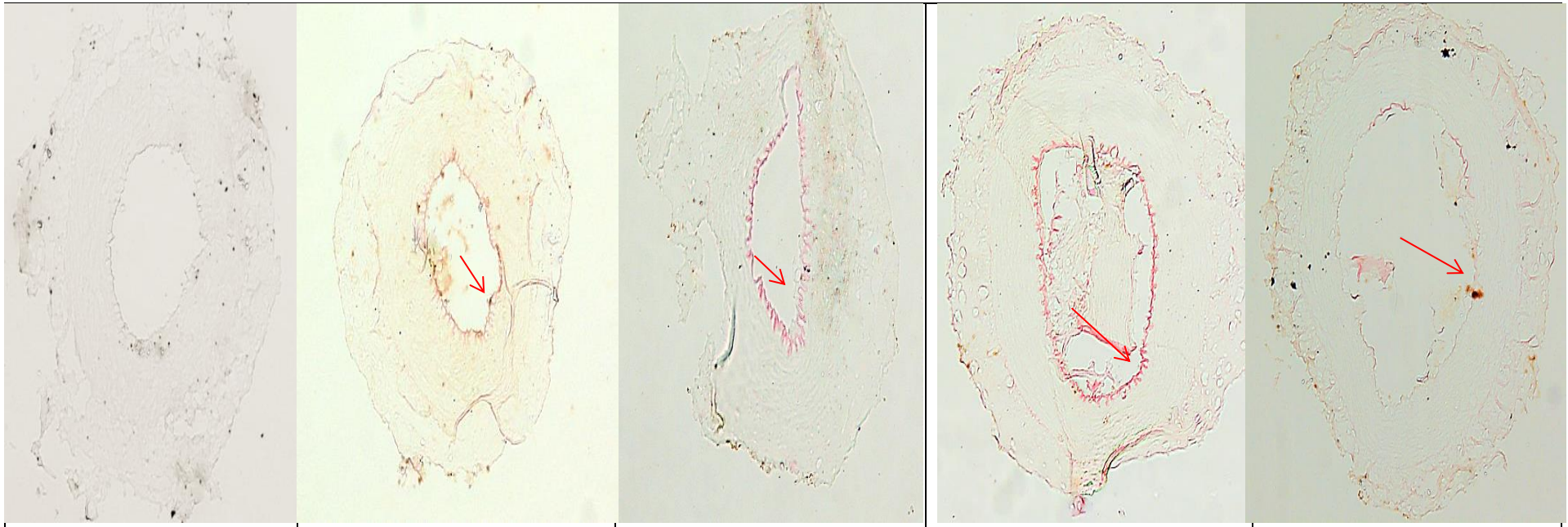


Figure 3.6.3 Graphical representation of IP receptor protein expression normalized to β-actin.

[a] indicates significant difference vs. normal 2-week rats; [b] indicates significant difference vs. diabetic 2-week rats; [c] indicates significant difference vs. normal 10-week rats using ANOVA followed by Bonferroni post hoc test..

N2WK: normal 2-week rats; D2WK: diabetic 2-week rats; N10WK: normal 10-week rats; D10WK: diabetic 10-week rats



Negative controls	Normal 2-week rats	Diabetic 2-week rats	Normal 10-week rats	Diabetic 10-week rats

Plate 3.1 Immunohistochemical staining photos showing the distributions of IP receptor in four groups: normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. Sections without primary antibodies were used as negative controls. The brown stain indicates expression of IP receptor proteins in the vessels (red arrows). Magnifications 100x.

3.4.3(b) PGIS protein expression in rat tail arteries

Figures 3.6.4 and 3.6.5 showed the expression of PGIS protein between normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. The expression of PGIS in in diabetic 10-week rats were significantly lower than normal 10-week rats ($p<0.001$).

Plate 3.2 represents immunohistochemical photos highlighting the distributions of PGIS protein in rat tail arteries of four groups: normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. The intensity of the PGIS protein was lower in diabetic 10-week rats compared to other groups.

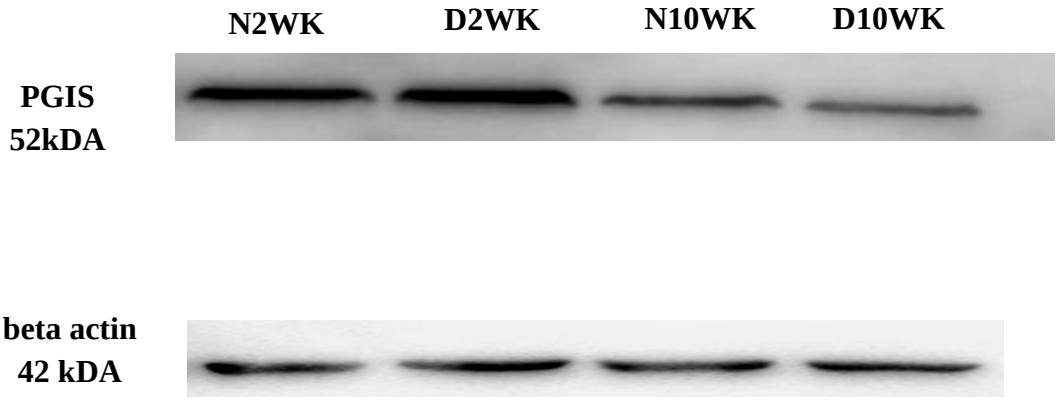


Figure 3.6.4 Representative Western blots illustrating the expression of PGIS protein in tail arteries of normal 2-week rats (N2WK), diabetic 2-week rats (D2WK), normal 10-week rats (N10WK) and diabetic 10-week rats (D10WK). n = 10 rats

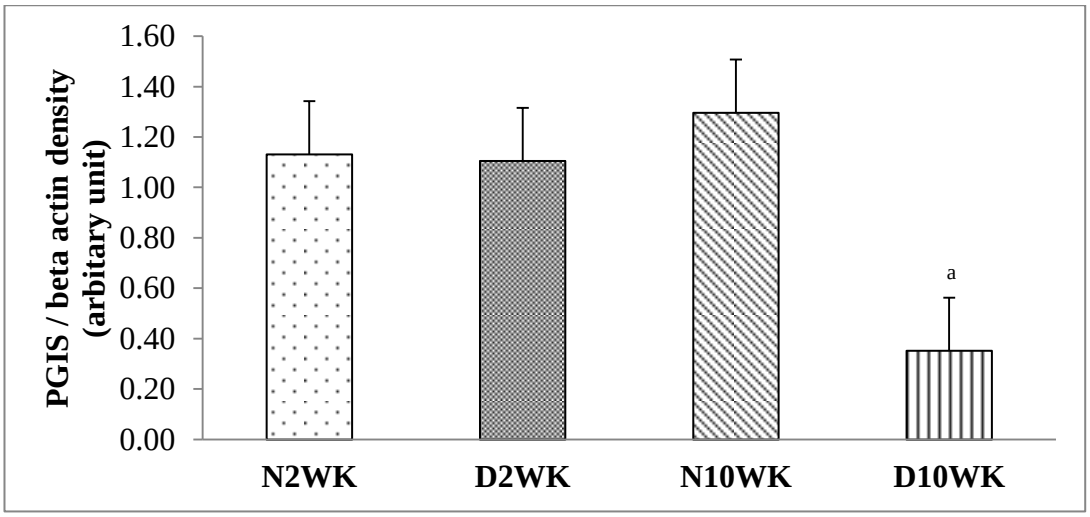
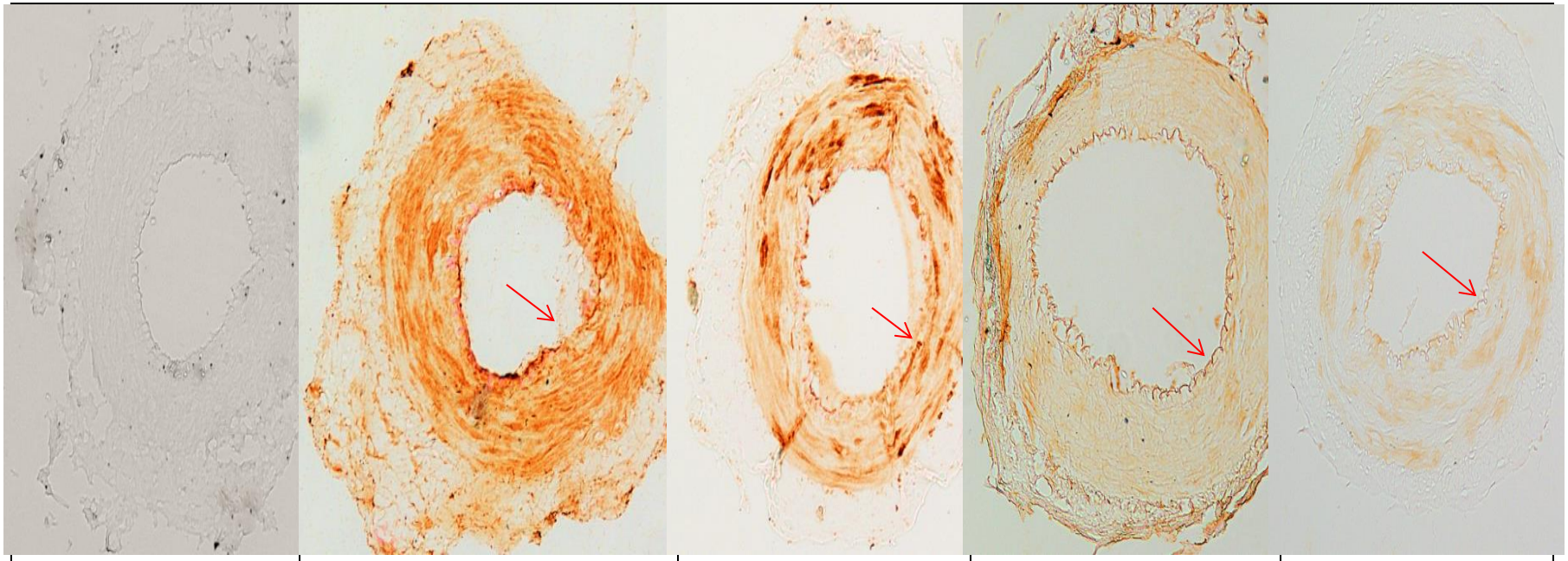


Figure 3.6.5 Graphical representation of PGIS protein expression normalized to β-actin.
[a] indicates significant difference vs. normal 10-week rats using ANOVA followed by Bonferroni post hoc test
N2WK: normal 2-week rats; D2WK: diabetic 2-week rats; N10WK: normal 10-week rats; D10WK: diabetic 10-week rats



Negative control	Normal 2-week rats	Diabetic 2-week rats	Normal 10-week rats	Diabetic 10-week rats

Plate 3.2 Immunohistochemical staining photos showing the distributions of PGIS protein in four groups: normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. Sections without primary antibodies were used as negative controls. The brown stain indicates expression of PGIS proteins in the vessels (red arrows) .Magnifications 100x.

3.4.4 Maximal EDH-mediated relaxation in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats

Figures 3.7 showed the maximal relaxation caused by EDH in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats.

The maximal relaxation contributed by EDH in normal 2-week rats was 37.22 (10.65) %; whereas relaxation in diabetic 2-week rats was 30.13 (13.80) %. In normal 10-week rats, maximal relaxation by EDH was 38.69 (17.71) %. EDH-mediated relaxation in diabetic 10-week rats was 19.80 (18.28) %. It was significantly lower compared to normal 2-week (p= 0.019) and normal 10-week rats (p= 0.009).

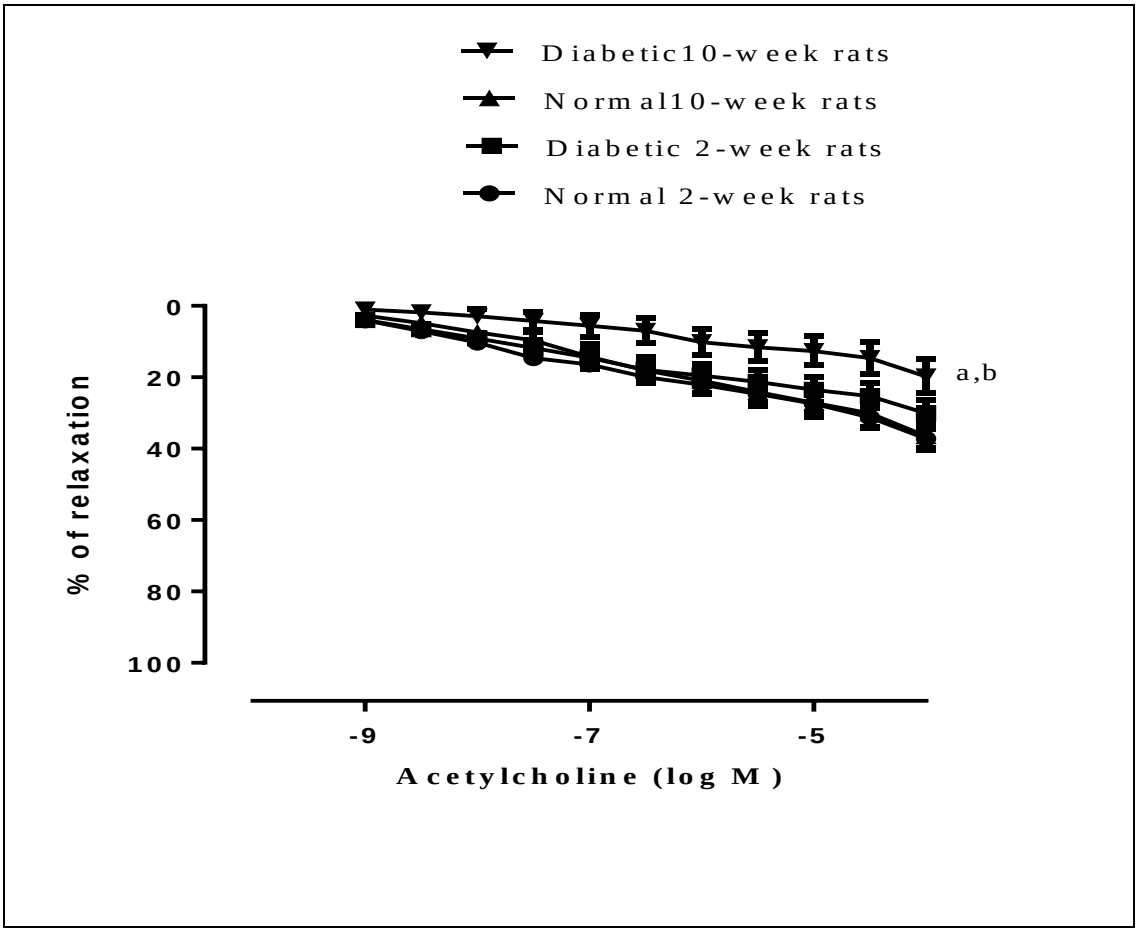


Figure 3.7 Concentration-response curves to EDH-mediated relaxations in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. The vessels were incubated with L-NAME and indomethacin. Relaxations were expressed as a percentage of the contraction induced by PE. [a] indicates significant difference vs. normal 2-week rats; [b] indicates significant difference vs. normal 10-week rats.

3.5 Endothelium-independent relaxation between the groups

3.5.1 Maximal-SNP mediated relaxation in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats

Figures 3.8 showed the maximal relaxation of endothelium-independent relaxation due to SNP in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats.

The maximal relaxation to SNP in normal 2-week rats was 86.40 (14.02) %. In diabetic 2-week rats, the maximal SNP-mediated relaxation was 70.27 (16.87) % with no significant differences seen with other groups. The maximal relaxation to SNP in normal 10-week rats was 75.56 (21.42) % with no significant differences seen with other groups. In diabetic 10-week rats, the maximal relaxation to SNP was 59.71 (24.27) %, which was significantly lower than normal 2-week rats [R_{max}: 59.71 (24.27) vs 86.40 (14.02) %, p=0.006]

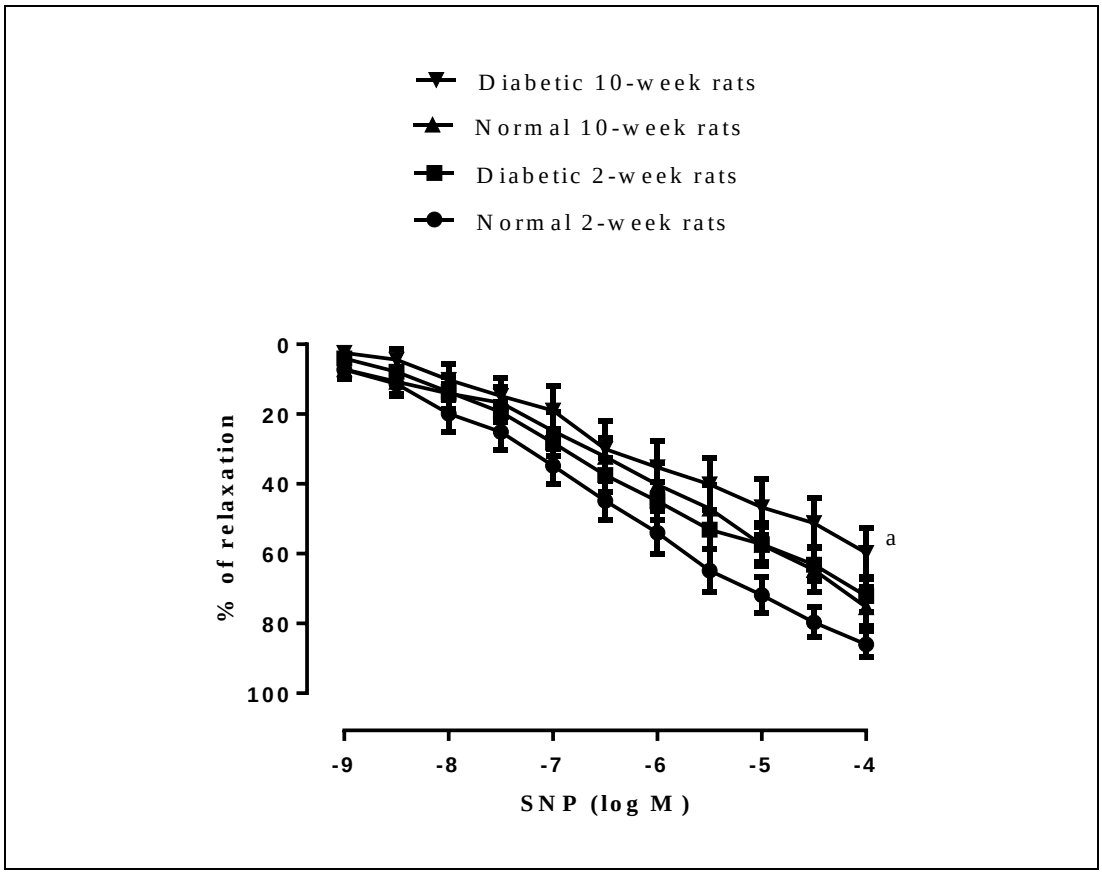


Figure 3.8 Concentration-response curves to SNP-mediated relaxations in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. The endothelial cells were removed from the lumen of the cells by crushing it with hair. Relaxations were expressed as a percentage of the contraction induced by PE. [a] indicates significant difference vs. normal 2-week rats.

3.6 Endothelium-dependent contraction within the groups.

3.6.1 Role of COX, COX-1, COX-2 and TXA2 receptor in endothelium-dependent contraction in normal 2-week rats

Figures 3.9 showed maximal contraction (E_{max}) in the tail arteries of normal 2-week rats in control solution, or after incubation separately with indomethacin (non-selective COX), valeryl salicylate (COX-1 inhibitor), NS398 (COX-2 inhibitor) or S18886 (TXA_2 receptor antagonist). Increases in tension were expressed as percentage of the reference contraction to 60 mM KCl obtained at the beginning of the experiment.

The overall endothelium-dependent contraction induced by calcium ionophore in solution without presence of blockers (control solution) was 34.80 (21.06) %. After blocking the vessels with indomethacin (non-selective COX inhibitor), the contraction was significantly reduced to 15.18 (12.19) %, (p=0.045). The contraction to calcium ionophore was significantly reduced to 14.00 (6.52) % when the vessels were incubated with valeryl salicylate (COX-1 inhibitor) (p=0.019). Blocking the vessels with NS398 (COX-2 inhibitor) showed no significant difference between the control solution. In the vessels incubated with S18886 (TXA_2 receptor antagonist), the contraction reduced to 15.27 (7.3) %, which was significantly lower compared with the vessel in control solution. No differences were seen in the contraction to calcium ionophore among vessels incubated with indomethacin, valeryl salicylate, NS398 and S18886.

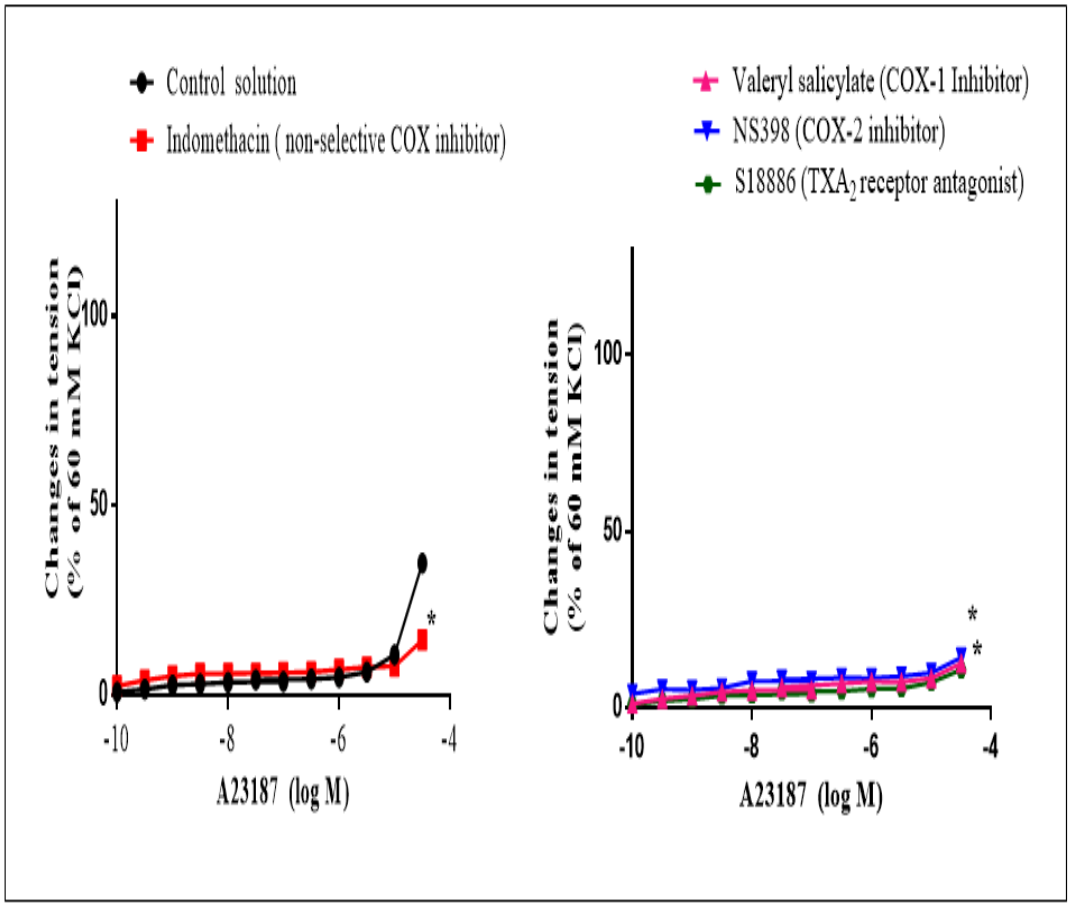


Figure 3.9: Concentration-response curves of calcium ionophore in tail arteries of normal 2-week rats in the control solution, or incubated with indomethacin (non-selective COX inhibitor), valeryl salicylate (COX-1 inhibitor), NS398 (COX-2 inhibitor) and S18886 (TXA_2 receptor antagonist)

* indicates significant difference vs. control solution using ANOVA followed by Bonferroni post hoc test

3.6.2 Role of COX, COX-1, COX-2 and TXA2 receptor in endothelium-dependent contraction in diabetic 2-week rats

Figures 4.0 showed maximal contraction (E_{max}) in the tail arteries of diabetic 2-week rats in control solution, or after incubation separately with indomethacin (non-selective COX), valeryl salicylate (COX-1 inhibitor), NS398 (COX-2 inhibitor) or S18886 (TXA_2 receptor antagonist). Increases in tension were expressed as percentage of the reference contraction to 60 mM KCl obtained at the beginning of the experiment

The overall contraction induced by calcium ionophore in solutions without presence of blockers (control solution) was 71.80 (46.02) %. However, after blocking the vessels with indomethacin (non-selective COX inhibitor), the contraction was reduced to 20.01 (18.62) %, (p=0.007). The contraction by calcium ionophore in a vessel incubated with valeryl salicylate (COX-1 inhibitor) was significantly reduced to 11.00 (9.28) %, (p<0.001) when compared to control solution. The contraction contributed by calcium ionophore after blocking the vessels with NS398 was significantly reduced to 13.47 (6.51) %, (p=0.002) compared to control solution. After the incubation with S18886, the contraction was significantly reduced to 8.13 (6.58) % compared with control solution (p<0.001).

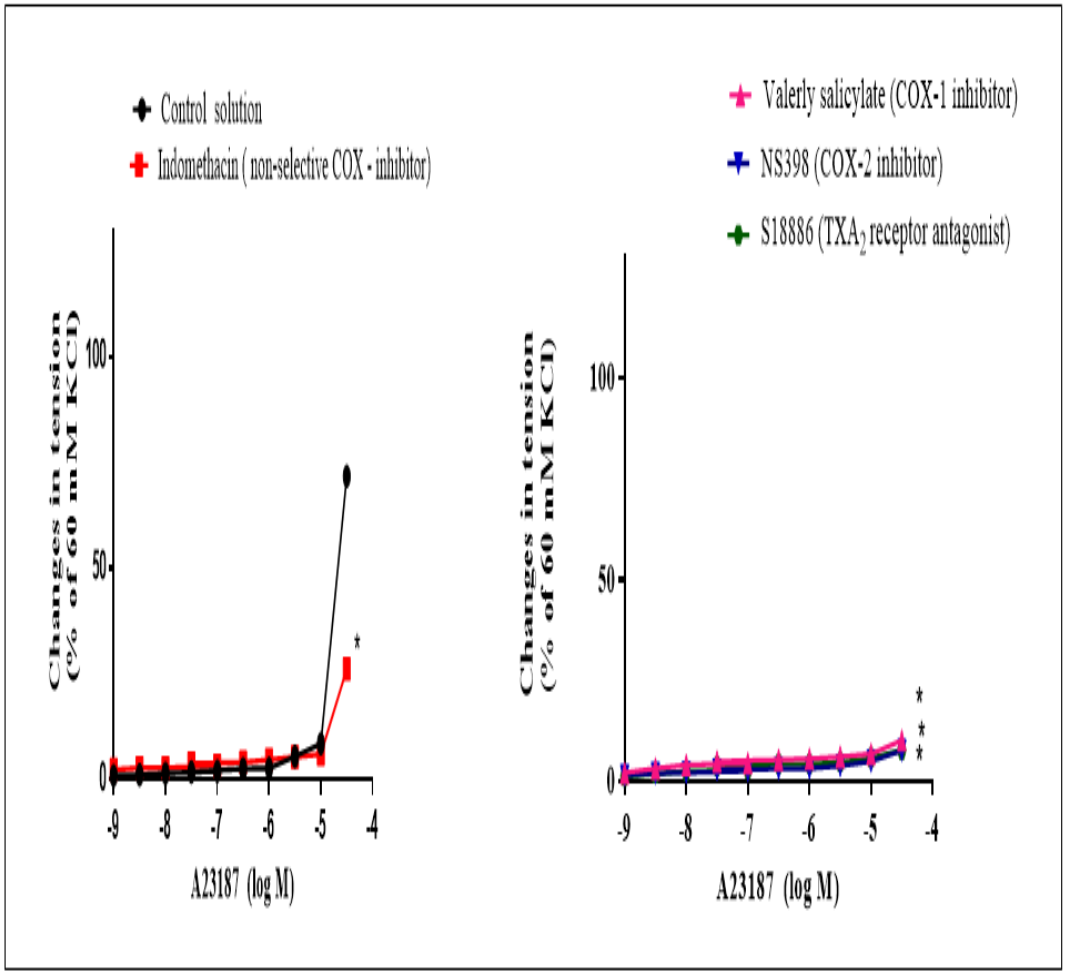


Figure 4.0 Concentration-response curves of calcium ionophore in tail arteries of diabetic 2-week rats in the control solution, or incubated with indomethacin (non-selective COX inhibitor), valeryl salicylate (COX-1 inhibitor), NS398 (COX-2 inhibitor) and S18886 (TXA_2 receptor antagonist)
* indicates significant difference vs. control solution using ANOVA followed by Bonferroni post hoc test

3.6.3 Role of COX, COX-1, COX-2 and TXA2 receptor in endothelium-dependent contraction in normal 10-week rats.

Figures 4.1 showed maximal contraction (E_{max}) in the tail arteries of normal 10-week rats in control solution, or after incubated separately with indomethacin (non-selective COX), valeryl salicylate (COX-1 inhibitor), NS398 (COX-2 inhibitor) or S18886 (TXA_2 receptor antagonist). Increases in tension were expressed as percentage of the reference contraction to 60 mM KCl obtained at the beginning of the experiment.

The overall endothelium-dependent contraction induced by calcium ionophore in control solution was 68.33 (57.80) %. However, after blocking the vessels with indomethacin, the contraction was reduced to 17.36 (16.67), (p=0.041). After incubating the vessels with valeryl salicylate, the contraction was reduced to 7.21(7.91) and was significantly lower to contraction in control solution (p=0.010). The maximal contraction in vessels blocked by NS398 was significantly reduced to 12.60 (10.64) %. This contraction was significantly lower than in control solution (p=0.021). The contraction caused by calcium ionophore after incubating the vessels with S18886 was 14.53 (12.95) where the contractions were reduced when compared with control solution (p=0.027).

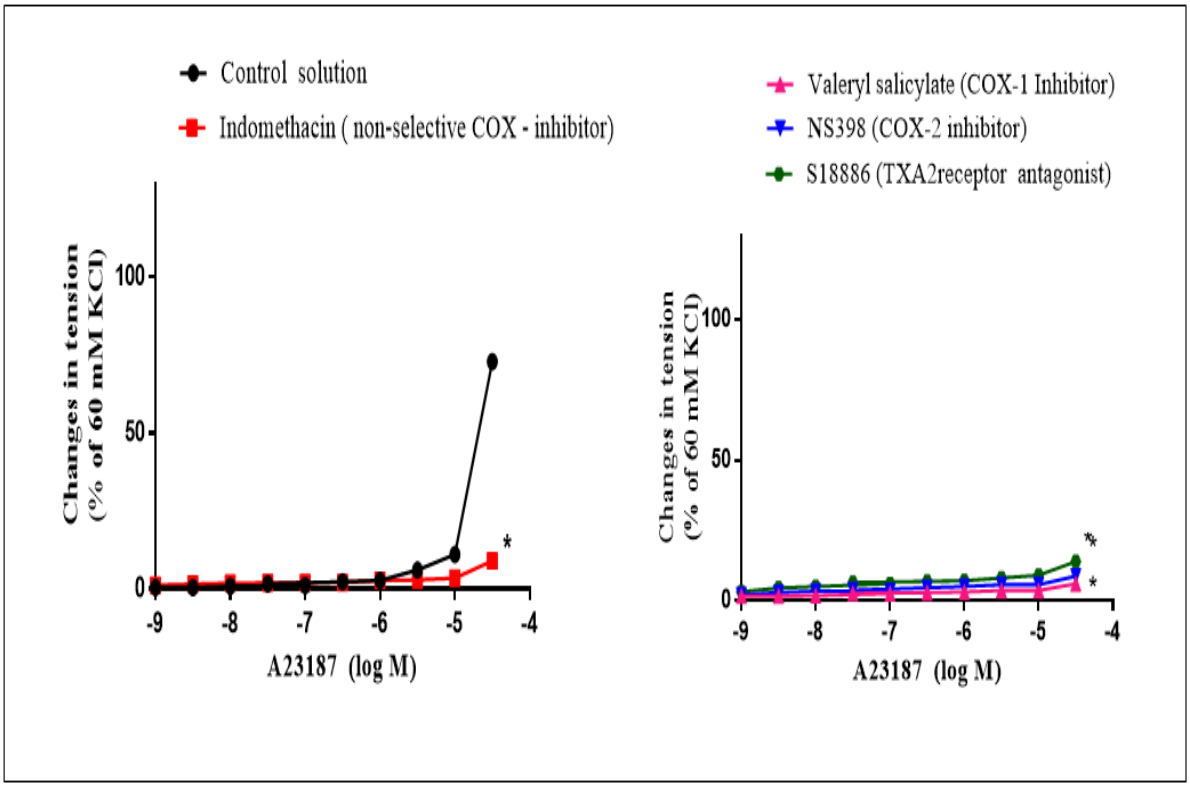


Figure 4.1 Concentration-response curves of calcium ionophore in tail arteries of normal 10-week rats in the control solution, or incubated with indomethacin (non-selective COX inhibitor), valeryl salicylate (COX-1 inhibitor), NS398 (COX-2 inhibitor) and S18886 (TXA2 receptor antagonist)

* indicates significant difference vs. control using ANOVA followed by Bonferroni post hoc test

3.6.4 Role of COX, COX-1, COX-2 and TXA_2 receptor in endothelium-dependent contraction in diabetic 10-week rats

Figures 4.2 showed maximal contraction (E_{max}) in the tail arteries of diabetic 10-week rats in control solution, or after incubated separately with indomethacin (non-selective COX), valeryl salicylate (COX-1 inhibitor), NS398 (COX-2 inhibitor) or S18886 (TXA_2 receptor antagonist). Increases in tension were expressed as percentage of the reference contraction to 60 mM KCl obtained at the beginning of the experiment.

The overall endothelium-dependent contraction induced by calcium ionophore in solutions without presence of blockers (control solution) was 113.73(51.32) %. However, after blocking the vessels with indomethacin (COX inhibitor), the contraction was reduced to 16.79 (13.28) % which was significantly lower when compared to control solution (p<0.001). After incubating the vessels with valeryl salicylate, the contraction was reduced to 14.60 (9.87) %. This contraction was lower than in control solution (p<0.001). In vessels incubated with NS398, the contraction was significantly reduced to 13.60 (10.05) % compared to control solution (p<0.001). When the vessels was incubated with S18886, the maximal endothelium contraction was 9.73 (10.94) % where the contractions were reduced when compared with control solution (p<0.001).

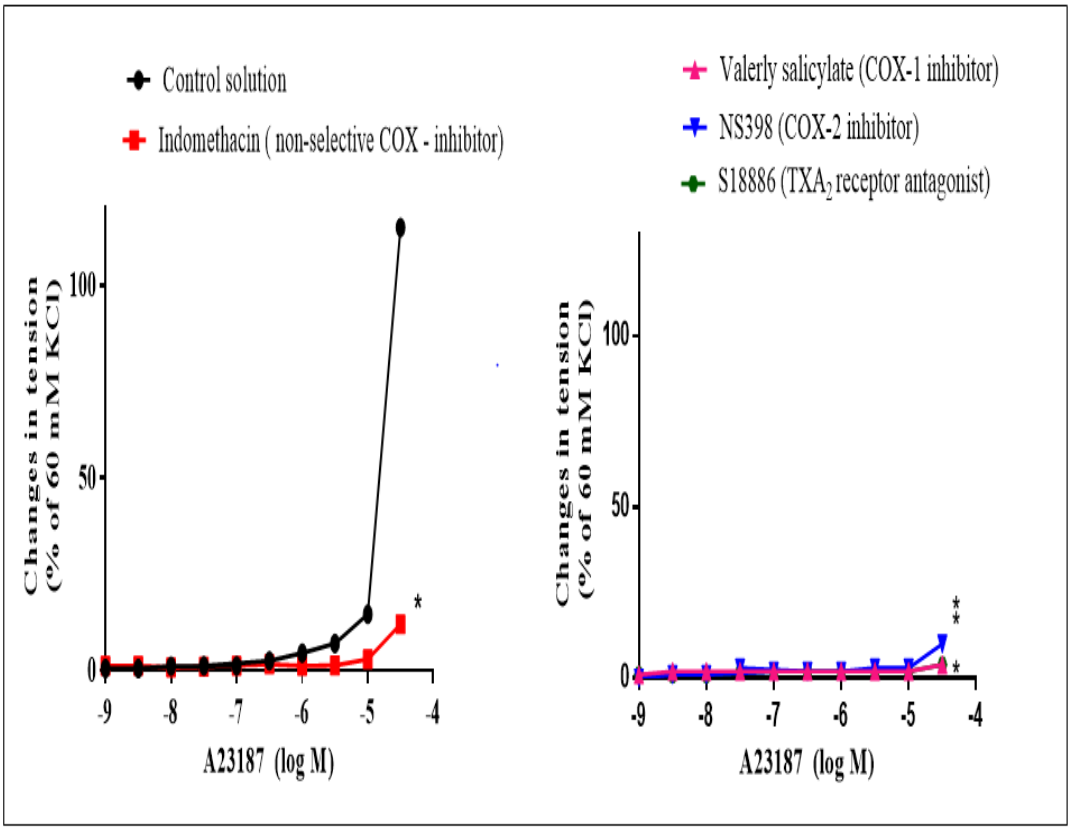


Figure 4.2 Concentration-response curves of calcium ionophore in tail arteries of diabetic 10-week rats in the control solution, or incubated with indomethacin (non-selective COX inhibitor), valeryl salicylate (COX-1 inhibitor), NS398 (COX-2 inhibitor) and S18886 (TXA_2 receptor antagonist)
* indicates significant difference vs. control using ANOVA followed by Bonferroni post hoc test

3.7 Endothelium-dependent contraction between the groups.

3.7.1 Maximal contraction to calcium ionophore in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats.

Figures 4.3 showed maximal contractions (E_{max}) to calcium ionophore in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats.

The maximal contraction in normal 2-week rats was 34.80 (21.0) %. In diabetic 2-week rats, the maximal contraction was 71.80 (46.02) % where there was trend in higher contraction when compared to contraction produced by normal 2-week vessels (p=0.058). The maximal contraction in normal 10-week rats was 68.30 (57.80) %. In diabetic 10-week rats, the maximal contractions was 113.73 (51.32) which was greater compared to normal 2-week rats (p<0.001).

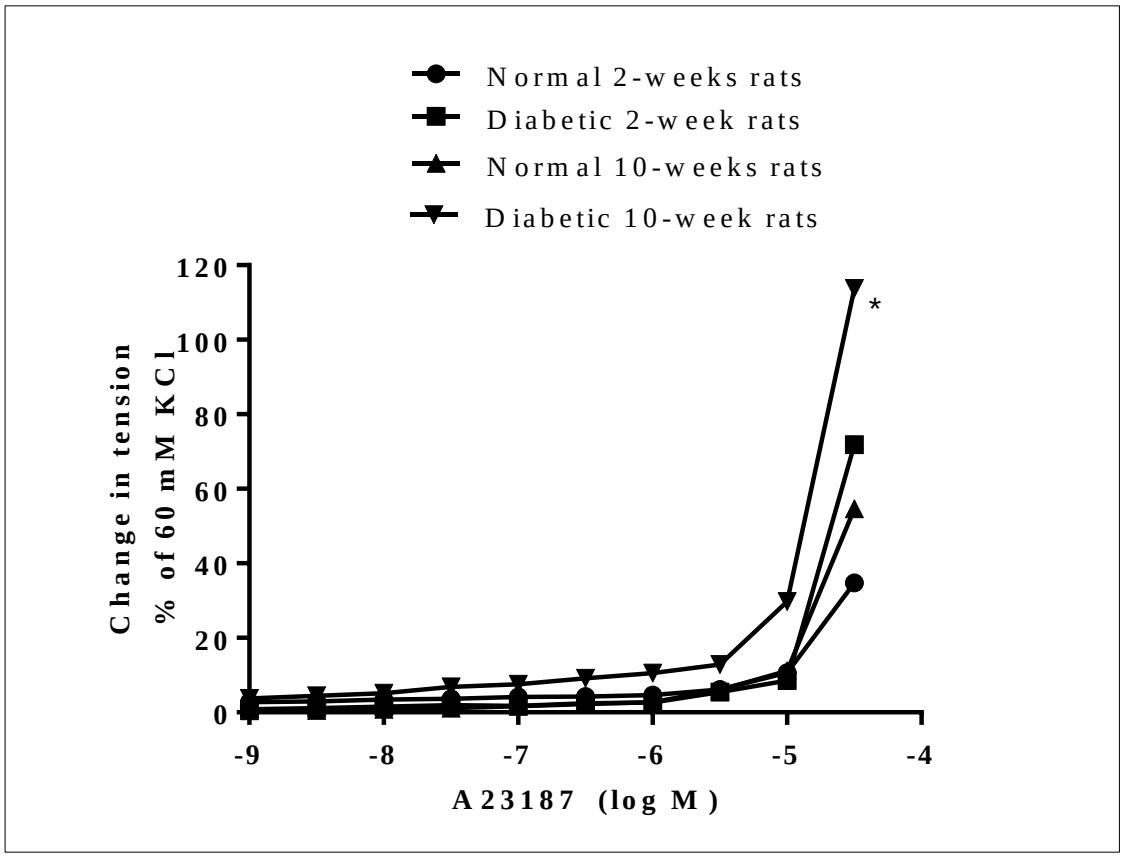


Figure 4.3 Concentration-response curves to calcium ionophore in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. * indicates significant difference vs. normal 2-week rats. There was a trend of increased contraction in diabetic 2-week rats when compared with normal 2-week rats (p=0.058).

3.7.2 Maximal COX-mediated contraction to calcium ionophore in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats.

Figure 4.4 showed the maximal COX-mediated contraction in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. COX-mediated contraction was defined as total endothelium-dependent contraction produced by calcium ionophore in control solution minus with the contraction produced by the vessels incubated with indomethacin.

COX- mediated contraction in normal 2-week rats was 19.75 (20.35) %. In diabetic 2-week rats, COX-mediated contraction was 53.00 (41.59) % with trend of higher contraction compared to normal 2-week rats (p=0.060). COX-mediated contraction in normal 10-week rats was 52.31 (53.03) %. In diabetic 10-week rats, COX- mediated contraction was significantly higher than normal 2-week rats [E_{max}: 96.94 (54.67) % vs 19.75 (20.35) %, p<0.001].

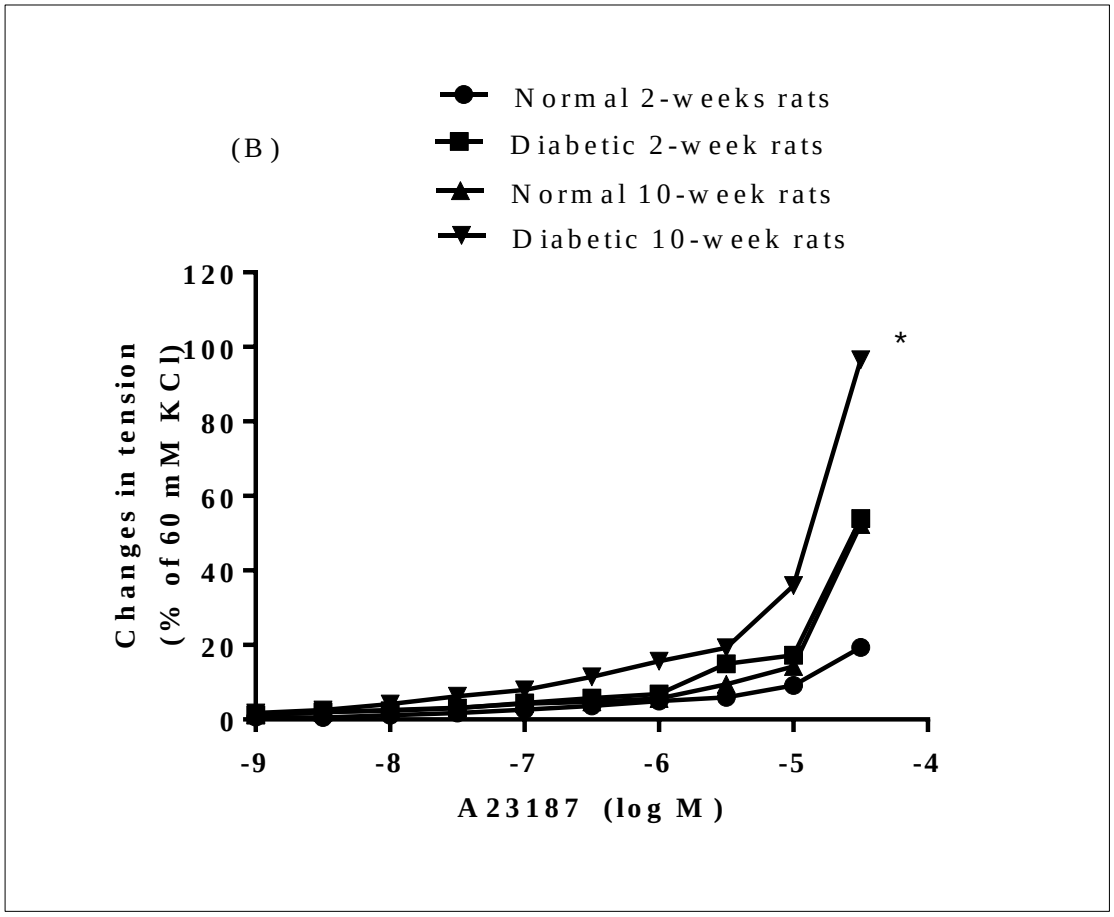


Figure 4.4 Concentration-response curves to COX-mediated contraction in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. E_{max} was analysed using ANOVA followed by Bonferroni post hoc test. * indicates significant difference vs. normal 2-week rats.

There was trend of increased contraction in diabetic 2-week rats compared to normal 2-week rats (p=0.060)

3.7.3 Maximal COX-1 mediated contraction to calcium ionophore in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats.

Figure 4.5 showed maximal COX-1 mediated contraction in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. COX-1 mediated contraction was defined as total endothelium-dependent contraction produced by calcium ionophore in control solution minus with the contraction produced by the vessels incubated with valeryl salicylate.

COX-1 mediated contraction in normal 2-week rats was 25.33 (17.52) %. In diabetic 2-week rats, COX-1 mediated contraction was 60.73 (46.23) %, whereas in normal 10-week rats, the contraction was 60.40 (58.71) %. In diabetic 10-week rat, COX-1 mediated contraction was significantly higher than normal 2-week rats [E_{max}: 99.13 (46.25) % vs 25.33 (17.52) %, $p<0.001$].

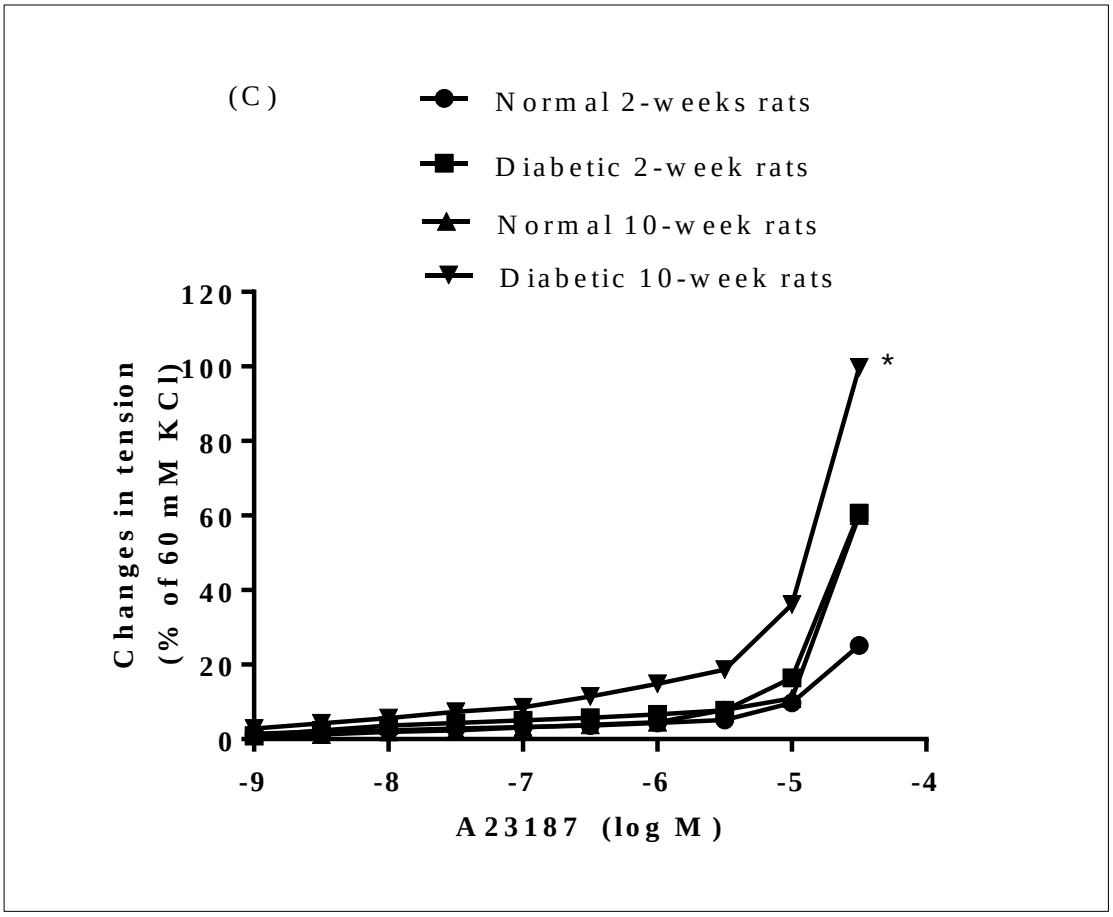


Figure 4.5 Concentration-response curves to COX-1 mediated contraction in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. E_{max} was analysed using ANOVA followed by Bonferroni post hoc test.* indicate significant difference was seen vs. normal 2-week rats

3.7.3(a) COX-1 protein expression in rat tail arteries

Figures 4.5.1 and 4.5.2 showed the expression of COX-1 protein in tail arteries of normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. No significant differences were seen between all experimental groups.

Plate 3.3 represents immunohistochemical staining photos highlighting the distributions of COX-1 protein in four groups: normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. No differences were seen in the intensity of the COX-1 in all experimental groups.

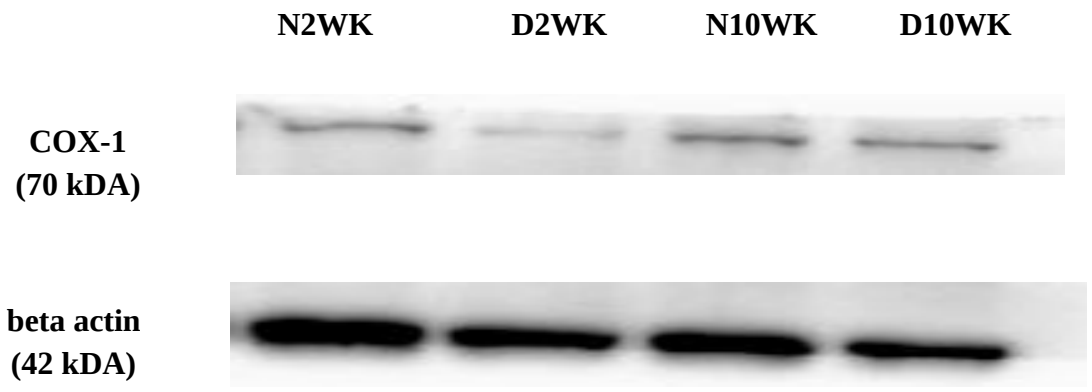


Figure 4.5.1 Representative of Western blots illustrating the expression of COX-1 protein in the tail arteries of the normal 2-week rats (N2WK), diabetic 2-week rats (D2WK), normal 10-week rats (N10WK) and diabetic 10-week rats (D10WK).
n =10 rats

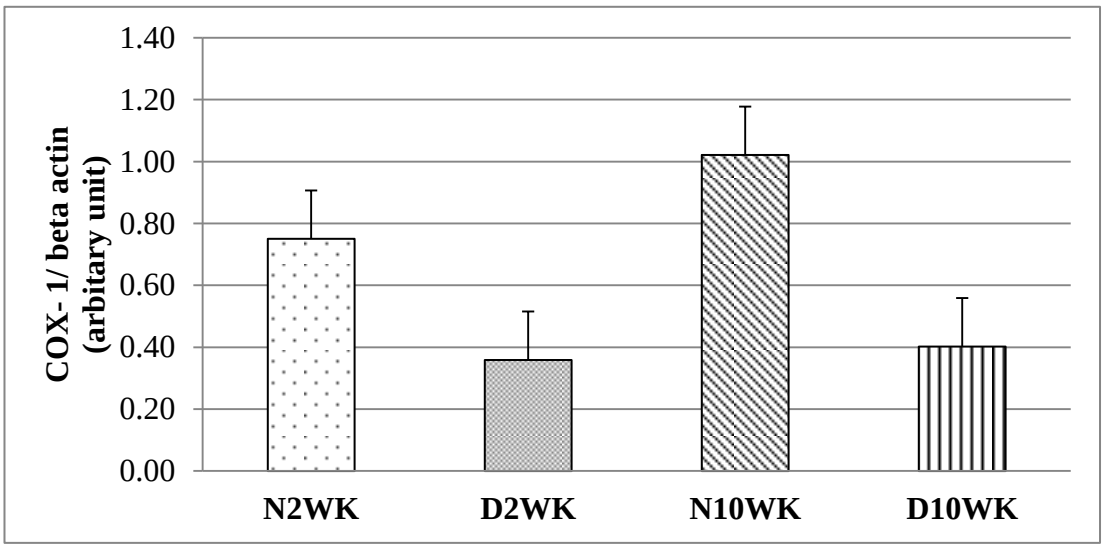
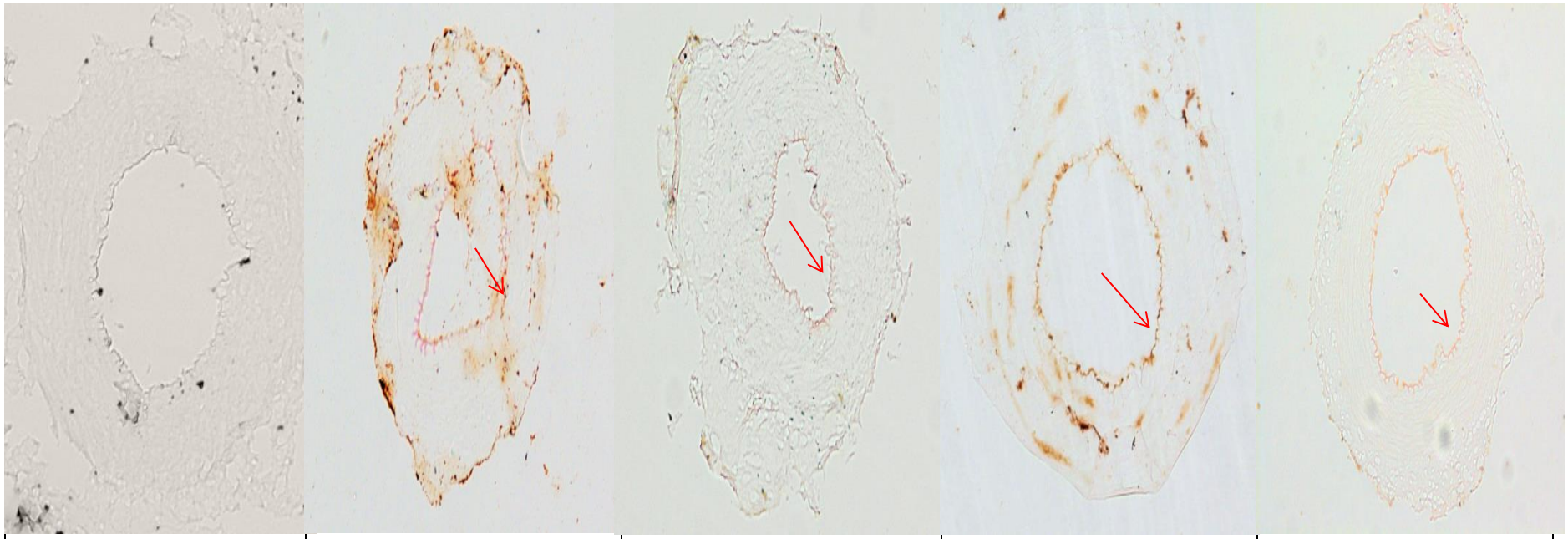


Figure 4.5.2 Graphical representation of the COX-1 protein expression normalized to β-actin. No significant difference was seen between all experimental groups

N2WK: normal 2-week rats; D2WK: diabetic 2-week rats; N10WK: normal 10-week rats; D10WK: diabetic 10-week rats



Negative control	Normal 2-week rats	Diabetic 2-week rats	Normal 10-week rats	Diabetic 10-week rats

Plate 3.3 Immunohistochemical staining photos showing the distributions of COX-1 protein in four experimental groups: normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. Sections without primary antibodies were used as negative controls.. The brown stain indicates expression of COX-1 proteins in the vessels (red arrows). Magnifications 100x.

3.7.4 Maximal COX-2 mediated contraction in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats

Figure 4.6 showed maximal COX-2 mediated contraction in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. COX-2 mediated contraction was defined as total endothelium-dependent contraction produced by calcium ionophore in control solution minus with the contraction produced by the vessels incubated with NS398.

COX-2 mediated contraction in normal 2-week rats was 17.80 (15.37) %. In diabetic 2-week rats, COX-2 mediated contraction was 56.80 (42.12) %, whereas in normal 10-week rats, the contraction was 55.20 (57.93) %. In diabetic 10-week rats, COX-2 mediated contraction was significantly higher than normal 2-week rats [E_{max}: 99.87 (52.41) % vs. 17.80 (15.37) %, $p<0.001$]. There were trend of increased contraction in diabetic 10-week rats compared to both normal 10-week ($p=0.053$) and diabetic 2-week rats ($p= 0.068$)

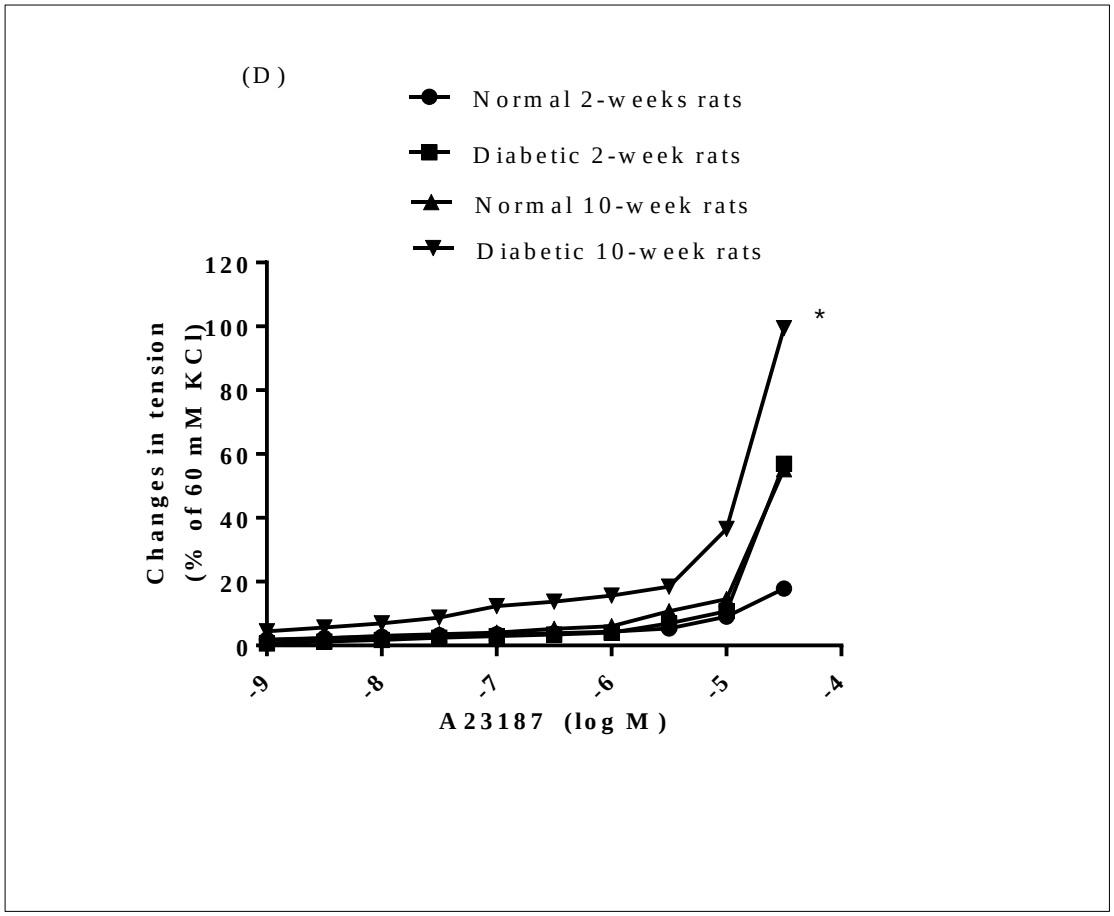


Figure 4.6 Concentration-response curves to COX-2 mediated contraction in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. E_{max} was analysed using ANOVA followed by Bonferroni post hoc test. * indicates significant difference vs. normal 2-week rats.

There was a trend of increase contraction in diabetic 10-week rats compared to diabetic 2-week rats (p=0.068). Trend of increased contraction was also seen in diabetic 10-week rats compared to normal 10-week rats (p=0.053).

3.7.4(a) COX-2 protein expression in rat tail arteries

Figures 4.6.1 and 4.6.2 showed the expression level of COX-2 protein in tail arteries of normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. No significant differences were seen between all experimental groups. There was a trend of increased expression of COX 2 in diabetic 10-week rats compared to normal 2-week rats (p=0.095).

Plate 3.4 represents immunohistochemical staining photos highlighting the distributions of COX-2 protein in four experimental groups: normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. No differences were seen in the intensity of the COX-2 protein in all groups.

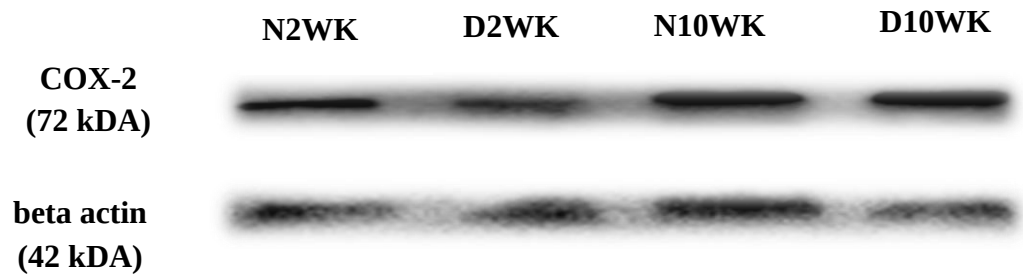


Figure 4.6.1 Representative of Western blots illustrating the expression of COX-2 protein in the tail arteries of the normal 2-week rats (N2WK), diabetic 2-week rats (D2WK), normal 10-week rats (N10WK) and diabetic 10-week rats (D10WK).
n=10 rats

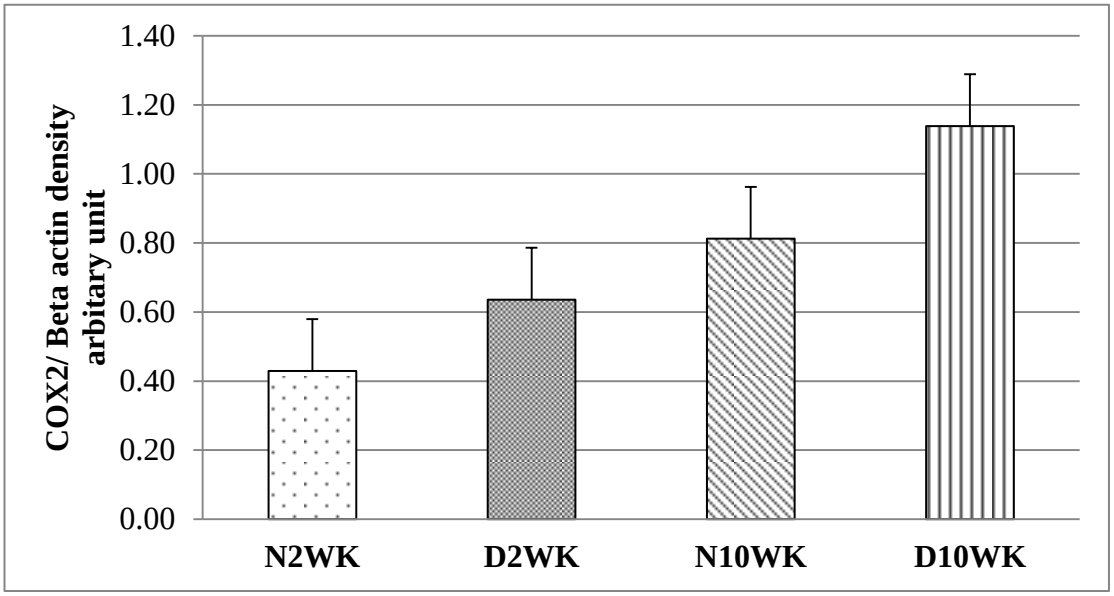
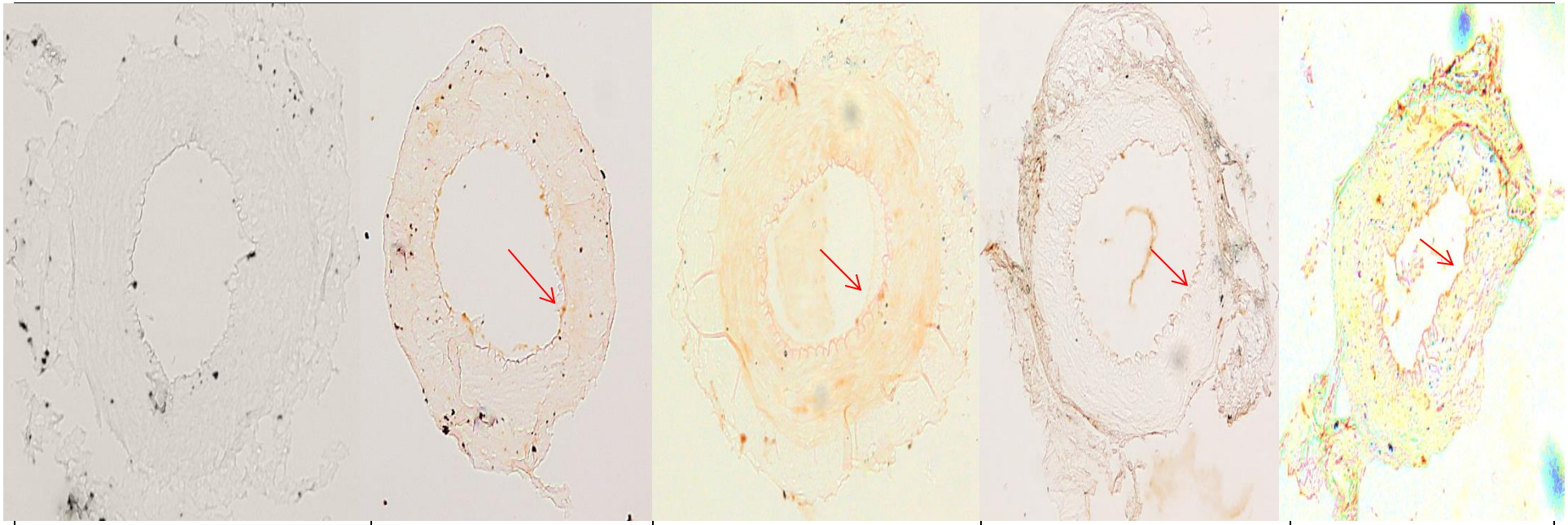


Figure 4.6.2 Graphical representation of the COX-2 protein expression normalized to β-actin.
There was trend of increased expression in diabetic 10-week rats compared to normal 2-week rats (p=0.095)

N2WK: normal 2-week rats; D2WK: diabetic 2-week rats; N10WK: normal 10-week rats; D10WK: diabetic 10-week rats



Negative Control	Normal 2-week rats	Diabetic 2-week rats	Normal 10-week rats	Diabetic 10-week rats

Plate 3.4 Immunohistochemical staining photos showing the distributions of COX-2 protein in four groups: normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. Negative control was not incubated with primary antibodies. The brown stain indicates expression of COX-2 proteins in the vessels. Magnifications 100x.

3.7.5 Maximal TXA_2 receptor-mediated contraction in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats

Figure 4.7 showed maximal TXA_2 receptor-mediated contraction in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. TXA_2 receptor-mediated contraction was defined as total endothelium-dependent contraction produced by calcium ionophore in control solution minus with the contraction produced by the vessels incubated with S18886.

Maximal TXA_2 receptor-mediated contraction in normal 2-week rats was 25.13 (22.13). In diabetic 2-week rats, TXA_2 receptor-mediated contraction was 62.13 (50.70) % with trend of increased contraction seen compared to normal 2-week rats (p=0.090). In normal 10-week rats, the TXA_2 receptor-mediated contraction was 50.47(64.27) %. In diabetic 10-week rats, TXA_2 receptor-mediated contraction was 103.40 (53.82) and significantly higher than normal 2-week rats (p<0.001).

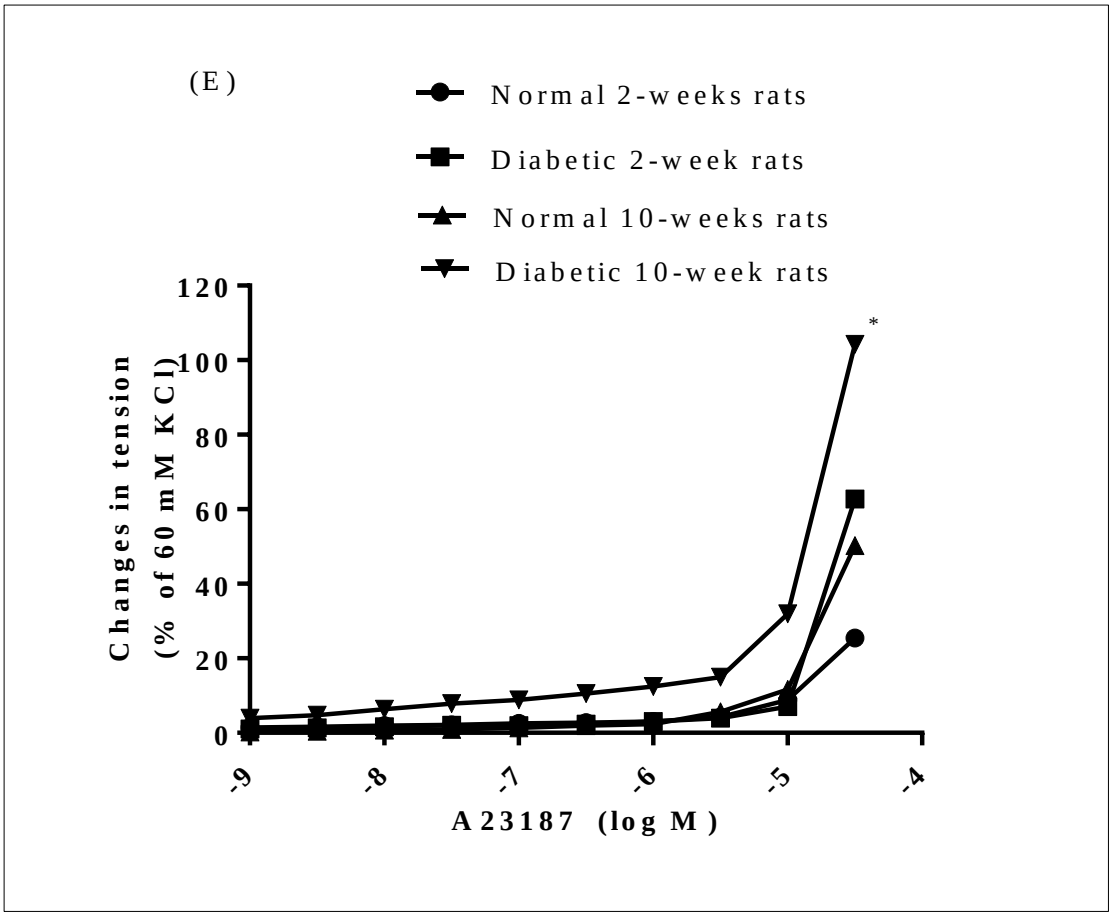


Figure 4.7 Concentration-response curves to TXA_2 receptor-mediated contraction in normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. E_{max} was analysed using ANOVA followed by Bonferroni post hoc test. *indicate significant difference vs. normal 2-week rats
There was trend of increased contraction in diabetic 2-week rats compared to normal 2-week rats (p=0.090)

3.7.5(a) TXA_2 receptor protein expression in rat tail arteries

Figures 4.7.1 and 4.7.2 showed the expression level of TXA_2 receptor protein in tail arteries of normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-weeks rats. There was a trend of increased expression of TXA_2 receptor protein in diabetic 10-week rats compared to normal 2-week rats (p=0.083).

Plate 3.5 represents immunohistochemical staining photos highlighting the distributions of TXA_2 receptor protein in four groups: normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. The intensity of the TXA_2 receptor protein was higher in diabetic 10-week rats compared to other groups.

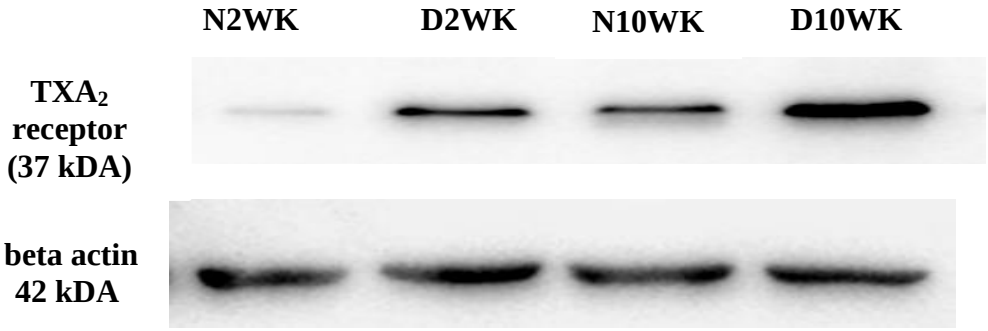


Figure 4.7.1 Representative of Western blots illustrating the expression of TXA_2 receptor protein in the tail arteries of normal 2-week rats (N2WK), diabetic 2-week rats (D2WK), normal 10-week rats (N10WK) and diabetic 10-week rats (D10WK). n =10 rats

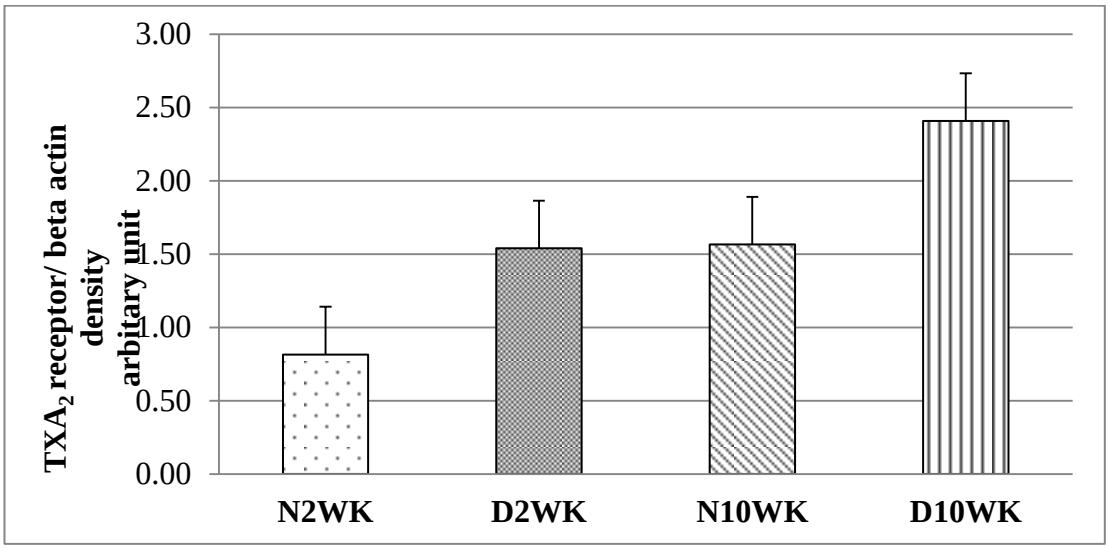
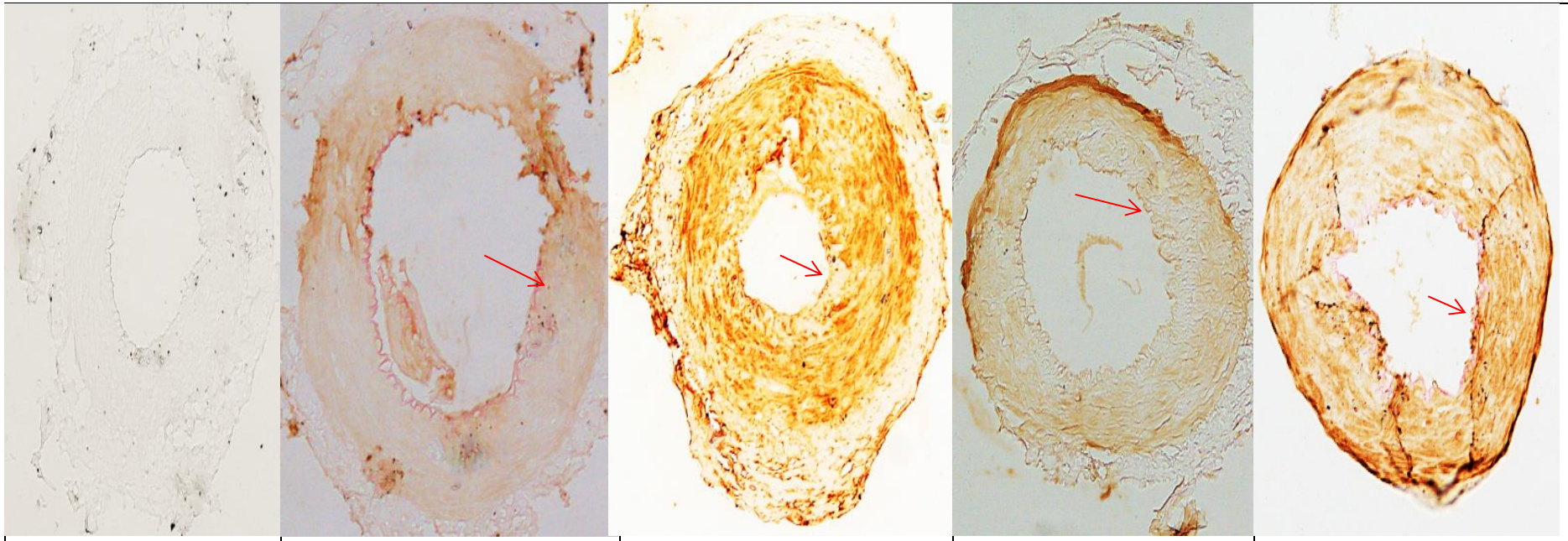


Figure 4.7.2 Graphical representation of the TXA_2 receptor protein normalized to β-actin.
There was a trend of increased expression in diabetic 10-week rats compared to normal 2-week rats (p=0.083)

N2WK: normal 2-week rats; D2WK: diabetic 2-week rats; N10WK: normal 10-week rats; D10WK: diabetic 10-week rats



Negative control	Normal 2-week rats	Diabetic 2-week rats	Normal 10-week rats	Diabetic 10-week rats

Plate 3.5 Immunohistochemical staining photos showing the distributions of TXA_2 receptor protein in four groups: normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. Sections without primary antibodies were used as negative controls. The brown stain indicates expression of TXA_2 receptor protein in the vessels (red arrows). Magnifications 100x.

3.7.5(b) TXA_2 synthase protein expression in rat tail arteries

Figures 4.7.3 and 4.7.4 showed the expression level of TXA_2 synthase protein in tail arteries of normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. No significant differences were seen between all groups.

Plate 3.6 represents immunohistochemical photos highlighting the distributions of TXA_2 synthase protein in four groups: normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. No prominent change was seen between the groups.

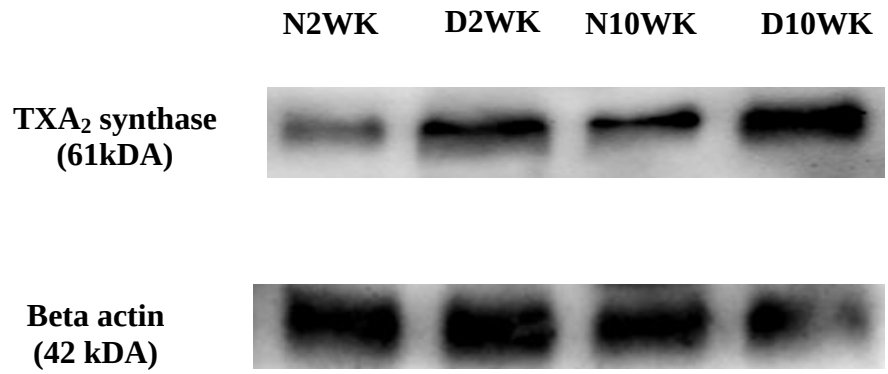


Figure 4.7.3 Representative of Western blots illustrating the expression of the TXA_2 synthase protein in tail arteries of the normal 2-week rats (N2WK), diabetic 2-week rats (D2WK), normal 10-week rats (N10WK) and diabetic 10-week rats (D10WK). n =10 rats

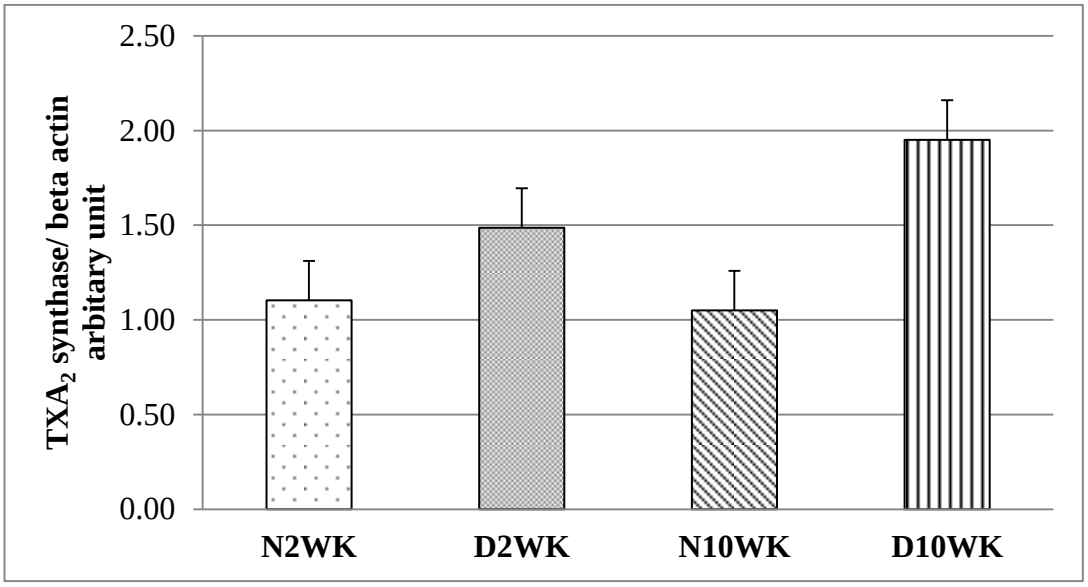
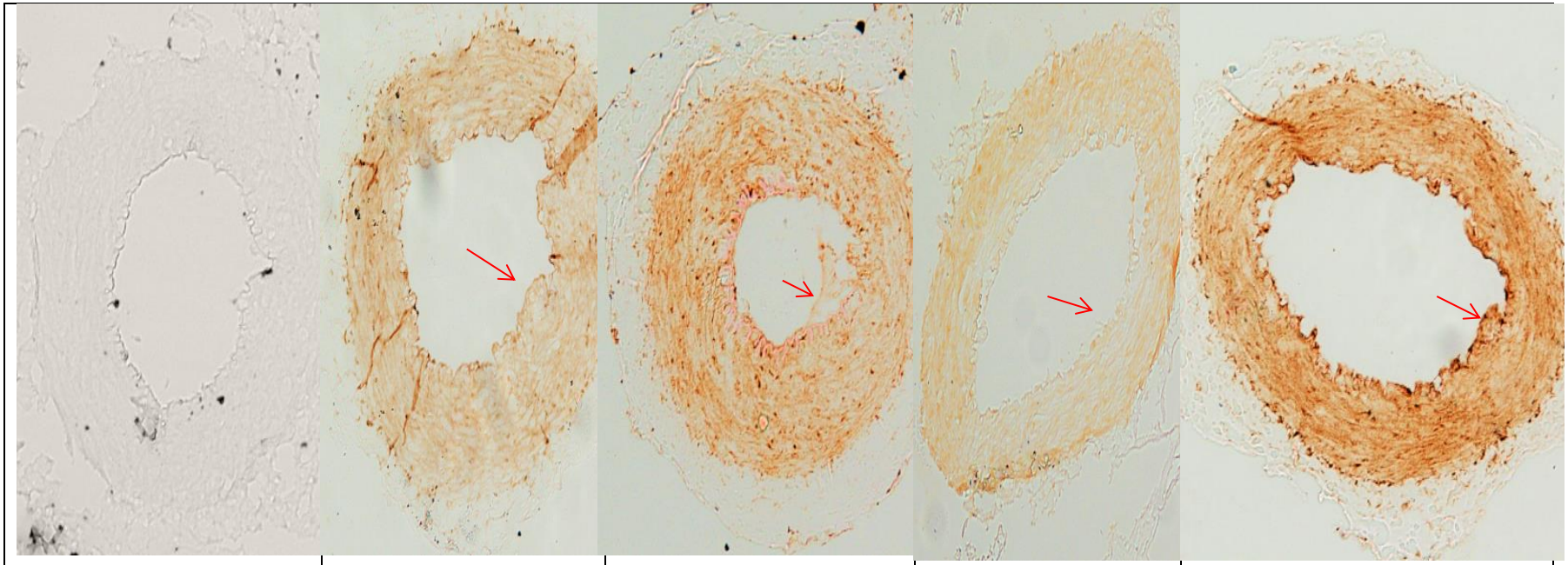


Figure 4.7.4 Graphical representation of the TXA_2 synthase protein expression normalized to β-actin. No significant differences were seen between the groups

N2WK: normal 2-week rats; D2WK: diabetic 2-week rats; N10WK: normal 10-week rats; D10WK: diabetic 10-week rats



Negative Control	Normal 2-week rats	Diabetic 2-week rats	Normal 10-week rats	Diabetic 10-week rats

Plate 3.6: Immunohistochemical staining photos showing the distributions of TXA_2 synthase protein in four groups: normal 2-week rats, diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats. Sections without primary antibodies were used as negative controls. The brown stain indicates expression of TXA_2 synthase protein in the vessels (red arrows). Magnifications 100x

CHAPTER 4

DISCUSSION

4.1. General discussion

In this study, the early (2 weeks) and late (10 weeks) effects of diabetes on the individual factor responsible for causing microvascular endothelium-mediated relaxation and endothelium-mediated contraction were evaluated. The enzymes and receptors involved in mediating these responses were also investigated through the methods of Western blotting and immunohistochemistry. Within group analysis of endothelium-dependent relaxation showed that NO, prostacyclin and EDH contributed to relaxation in normal and diabetic rats. NO is the primary vasodilator in all three groups except in diabetic 10-week rats. Significant reduction in ACh-mediated relaxation was seen as early as 2 weeks after diabetes induction with marked impairment noted in diabetic 10-week rats. Also, with the progression of diabetes was seen to worsen ACh-mediated relaxation. NO-mediated relaxation was blunted in diabetic 10-week rats with a trend of reduced NO-mediated relaxation in diabetic 2-week rats (p=0.09). This showed that NO production was affected early in diabetes, compared to other endothelium-derived vasodilators. With the progression of diabetes, the production of NO was markedly diminished at 10 weeks diabetes. Impairment in both EDH and prostacyclin-mediated relaxations were only seen later in diabetic 10-weeks rats with no change observed at diabetic 2-weeks rats. Western blot and immunohistochemistry results supported the findings of functional studies; where the expression and distribution of eNOS protein was significantly lower in diabetic 10-week rats. The expression and distributions of IP receptor and PGIS

enzyme were significantly lower in diabetic 10-week rats which are consistent with the functional study.

Endothelium-dependent contraction showed significant increase in diabetic 10-week rats with a trend of increase in diabetic 2-week rats (p=0.058) compared to normal 2-week rats. Within groups analysis showed that endothelium-dependent contractions were mediated by COX-1 and TXA_2 receptor in all the groups. COX-2 contributes to endothelium-dependent contraction in all other three groups (diabetic 2-week rats, normal 10-week rats and diabetic 10-week rats) but not in normal 2- week rats. Unlike NO, being the predominant vasodilator in endothelium-dependent relaxation up until early diabetes and both in normal groups, there were no significant difference between contributions of COX-1, COX-2 and TXA_2 receptor in endothelium-dependent contraction in all the groups.

Between the groups analysis showed significant increase in endothelium-dependent contraction mediated by COX-1, COX-2 and TXA_2 receptor in diabetic 10-week rats compared to normal 2-week rats. Trend of increased endothelium-dependent contraction mediated by COX (p=0.060) and TXA_2 receptor (p=0.090) were also seen in diabetic 2-week rats compared to normal 2-week rats. Western blot and immunohistochemistry showed no difference in COX-1 protein expression and distribution among all the groups. Trend of increased expression of COX-2 and TXA_2 receptor were seen in diabetic 10- week rats. Findings from this study showed that endothelium-dependent contraction increased with the diabetes and this increase is mainly due to the increase in both COX-1 and 2 activities together with activation of TXA_2 receptor that eventually leads to vasoconstriction. Findings from functional study also showed that endothelium-dependent relaxations were impaired earlier

than endothelium-dependent contractions as microvascular impairment in relaxation started to be evidenced early in diabetic 2-week rats.

4.2 Weight measurement, fasting blood glucose and vascular sensitivity to PE and KCl contraction

In this study, rats from both diabetic groups showed more weight lost compared to normal rats. As per our studies, other studies also reported weight lost following diabetic inductions (Zhang *et al.*, 2012; Mokhtar *et al.*2016) indicating their diabetic conditions. The fasting blood glucose in both diabetic groups were higher compared to the normal groups, which confirmed the diagnosis of diabetes. Diabetic rats in both groups were also seen to show polyuria (frequent urination) compared to normal rats. This necessitates more frequent bedding change for the diabetic rats. STZ which was used to induce diabetes in rats causes the damage to pancreatic β cells, resulting in hypoinsulinemia and hyperglycemia. This causes insufficient insulin which prevents the body from getting glucose from the blood into the body's cells to use as energy. When this occurs, the body starts burning fat and muscle for energy, causing a reduction in overall body weight.

There was no significant difference between normal 2-week, diabetic 2-week, normal 10-week and diabetic 10-week rats in their contraction to KCl indicating that vascular smooth function were preserved in all the groups. Similarly, contractions to PE also showed no significant difference between normal and diabetic groups showing that no impairment seen in vascular smooth muscle function.

4.3 Endothelium-dependent relaxation

4.3.1 Within group endothelium-dependent relaxation

When ACh was introduced to the endothelial cells, it binds to muscarinic receptors at the endothelial cells. This causes calcium release from endoplasmic reticulum and Ca^{2+} influx through opening of calcium channel at endothelial cell membrane which eventually increases intracellular Ca^{2+} concentration and leads to production of vasodilators such as NO, EDH and prostacyclin. The present study showed that endothelium-dependent relaxation in the tail arteries of normal-2 week rats was mainly mediated by NO; the percentage of NO contributing to endothelium-dependent relaxation was 63.87% followed by prostacyclin (41.26%) and EDH (37.22%). In diabetic 2-week rats, NO-mediated relaxation was 53.67% followed by prostacyclin (35.10 %) and EDH (30.13%). In normal 10-week rats; NO was still the main vasodilator contributing to 55.49 % of endothelium-dependent relaxation followed by EDH (36.53%) and prostacyclin (33.70%). In diabetic 10-week rats, there was a significant reduction in endothelium- dependent relaxation, where no significant differences were seen among NO (31.31%) prostacyclin (20.38%) and EDH (19.80%) indicating that the productions of all the vasodilators impaired with diabetes progression.

This study showed that there were significant differences in NO-mediated relaxations compared to prostacyclin and EDH in all three groups: normal 2-week rats, diabetic 2-week rats, normal 10-week rats indicating that NO was the predominant vasodilator up until early diabetes rat. However, NO production was seen affected with diabetes progression and no longer being the major vasodilator at the later stage of disease. No significant difference was seen between relaxation mediated by prostacyclin and EDH in all the four groups.

4.3.2. Between group endothelium-dependent relaxation

4.3.2(a) ACh-mediated relaxation

This present study was conducted to evaluate endothelium-dependent relaxation in the early phase of diabetes (2 weeks) and late phase of diabetes (10 weeks). In this study, impairment in endothelium-mediated relaxation started to be evident in the microcirculation of Sprague Dawley rats even in the early phase of diabetes 2 weeks after diabetes induction. Other than that, this study also showed that age did not play an important role in endothelium-mediated relaxation compared to rats having diabetes, as there was no significant difference between normal 2-week and normal 10-week rats. Significant reduction in ACh-mediated relaxation between diabetic 2-week and diabetic 10-week rats showing that microvascular endothelium-mediated relaxation worsened with the progression of diabetes. The impairment in diabetic 10-week rats was more pronounced compared to diabetic 2-week rats, which was due to the impairment in vasodilation mediated by all the vasodilators studied, namely NO, prostacyclin and EDH.

Impairment in ACh-mediated relaxation in both 2- and 10-week of diabetic vessel was consistent with another study conducted by Rodriguez-Manas *et al*., (2003). In this study, early and intermediate products of non-enzymatic protein glycosylation (Amadori adducts), oxidative stress, and endothelial function were evaluated in streptozotocin-induced diabetic rat's mesenteric arteries. Endothelial function was tested at several phases of streptozotocin-induced diabetes (from week one until nine weeks); impairment in ACh-induced relaxations was seen after 2 weeks of diabetes both in the mesenteric microvessels and aortic segments. The difference between the above study and the current study is that the current study

investigated on the role of individual vasodilators which includes NO, EDH and prostacyclin whereas the study by Rodriguez-Manas *et al*.(2003) only evaluated the ACh-mediated relaxations.

4.3.2(b) NO-mediated relaxation

From this study, it can be seen that NO was the major contributor for relaxation in all three groups but not during late diabetes. NO contributed 30-65% of endothelium-dependent relaxation in the tail arteries of normal and diabetic rats. In this study, NO-mediated relaxations were impaired in diabetic 10-week rats compared to normal 2-week rats and normal 10-week rats. NO-mediated relaxation was also seen to be greatly diminished in diabetic 10-week vessels compared to diabetic 2-week showing that with the progression of diabetes; the production of NO was severely impaired. A trend of reduced NO-mediated relaxation was also seen in diabetic 2-week rats when compared to normal 2-week rats suggesting that impairment in NO-mediated relaxation started to occur from the early phase of diabetes. This observation is supported by Western blot and immunohistochemistry results, where the expression and distribution of eNOS enzyme were significantly reduced in diabetic 10-week compared to normal 2-week and normal 10-weeks rats. The expression of eNOS was significantly lower in diabetic 10-week compared to diabetic 2-week and normal groups. The expression of eNOS was significantly lower in diabetic 10-week compared to diabetic 2-week rats showing that reduction in NO synthase becomes more pronounced with prolonged duration of diabetes.

Similar findings were seen by a previous work by Mokhtar *et al.* (2016). In that study, NO-mediated relaxation was impaired and associated with reduced expression of eNOS in the tail artery of STZ induced diabetic rats. That study was

performed in six weeks diabetic rats, whereas our studies involved 2 and 10 week of diabetic rats evaluating the early and late response of diabetes in the tail arteries. The same researcher also reported that NO-mediated relaxation was severely impaired in subcutaneous arteries from lower limbs of diabetic patients compared to non-diabetic patients (Mokhtar *et al*., 2014); their functional study results were supported by biochemistry results showing lowered eNOS protein expression in the arteries of diabetic humans.

Impairment in NO-mediated relaxation was also seen in another study in mesenteric arteries of 10 weeks diabetic rats. In this study, the researchers reported that the expression of total eNOS protein was not different between diabetic and normal rats. The total expression of eNOS remained unchanged; however, the dimer form of eNOS were significantly reduced in diabetic groups compared to normal rats (Leo *et al*.,2011). eNOS may exist in dimer and monomer forms in the endothelial cells. eNOS dimerization is essential to ensure the normal function of eNOS (Chen *et al.*, 2014). The dimer form of eNOS is regarded as the active form, whereas the monomer form is considered inactive. In eNOS dimers, one eNOS monomer reductase domain transfers an electron to another eNOS monomer's heme center of the oxygenase domain, which catalyses the conversion of L-arginine to L-citrulline, producing NO. However when the eNOS is in the monomer form, it is not able to deliver the electrons to the heme center of another eNOS monomer. This causes the electrons to be transferred to a different position on the same eNOS monomer. This condition provides the interaction with oxygen and the formation of superoxide (O_2^-) free radicals (Xu *et al.*, 2007; Schulz *et al.*, 2008b; Chen *et al.*, 2014). In Leo's study, increased superoxide levels was seen in diabetic mesenteric arteries, thus the researchers suggested that reduction in dimer expression of eNOS in

diabetes rats was due to increased superoxide levels which leads to the degradation of NO by superoxide (eNOS uncoupling). The difference between the current study and Leo's study is the type of vessels studied; we used tail artery instead of mesenteric artery. It was reported that the source of NO production differs in mesenteric and tail artery (Sousa et al., 2015). In mesenteric artery, NO was mainly produced through nNOS pathway, while in tail artery, the synthesis of NO was via eNOS pathway. This difference can explain why the total expression of eNOS was not affected in Leo's study, though there was impairment in NO-mediated relaxation.

There are a number of mechanisms that may contribute to the impairment in NO-mediated relaxation in diabetes. Firstly, oxidative stress contributes to the inactivation of NO production in the early stage of diabetes and worsened with the progression of diabetes. The increase of reactive oxygen species (ROS) can contribute to the imbalance between vasodilators and vasoconstrictor mainly by inactivating the production of NO (Schieber and Chandel, 2014; Pierini and Bryan, 2015). A number of enzymes such as NADPH oxidases, xanthine oxidase, and enzymes of the mitochondrial respiratory chain are capable of generating ROS (Souza *et al.*, 2001; Forstermann and Sessa, 2012; Daiber *et al.*, 2017). NADPH oxidases are identified as the primary source for ROS generation (Beswick Richard *et al.*, 2001) where it can trigger endothelial NOS (eNOS) uncoupling (Landmesser *et al.*, 2003), xanthine oxidase activity (McNally *et al.*, 2003; Landmesser *et al.*, 2007), and mitochondrial enzymes to produce more ROS (Doughan Abdulrahman *et al.*, 2008; Wenzel *et al.*, 2008; Swenja *et al.*, 2014). Studies had shown that the production of NO was diminished when the endothelial cells were exposed to elevated glucose level (Hoshiyama *et al.*, 2003). During hyperglycaemia in diabetes, healthy cells lose their functions in balancing the cellular redox environment and

favour excess generation of ROS (Circu and Aw, 2010). Excess ROS will further inactivate NO by rapid oxidation. Besides that, increased production of superoxide anion (O_{2-}) and NO cause the formation of peroxynitrite ($ONOO^-$) within the vascular wall (Krötz *et al.*, 2004). Formation of peroxynitrite oxidized the NOS cofactor, tetrahydrobiopterin (BH4) which is necessary for NO synthesis. The presence of both peroxynitrite and superoxide itself can oxidize BH4 leading to BH4 deficiency (Bagi *et al.*, 2004; Schulz *et al.*, 2008a; Chen *et al.*, 2010b; Gillis *et al.*, 2018). Apart from that, generation of peroxynitrite also reduced cellular transport of L-arginine, an eNOS substrate which further promotes eNOS uncoupling (Förstermann and Sessa, 2012). Studies also showed that deficiency of L-arginine and overabundance of superoxide affects NO bioavailability (Hoshiyama *et al.*, 2003; Yang and Ming, 2013). Therefore the production of NO is dependent on the availability of BH4 and also *L*-arginine (Luiking *et al.*, 2012; Yang and Ming, 2013). Consequently, all these events will cause eNOS uncoupling which in turn generates more peroxynitrite formation and further causes inactivation of NO.

Secondly, chronic hyperglycemia also increases the formation of advanced glycation end products (ADMA) which contributes to endothelial dysfunction (Singh *et al.*, 2014). ADMA is a competitive inhibitor of eNOS where the increase in ADMA will compete with L-arginine for eNOS binding. This causes eNOS uncoupling which leads to NO depletion and an increase in superoxide formation (Sydow and Munzel, 2003; Pluta, 2008; Forstermann and Sessa, 2012). The formation of ADMA also increases with rising levels of oxidative stress. This might be due to the decrease in dimethylarginine dimethylaminohydrolase (DDAH) activity which normally degrades ADMA production in the cells (Alacam *et al.*, 2013). An increase in nitrosative stress also causes nitrosylation of DDAH enzymes

and thus increases ADMA concentration (Leiper *et al.*, 2002). Emerging studies suggested that S-glutathionylation of eNOS as another mechanism for eNOS uncoupling (Chen *et al.*, 2010a; Guerby *et al.*, 2019). Under oxidative stress, cysteine (Cys) residues on eNOS are oxidized, leading to glutathionylation of eNOS contributing reduced NO generation.

Thirdly, hyperglycemia also increases the synthesis of a molecule called diacylglycerol (DAG) which further intensifies the activations of PKC kinase pathway (Beckman Joshua *et al.*, 2002; Gutterman David, 2002; Brownlee, 2005). Several studies showed that stimulation of PKC by DAG causes sudden influx of calcium intracellularly which mediates contraction in isolated blood vessels (Khalil and van Breemen, 1988; Horowitz et al., 1996; Kanashiro and Khalil, 1998; Khalil, 2013). PKC activation also turns off the production of eNOS while the production of other vasoconstrictors such as Ang II and endothelin increases (Ohara *et al.*, 1994; Hirata *et al.*, 1995; Ohara *et al.*, 1995; Brownlee, 2005). Apart from that, PKC activation also causes NO degradation since its activation causes free radical generation.

Finally, recent studies showed that hyperglycemia via activation of the hexosamine pathway impaired NO-dependent relaxation. In this pathway, hyperglycemia leads to an increase of protein O-GlcNAcylation. O-GlcNAcylation of eNOS decreases its activity which leads to depletion of NO-mediated relaxation (Beleznai and Bagi, 2012; Issad, 2015; da Costa *et al.*, 2018). It also increases the production of superoxide which further impairs the endothelium-mediated relaxation (Du *et al.*, 2000).

4.3.2(c) EDH-mediated relaxation

In this study, EDH contributed approximately 19 to 38 % of endothelium-dependent relaxation in tail arteries of normal and diabetes rats. EDH-mediated relaxations were significantly reduced in diabetes 10-week rats when compared to both normal groups. Impairment in EDH-mediated relaxation was also seen in other studies involving mesenteric arteries (Leo *et al.*, 201,Fukao *et al.*, 1997; Wigg *et al.*, 2001; Matsumoto *et al.*, 2003b; Matsumoto *et al.*, 2005; Matsumoto *et al.*, 2006b). However, in a different study by Mokhtar *et al.* (2014), EDH-mediated relaxation in the tail arteries of SD rats were seen comparable between diabetic and control groups which can be related to the duration of diabetes which was six weeks compared to our study which was longer than theirs. In another study, augmented EDH-mediated relaxation was seen in mesenteric artery of diabetes rats which was due to the compensatory response as the NO-mediated vasodilatation being reduced (Shi *et al.*, 2006). In Shi's study, the difference in the variability of vascular parts chosen may contribute to the difference in EDH role in relaxation. Besides using mesenteric artery, Shi *et al*. (2006) also investigated the role of EDH in carotid and femoral arteries in 12 weeks of diabetic rats. In that study, augmented EDH responses were seen in mesenteric and femoral arteries but not in carotid artery. The difference in the findings could be due to a number of factors such as different vascular bed chosen, duration of diabetes in animal models and severity of hyperglycemia. In the current study, the FBG was higher at approximately 30 mmol/L whereas in Shi's study, the FBG level was lower at 20.8 mmol/L. Studies had previously reported that severity of hyperglycemia affects EDH-mediated relaxation (Pieper, 1999; MacKenzie *et al.*, 2008; Tajbakhsh and Sokoya, 2014; Goto and Kitazono, 2019).

There are multiple mechanisms that may cause impairment in EDH-mediated relaxation in diabetes; the contributing factors are still being studied. One of the mechanisms is via defects in the the adenosine 3',5'-cyclic monophosphate cyclic (cAMP) signalling pathway which abolishes EDH responses. cAMP that is produced in endothelial cells disperse to the underlying smooth muscle via myo-endothelial gap junctions. This causes hyperpolarisation of the VSMC membrane mainly through PKA-dependent activation of K^+ channels (Garland *et al.*, 2011; Mokhtar and Rasool, 2015). cAMP levels was found to be reduced in mesenteric arteries of diabetic rats. Abnormalities in the cAMP signalling cause reduction in cAMP levels and increase in phosphodiesterase 3 (PDE) activities (Matsumoto *et al.*, 2003b; Matsumoto *et al.*, 2006a). The use of PDE inhibitor was seen to supress the PDE activity which enhances EDH-mediated vasodilatation (Matsumoto *et al.*, 2003c; S Moreira *et al.*, 2018). The reduction in cAMP concentration leads to the impairment of EDH-mediated vasodilatation (Matsumoto *et al.*, 2003a). Impairment of cAMP-mediated vasodilatation most likely occurs due to the decline in PKA activity (Matsumoto *et al.*, 2003c; Matsumoto *et al.*, 2004). Hyperglycemia leads to modification of PKA content, alterations in its subcellular localization, reduction of PKA substrates and decreased or altered β-adrenergic signalling which contributes to reduced activity of PKA and consequently abolished EDH-mediated relaxation (Matsumoto *et al.*, 2004)

Diabetes has been shown to increase lysophosphatidylcholine (LPC) (Rabini *et al.*, 1994) which inhibited EDH-mediated relaxations (Fukao *et al.*, 1995; Matsumoto *et al.*, 2006b). Huge increase of plasma LDL-C in diabetic rats causes sudden rise of superoxide anions due to the decreased activity of vascular superoxide dismutase (SOD) (Makino *et al.*, 2000; Matsumoto *et al.*, 2006b). This sudden rise

of superoxide anions in turn oxidizes LDL-C generating LPC (Kobayashi and Kamata, 1999). LPC is then transported to the endothelial surface where it prevents EDH-mediated relaxation by reducing the endothelial KCa channel activities (Matsumoto *et al.*, 2006b). In another study, as a consequence of LPC released from oxidized low-density lipoprotein (ox-LDL), the intracellular calcium release and also Ca^{2+} influx from the extracellular space were seen compromised in aortic endothelial cells from STZ-induced diabetic mice. In this study, LPC was seen to impair EDH-mediated responses by decreasing endothelial Ca^{2+} rise in STZ-induced diabetes. However, the relaxation was improved by lowering plasma LDL level with cholestyramine. This study proposed that LPC may have significant role over Ca^{2+} mobilization in the aortic endothelium of diabetic mice (Kamata and Nakajima, 1998).

Other than that, abnormalities or dysregulations in Ca^{2+} signalling pathways (reduction in the intracellular Ca^{2+} release and/or extracellular Ca^{2+} influx) in endothelial cells has also been reported to be associated with impairment in EDH-mediated responses. Hyperglycemia increases the production of oxidative stress and also stimulates excessive protein kinase C (PKC) activation which in turn causes disruption or alteration in intracellular Ca^{2+} mobilization (Kugiyama *et al.*, 1992).

Besides that, impairment in EDH-mediated relaxation was also linked to modification in the function and/or expression of potassium channels (SKCa and IKCa channels). Reduced responses to KCa channel activators were seen in mesenteric arteries of STZ-induced rodents (Wigg *et al.*, 2001; Morikawa *et al.*, 2005; Absi *et al.*, 2013). The decrease in the downstream KCa channel activation in diabetes vessels can be due to the changes in endothelial Ca^{2+} mobilization (Kamata and Nakajima, 1998; Estrada Irene *et al.*, 2012; Gokina *et al.*, 2013). Moreover, the

degradation of endothelial glycocalyx (external layer of the endothelial cells) (Dogné *et al.*, 2018) may also result in the impairment of EDH-mediated responses through a reduction of the SKCa channel input to EDH (Dogné *et al.*, 2016). Again, an increase in ROS production is another factor seen to reduce the KCa channel function via the downregulation of KCa channel expression in some diabetic vascular endothelial cells (Choi *et al.*, 2013a; Choi *et al.*, 2013b). Advanced glycation end products (AGEs) is also seen to stimulate ROS generation and reduces the KCa channel function by dysregulating the intracellular Ca^{2+} mobilization (Naser *et al.*, 2013) or by downregulating the expression of KCa channel proteins (Zhao *et al.*, 2014).

Current studies also proposed that nonselective cation channels of the transient receptor potential (TRP) family in endothelial cells may serve a pivotal role in agonist-stimulated Ca^{2+} influx where it causes endothelium-dependent vasorelaxation (Earley and Brayden, 2015; Behringer and Hakim, 2019; Goto and Kitazono, 2019). Recent studies emphasized that the downregulation of endothelial TRP vanilloid type 4 (TRPV4) and small-conductance of Ca^{2+} -Activated K^{+} impaired endothelium-dependent hyperpolarization (Seki *et al.*, 2017; Boudaka *et al.*, 2019; Goto and Kitazono, 2019). The presence of high glucose was seen to downregulate the protein expression of TRPV4 channels, thus tempering the agonist-stimulated Ca^{2+} influx in retinal microvascular endothelial cells (Monaghan *et al.*, 2015). Protein expression of endothelial TRPV4 channels was also reported to be reduced in retinal arterioles (Monaghan *et al.*, 2015) and mesenteric arteries (Ma *et al.*, 2013) of STZ-induced diabetic rats, where this expression of protein was linked to impaired EDH-mediated relaxation (Ma *et al.*, 2013).

4.3.2(d) Prostacyclin-mediated relaxation

In this study, prostacyclin-mediated relaxation contributed to only approximately 20-45% of endothelium-dependent mediated relaxations in normal and diabetic rats. Results from this study showed that prostacyclin-mediated relaxation were severely impaired in diabetic 10-week rats when compared with normal 2-week rats. A trend of reduced prostacyclin-mediated relaxation was also seen between diabetic 10-week rats compared to normal 10-weeks rats. No difference was noted in diabetic 2-week rats when compared to both normal groups. However significant difference was seen between diabetic 2-week rats and diabetic 10-week rats indicating that duration of diabetes affects prostacyclin-mediated relaxation, where impairment in its relaxation was seen with the progression of diabetes. Western blot and immunohistochemistry results showed that both expressions of IP and PGIS in diabetic 10-week rats were significantly lower compared to normal groups. The expression of IP receptor was significantly reduced in diabetic 10-week rats compared to diabetic 2-week rats showing reduction in IP receptor occurs with prolonged duration of diabetes. The expression of PGIS in diabetic 10-week was significantly lower compared to normal 10-week rats. Several studies in human microcirculation (Meeking *et al.*, 2000; Bolego *et al.*, 2006; Szerafin *et al.*, 2006; Mokhtar and Rasool, 2015) and large vessels of animal studies (Shen *et al.*, 2003; Kamata *et al.*, 2006; Toniolo *et al.*, 2013) showed impairment in prostacyclin-mediated relaxation supporting our observation. However, human studies were all studied in long term diabetes patients

However, a study by Mokhtar *et al.*, 2016 reported that prostacyclin-mediated relaxations are not impaired in the small arteries of STZ-induced diabetic rats (Mokhtar *et al.*, 2016). The difference in findings with Mokhtar study could be

related to the duration of diabetes. Mokhtar *et al.*(2016) reported that prostacyclin-mediated relaxation was not impaired in 6 weeks tail artery of STZ diabetes induced rats, while the current study was performed in 10 weeks tail arteries. Mokhtar *et al.*, (2016b) also reported increase in COX-1 and COX-2 protein expressions in tail arteries of diabetic rats compared to controls. Even though no compensatory rise or impairment reported in prostacyclin-mediated relaxation in six weeks vessels, an increase in both COX-1 and COX-2 expressions in diabetic vessels indicated that prostacyclin might be involved in the attempt to preserve vascular relaxation which is impaired with the progression of diabetes. Similar to Mokhtar's study, trend of increased expression in COX-2 enzyme seen in diabetic rats which is supported by significant increase in COX-2 mediated contraction in diabetic 10-week rats. The increase in COX-2 expression in current study may lead to the increase in prostaglandin productions that may cause contraction instead of relaxation. The increase in COX-2 expression could be due to the increase in inflammation level with the progression of diabetes. In the current study, there was no change in COX-1 expression between all the groups.

A study by Kamata *et al.* (2006) on large vessels showed that apart from NO and EDHF, prostacyclin plays a significant role in relaxation in diabetic rats; however no contribution of prostacyclin-mediated relaxation was seen in control rats. In that study, endothelium-dependent relaxation in the renal vascular arteries of STZ-induced diabetic rats was compromised when the vessels treated with tranylcypromine (prostacyclin-synthesis inhibitor). That study demonstrated that an increase in prostacyclin relaxation may be due to compensatory vasodilatory mechanism, when other productions of vasodilators were reduced with the development of diabetes.

Prostacyclin was also seen to contribute to endothelium-dependent relaxation in the aorta of early stage STZ-induced diabetic mice (Shen *et al.,* 2003). In that study, an increase in the blood serum of 6-keto-PGF1α (vasodilator prostacyclin) was reported in the initial stage of STZ-induced diabetic mice (Shen *et al.*, 2003), where this increase was linked to enhanced ACh-induced relaxation.

A few mechanisms were suggested to contribute to the impairment of prostacyclin-mediated relaxation in diabetes. Firstly, with the progression of diabetes, the production of prostacyclin was found to be disrupted which could be due to the increasing level of oxidative stress. Hyperglycemia causes the formation of peroxynitrite, which prevents the activity of PGIS via tyrosine nitrosylation-dependent reaction (Cooke and Davidge, 2002; Zou *et al.*, 2002; Zou, 2007; He *et al.*, 2010). Endothelium-dependent vasodilation is compromised in mesenteric arteries from superoxide dismutase knockout mice (animal model of oxidative stress); due to alterations in NOS and prostacyclin-dependent relaxation responses. The relaxation was partly normalized after incubation with exogenous SOD (Cooke and Davidge, 2003).

Secondly, under certain circumstances such as ageing and hypertension, prostacyclin might cause endothelial contraction instead of relaxation. Prostacyclin seems to play a role for contraction which accounts for endothelial dysfunction seen in those conditions such as in hypertension (Blanco-Rivero *et al.*, 2005; Gluais *et al.*, 2005a; Xavier *et al.*, 2008; Vanhoutte Paul, 2011; Liu *et al.*, 2012; Zhou *et al.*, 2013; Luo *et al.*, 2016). The contraction, rather than relaxation by prostacyclin may occurs because of the low expression or dysfunction of IP receptors; in this situation prostacyclin acts on TXA_2 receptors to cause contraction. This is also seen in our

study, where the expression of IP receptors and PGIS were significantly reduced in prolonged diabetes.

4.4 Endothelium-independent relaxation: SNP-mediated relaxation

In this study, SNP was used to determine endothelium-independent relaxation. This experiment was carried out to test whether diabetes affects the vascular smooth muscle function in early (2 weeks) and late duration (10 weeks) of diabetes. In the present study, the response of VSMC to SNP (NO donor) was significantly reduced in diabetic 10-week rats compared to normal 2-week rats indicating diminished VSMC sensitivity to NO. However, no change was seen in diabetic 2-week indicating that VSMC was still preserved in the early phase of diabetes. VSMC was also seen not affected following one week of diabetic induction in the mesenteric artery (Zhang *et al.*, 2012). Similar findings were also seen in the tail arteries of six weeks diabetic type 1 rats (Mokhtar *et al.*,2016) and also in aorta of four weeks type 2 diabetic rats (Viswanad *et al.*, 2006). From this we can conclude that a shorter duration of diabetes did not affect the sensitivity of VSMC.

In this study, SNP-mediated relaxation was seen impaired in diabetes 10-week rats showing diminished in VSMC sensitivity. This observation was supported by other studies that showed impairment in SNP-mediated relaxation in mesenteric artery (Zhang *et al.*, 2012) and aorta (Brown *et al.*, 2001; Csanyi *et al.*, 2007) of eight weeks diabetic rats. This showed that with diabetes progression, the cylic guanosine monophosphate (cGMP pathway) was affected and thereby diminished the sensitivity to VSMC.

4.5 Endothelium-dependent contraction

4.5.1 Within group endothelium-dependent contraction

In this study, calcium ionophore (A23187) was used to evoke endothelium-dependent contractions. Calcium ionophore directly causes the entry of calcium ions across the vascular membrane where the increase in intracellular calcium ion concentration activates phospholipase A2 (PLA_2) leading to production of arachidonic acids (AA) in endothelium. AA is later catalysed by the cyclooxygenase (COX) enzymes to produce prostanoids such as thromboxane A_2. TXA_2 will then diffuse to the underlying vascular smooth muscle to activate TXA_2 receptor which eventually causes the contraction vascular smooth muscle. An increase in endothelial calcium levels is one of the key factors that lead to endothelium-dependent contraction.

In this study, to investigate endothelium-dependent contraction, the experiments were performed in the presence of L-NAME, an NOS inhibitor to inhibit NO production thus optimize endothelium-dependent contraction. To investigate the involvement of cyclooxygenase enzymes and TXA_2 receptor in each group, the tail arteries from each group were separately incubated in indomethacin (non-selective COX inhibitor), valeryl salicylate (COX-1 inhibitor), NS398 (COX-2 inhibitor), S18886 (TXA_2 receptor antagonist) and the resulting contractions measured.

In normal 2-week rats, the endothelium-dependent contraction by calcium ionophore was 34.80%. The contraction was significantly reduced to 15.18% when incubated with indomethacin. In the vessels incubated with valeryl salicylate, the endothelium-dependent contraction was significantly reduced to 14.00%, whereas no difference was seen in the contraction of arteries incubated in NS398; showing that

COX-2 did not play a significant role in endothelium-dependent contraction in arteries of young rats. Inhibition with TXA_2 receptor antagonist significantly reduced the contraction to 15.27%, indicating that endothelium-dependent contractions involved activation of TXA_2 receptor.

In diabetic 2-week rats, the endothelium-dependent contraction induced by calcium ionophore was 71.80% which was reduced to 20.01% after blocking with indomethacin. The endothelium-dependent contraction by calcium ionophore in a vessel incubated with valeryl salicylate was significantly reduced to 11.00%; whereas the contraction after blocking the vessels with NS398 was significantly reduced to 13.47%. The endothelium-dependent contraction was significantly reduced to 8.13% after blocking with S18886. These findings showed that endothelium-dependent contraction with short duration of diabetes involved both COX-1 and COX-2, and due to activation of TXA_2 receptor.

The endothelium-dependent contraction induced by calcium ionophore in normal 10-week rats was 68.33% which was later reduced to 17.36% after incubating with indomethacin. The endothelium-dependent contraction was reduced to 7.21% after blocking with valeryl salicylate. Blocking with NS398 significantly reduced endothelium-dependent contraction to 12.60%, indicating that COX-2 play a role in endothelium-dependent contraction in normal 10-week. This was in contrast with normal 2-week rats where COX-2 was not seen to mediate the endothelium-dependent contraction. In normal 10-week rats, COX-2 was seen to contribute to endothelium–dependent contraction suggesting that with aging, COX-2 plays a role in endothelium-dependent contraction. After incubating the vessels with S18886, the endothelium-dependent contraction was reduced to 14.53%.

In diabetic 10-week rats, the endothelium-dependent contraction induced by calcium ionophore was 113.73% which was reduced to 16.79% after blocking the vessels with indomethacin. The endothelium-dependent contraction was reduced to 14.60% after incubating the vessels with valeryl salicylate while the contraction was significantly reduced to 13.60% when incubated with NS398. The endothelium-dependent contraction was reduced to 9.73% when the vessel was incubated with S18886.

Overall from the above findings, it was noted that incubation with inhibitors of COX-1, COX-2 (except in normal 2-week rats) and TXA_2 receptor antagonist caused significant reductions in endothelium-dependent mediated contraction compared to control solution indicating active involvement of COX enzymes and activation of TXA_2 receptors in mediating endothelium-dependent contraction.

We could not find similar studies that reported on the roles of COX, and TXA_2 receptor on vascular contraction response in the diabetic microvasculature. However, a similar study has been reported in large vessels of rats (Shi *et al.*, 2007), where endothelium-dependent contraction in the femoral artery was mediated by cyclooxygenase products. Shi *et al.* (2007) reported that endothelium-dependent contraction to calcium ionophore was increased in diabetic four weeks rats; the contraction was prevented by indomethacin and S18886, suggesting that endothelium-dependent contraction involved cyclooxygenase enzymes and activation of TXA_2 receptors. In twelve weeks of diabetic rats, endothelium-dependent contraction was augmented which was abolished by indomethacin and valeryl salicylate, and not to COX-2 inhibition. This observation was supported by their Western blot results where the protein expression of COX-1 was increased, while the expression of COX-2 remains unaffected. TXA_2 receptor antagonist was

seen to cause partial inhibition of the contraction, whereas combination of TXA_2 receptor antagonist with prostaglandin E_2 receptor (EP1-receptors) blockers abolished the contractions, indicating that the EDCFs activated both TXA_2 and EP1-receptors. In this current study, COX-1 and COX-2 mediated the endothelium-dependent contractions in normal 10 weeks and in both diabetic groups. However, COX-2 was seen not to mediate endothelium-dependent in normal 2 weeks. This current study also showed that TXA_2 receptor antagonist abolished the endothelium-dependent contraction in all the groups without the need of prostaglandin E_2 receptor. These dissimilarities between current study with Shi *et al.*(2007) likely due to the type of the vessels used. In the current study, tail artery was used which is considered microvasculature, whereas in their study, femoral artery, a large vessel was used. Besides that, in that study, the diabetes duration was 4 and 12 weeks whereas in our study the duration was 2 and 10 weeks.

Another study that shared similar findings with Shi, reported that COX-1 mediated thromboxane dependent contraction in mesenteric arteries of normal Wistar Kyoto rats aged between 8 to 10 weeks (Bolla *et al.*, 2004). In this study, only COX-1 was seen to mediate the contractions, while COX-2 did not play a significant role in endothelium-mediated contraction. The current study also supports the findings where only COX-1 was seen to mediate endothelium-contraction in normal 2-week rats. However, in the current study, in normal 10 week- rats, both COX-1 and COX-2 were seen to mediate endothelium-dependent contraction. The role of TXA_2 was also evaluated in Bolla's study and it was found that TXA_2 antagonist contributes to partial inhibition of endothelium contraction whereas combination use with prostaglandin E_2 receptor antagonist fully inhibited endothelium-dependent contraction (Bolla *et al.*, 2004). In the current study,

complete inhibition of endothelium contraction by TXA_2 antagonist was seen in normal 2-week and normal -10 week rats. The differences between the studies were the involvement of COX-2 in normal 10-week rats and complete inhibition by TXA_2 antagonist in both normal 2-week and normal 10-week rats. These differences may be due to the species; current study used Sprague Dawley whereas normal Wistar Kyoto was used by Bolla *et al*. (2004). Besides, in Bolla's study, the mean age of the rats was 9 weeks whereas in the current study, the mean age of both rats from normal groups was 13 and 20 weeks which are much older than Bolla's study**.** In Bolla's study however, the roles of COX and TXA_2 in diabetes were not studied, therefore the comparison were only made between normal groups.

So far, from our knowledge, the roles of COX-1, COX-2 and TXA_2 receptors on endothelium-mediated contractions in diabetic microcirculations have not been reported. No studies also reported regarding early and late effect of diabetes on endothelium-dependent contraction in diabetic microvasculature.

4.5.2 Between groups endothelium-dependent contraction

4.5.2(a) Diabetes increases endothelial contracting factor

In this study, endothelium-dependent contraction to calcium ionophore was augmented in diabetic 10-week rats compared to normal 2-week rats. There was a trend of increased contraction in diabetic 2-week rats compared to normal 2-week rats ($p=0.058$). Within group analysis showed that endothelium-dependent contraction is mainly mediated by COX products and involved TXA_2 activation in all the groups. However, COX and TXA_2-receptor mediated contractions were seen augmented in diabetic-10 week rats compared to normal 2-week rats indicating that diabetes increases endothelium-dependent contraction. There was no significant

difference between normal 2-week and normal 10-week rats indicating that up to 10 weeks, aging do not play a significant role in affecting endothelium-dependent contraction. Aging plays a lesser role in affecting endothelium-mediated contraction compared to diabetes.

In diabetic 2-week rats, the trend observed was an increase in endothelium-dependent contraction together with a significant reduction in endothelium-dependent relaxation indicating that the microvascular endothelial functions started to be disrupted at the early diabetes stage. The augmented endothelium-dependent contraction with blunted relaxation response in diabetic 10-week rats showed that with the progression of diabetes, the microvascular endothelial function worsened. In this study, endothelium-dependent relaxation was seen to be earlier affected compared to endothelium-dependent contractions with diabetes progression.

There are a number of reasons that may contribute to the increase in endothelium-dependent contraction in the microcirculation of diabetes rats. Firstly, diabetes reduced the production of individual vasodilators and caused imbalance between availability of EDRF and EDCF. This was seen in this study where reduction in the production of vasodilators were observed which leads to impairment in endothelium-dependent relaxation. In this study, a trend of NO reduction occurs since 2 weeks of diabetes, and the impairment worsened at 10 weeks. The expression of eNOS was significantly reduced in diabetic 10-week rats causing reduced production of NO. Impairment in prostacyclin-mediated relaxation was also seen in diabetic 10-week rats with reduced expression of IP receptor and PGIS in diabetic 10-week rats. Several studies reported that prostacyclin, instead of mediating relaxation; it can cause contraction in aging and hypertension by acting on TXA_2 receptor (Ge *et al.*, 1995; Rapoport and Williams, 1996; Blanco-Rivero *et al.*, 2005;

Gluais *et al.*, 2005b). In the current study, prostacyclin-mediated relaxation was impaired in diabetic 10-week rats. The augmented endothelium-dependent contraction may be due to prostacyclin which may appear as vasoconstrictor in diabetic 10-weeks rats. EDH-mediated relaxation was seen to be abolished in diabetic 10-week rats. From the above observations, all individual vasodilators were significantly impaired in diabetic 10-week rats with the progression of diabetes. These reduction causes disruption between endothelium relaxing and contracting factors which eventually favour vasoconstriction and contribute to microvascular dysfunction.

Secondly, diabetes increases the production of oxidative stress via activation of protein kinase C (PKC) resulting in overproduction of oxidative stress (Kizub *et al.*, 2014). Activation of PKC not only causes impairment in endothelium-dependent relaxation but also increases endothelium-dependent contraction mediated by TXA_2 (Hattori *et al.*, 1999; Cosentino *et al.*, 2003; Liu *et al.*, 2009), prostaglandin E_2 (PGE_2) (Cosentino *et al.*, 2003; Ishida *et al.*, 2012) and endothelin (Hattori *et al.*, 1999). Other than that, oxidative stress was also seen to contribute to impairment in endothelial relaxation by inactivating vasodilators besides being one of the major sources of endothelium-dependent contraction (Gryglewski *et al.*, 1986; Rubanyi and Vanhoutte, 1986; Auch-Schwelk and Vanhoutte, 1992; Tschudi *et al.*, 1996; Miyagawa *et al.*, 2007; Macarthur *et al.*, 2008). In a few studies, oxidative stress was seen to induce contraction by stimulating the production of prostaglandin E_2 and F_{2a} which in turn causes endothelium-dependent contractions (Zou *et al.*, 1999; Bachschmid *et al.*, 2003; Gluais *et al.*, 2005b). Reactive oxygen species (ROS) also acts as endothelial contracting factor in the canine basilar artery (Katusic and Vanhoutte, 1989) and in rat renal artery (Gao and Lee, 2005). Free radicals formed

during oxidative stress diffuse to the underlying VSMC and trigger COX in the vascular smooth muscle cells to create more prostanoids which in turn activate TXA_2 receptors (Auch-Schwelk *et al.*, 1989; Katusic and Vanhoutte, 1989; Garcia-Cohen *et al.*, 2000; Yang *et al.*, 2002; Wang *et al.*, 2003; Shi *et al.*, 2008). In hypertensive animal models such as in mouse aorta and renal arteries of rats, production of ROS is seen to be increased which causes endothelial COX-2 to create more ROS and prostanoids which consecutively triggers TXA_2 receptors to cause VSMC contractions (Tang *et al.*, 2007; Tian *et al.*, 2012). When the endothelial cells are triggered with ACh or calcium ionophore (A23187), it causes a sudden outburst of endothelial free radical (Tang *et al.*, 2007). This outburst is seen to be prevented by indomethacin (COX inhibitors) proving that COX is one of the primary sources of superoxide anions in endothelial cells (Tang *et al.*, 2007). This incidence is also evidenced in human forearm vasculature, where both COX inhibitors and antioxidant inhibit endothelial dysfunction (Virdis *et al.*, 2013). The free radical, hydrogen peroxide is also seen to assist the contraction by inhibiting the translocation and degradation of TXA_2 receptor, increase its density at the vascular cell membrane (Feletou *et al.*, 2010). Apart from that, free radicals also play a harmful role by catalyzing nonenzymatic peroxidation of arachidonic acid which causes production of PG-like compounds known as isoprostanes. During oxidative stress, the production of isoprostane surpasses that of COX-derived PGs (Hardy *et al.*, 2000). Isoprostanes functions as biomarkers of oxidative stress (Buczynski *et al.*, 2009) and also serve as potent endogenous agonist at TXA_2 receptor which facilitates contractions (Feletou *et al.*, 2010; Bauer *et al.*, 2014)

Thirdly, apart from TXA_2 being one of the products derived from COX activity, COX enzymes are also able to generate other endoperoxides, includes

prostacyclin, prostaglandin D_2, prostaglandin E_2 and/or prostaglandin $F_{2\alpha}$, which contributes to endothelium-dependent contractions (Ito *et al.*, 1991; Asano *et al.*, 1994; Ge *et al.*, 1995; Ge *et al.*, 1999; Bos *et al.*, 2004; Hirao *et al.*, 2008; Vanhoutte and Tang, 2008). High concentrations of prostacyclin were reported to induce contraction of VSMC instead of relaxation through the activation of TXA_2 receptors in diseased conditions such as hypertension (Ge *et al.*, 1995; Rapoport and Williams, 1996; Blanco-Rivero *et al.*, 2005; Gluais *et al.*, 2005b). Prostaglandin F_{2a} is also found predominantly as EDCF in human renal arteries (Wong *et al.*, 2009; Tian *et al.*, 2012). In ageing, it was found that COX-2-derived prostaglandin F_{2a} appear to be the main EDCF in the aorta of hamsters (Wong *et al.*, 2009; Wong *et al.*, 2010). Therefore, the contribution of each prostaglandin differs amongst species and vascular beds. Finally, other endothelial contracting factors such as endothelin and angiotensin II (which were not studied in this study) were seen to increase endothelial contraction. Endothelin and angiotensin II levels have been reported to increase in diabetes (Mather *et al.*, 2002; Chawla *et al.*, 2010). In diabetic rats, treatment with endothelin blockers improved diabetes-induced aortic endothelial dysfunction (Kanie and Kamata, 2002). Studies also showed that overproduction of endothelin in diabetes could be due to the generation of oxidative stress which eventually leads to endothelial dysfunction (Amiri *et al.*, 2004). Overexpression of endothelin in small resistance arteries demonstrated that diabetes exaggerated endothelial dysfunction where decreased in eNOS expression and antioxidant capacity were seen with enhanced ROS production (Idris-Khodja *et al.*, 2016). Angiotensin II (Ang II) was reported to cause injury to endothelial cells and leads to endothelial dysfunction. It induces formation of vascular oxidative stress excessively following upregulation of NADPH oxidase (Tummala *et al.*, 1999; Walter *et al.*,

2008). Upregulation of NADPH oxidase causes disruption in homeostatic balance where it deplete NO production by generating reactive oxygen species (Ceconi *et al.*, 2002; Förstermann and Li, 2011; Ramalingam *et al.*, 2017).

4.5.2(b) Duration of diabetes increases endothelium-dependent contraction

Prolonged diabetes causes augmentation in endothelium-dependent contraction. Chronic hyperglycemia for a long period causes the endothelial cells to lose the ability to release vasodilators (Hein *et al.*, 2016) which was seen in the current study where impairment in endothelium-dependent relaxation in diabetic 10-week rats was greater than diabetic 2-week rats. Longer duration of diabetes also significantly increased oxidative stress (Yan, 2014). Over time, increased oxidative stress may cause permanent impairment of vasodilators functions by turning off their production and enhancing the effects of endothelial contracting factors such TXA_2, angiotensin II and endothelin. In the current study, NO- and prostacyclin-mediated relaxation were impaired to a greater degree in diabetic 10-week compared to diabetic 2-week rats showing that duration of diabetes affects the involvement of vasodilators.

In the current study, a trend of increased COX-2 mediated contraction was observed in diabetic-10 week rats compared to diabetic 2-week rats ($p=0.068$). This showed that COX-2 which is induced by inflammation increases with the progression of diabetes. Many studies indicated that inflammation level increases with the progression of diabetes (Wellen and Hotamisligil, 2005; Bagi *et al.*, 2006; Vinagre *et al.*, 2014; Tsalamandris *et al.*, 2019). Trend of increased in COX-2 expression in Western Blot was also seen in diabetic 10-week rats. Increased

expression of COX-2 will increase the production of prostanoids such as TXA_2 which will act on TXA_2 receptors and causes increased contraction

4.5.2(c) Role of COX enzymes and TXA_2 receptor in endothelium-dependent contraction

In this study, the contribution of individual COX enzymes and TXA_2 receptor in endothelium-dependent contraction between the four groups were evaluated.

COX-mediated contraction was significantly increased in diabetic 10-week rats compared to normal 2-week rats. A trend of increased COX–mediated contractions was also seen in diabetic 2-week rats compared to normal 2-week rats ($p=0.060$). These findings showed that the endothelium-dependent contraction was largely mediated by COX-products. It also showed that diabetes increases COX-mediated contractions which worsened with diabetes progression.

COX-1 mediated contraction was significantly higher in diabetic 10-week rats compared to normal 2-week rats. However, Western blot showed no significant difference in the density of COX-1 between diabetic and normal groups. Similar observation was seen in another study which reported that upregulation of COX-1 expression or a greater presence of the protein is not essential for EDCF-mediated responses (Tang and Vanhoutte, 2009). The author reported that the augmented respond of endothelium-dependent contraction are associated to the VSMC sensitivity to the PGH_2 and arachidonic acid, but not to an increased expression of COX-1 expression (Ge *et al.*, 1999)

COX-2 mediated contraction was significantly higher in diabetic 10-week rats compared to normal 2-week rats. Within group analysis showed that COX-2

mediated contraction was not seen in normal 2-week rats. A trend of increased in COX-2 mediated contraction was also seen in diabetic 10-week rats compared to normal 10-week rats (p=0.053). Besides that, a trend of increased COX-2 mediated contraction was observed in diabetic-10 week compared to diabetic 2-week rats (p=0.068). In western blot, a trend of increased expression of COX-2 (p=0.095) was seen in diabetic 10-week rats compared to normal 2-week rats indicating that COX-2 may be upregulated in prolonged diabetes. The increase in the activity and expression of COX-2 may be associated with increased inflammation in diabetic 10-week rats. These observations were also seen in diabetes animal models involving large vessels where enhanced COX-2 activity was seen accompanied by the increase in endothelium-dependent contraction (Quilley and Chen, 2003; Guo *et al.*, 2005; Xu *et al.*, 2019) and decrease in endothelium-dependent mediated relaxation (Nacci *et al.*, 2009; Vessières *et al.*, 2013). COX-2 enzyme is enhanced in inflammation and increase in inflammation level increases endothelial dysfunction and is associated with cardiovascular diseases.

TXA_2 receptor mediated contraction was significantly higher in diabetic 10-week rats compared to normal 2-week rats. A trend of increased TXA_2 receptor mediated contraction was also seen in diabetic 2-week compared to normal 2-week rats. This showed that endothelium-dependent contraction was significantly increased in diabetic rats and involved TXA_2 activation. Western blot showed a trend of increased expression in TXA_2 receptor in diabetic 10-week rats compared to normal 2-week rats, while no significant change was seen in TXA_2 synthase enzyme. Trend of increased TXA_2 expression in diabetic 10-week rats may contribute to more vasoconstrictors binding to TXA_2 receptors which are in line with increased TXA_2 receptor-mediated contraction. Immunohistochemistry results also indicated that the

intensity of TXA_2 receptor protein was prominent in diabetic 10-week rats compared to other groups. These observations showed that TXA_2 receptor significantly mediates endothelium-dependent contraction.

CHAPTER 5.0

CONCLUSION

5.1 Study conclusion

In this study, endothelium-dependent relaxation was significantly reduced in early diabetes at 2 week after induction. In endothelium-dependent relaxation, all the vasodilators investigated (NO, prostacyclin and EDH) contributed to relaxations in both normal and diabetic rats. NO is the predominant contributor of endothelium-dependent relaxation followed by prostacyclin and EDH. A trend of reduced of NO-mediated relaxation was seen in diabetic 2-week rats; significant reduction in NO-mediated relaxation was observed in diabetic 10-week rats. Impairment in prostacyclin and EDH-mediated relaxations were also seen in diabetic 10-week rats although no change was observed in 2 weeks diabetic rats. This shows that NO-mediated relaxation is impaired very early in diabetes compared to other vasodilators. In molecular studies, the expression and distribution of eNOS, IP receptor and PGIS were reduced in diabetic 10-week rats which are in line with findings of functional study. Endothelium independent-relaxation which indicates smooth muscle function was impaired in late diabetes (10 week rats) indicating that endothelium-independent relaxation was still preserved in early diabetes, but started to be compromised with prolonged diabetes.

In this study, at 10 weeks diabetes, increased endothelium-dependent contraction was seen indicating that more vasoconstriction responses occur with the progression of diabetes. Functional study showed participation of COX-1 and TXA_2 receptor in mediating endothelium–dependent contraction in all the groups. COX-2 was seen to mediate endothelium-dependent contraction in all the groups except in

normal 2-week rats. Thus it appears that endothelium-dependent contraction in young normal adult rats does not involved COX-2 mediated contraction. Endothelium-dependent contractions mediated by COX-1, COX-2 and TXA_2 receptor were seen increased in diabetic 10-week rats. Prolonged diabetes showed upregulation (increased expression) of COX-2 enzyme and TXA_2 receptor, as shown by increased expression of microvascular tissue COX-2 enzyme and TXA_2 receptor, which supports the functional observation of increased TXA_2 receptor and COX-2 mediated contractions in diabetic 10-week rats.

Overall from this study, the productions of individual vasodilators were decreased with diabetes progression. Increase in endothelium-dependent contraction was seen, where this contraction is mainly mediated by COX- products and involved TXA_2 activation. This study showed that prolonged diabetes worsened the imbalance between vasodilators and vasoconstrictor which favours vasoconstriction and eventually leads to impairment in microvascular endothelial function. Over prolonged diabetes, smooth muscle function was also seen impaired.

5.2 Study Novelty and Possible Clinical Implication

5.2.1 Endothelium-dependent relaxation

As far as known, this study would be the first study reporting on the early (2 weeks) and late effects (10 weeks) of diabetes on endothelial function in the tail artery, which represent the microcirculation of rats. Findings from this study show that endothelium-dependent relaxation is affected early in diabetes, compared to endothelium-dependent contraction. However, over time, endothelium-dependent contraction is also affected. Endothelium-dependent relaxation is worsened with progression of diabetes, which is associated with significant reduction in eNOS expression.

5.2.2 Endothelium-dependent contraction

As far as known, this study is the first that showed COX-1 and COX-2 enzymes and TXA_2 receptor played significant roles in endothelium-dependent contraction in microvasculature of diabetes rats, during early and late diabetes. Augmented responses in COX-1, COX-2 and TXA_2-mediated contractions were seen in late diabetes indicating that COX-mediated products are involved as vasoconstrictor which worsened endothelial function. This is associated with trend of increased vascular expressions in COX-2 enzyme and TXA_2 receptor. Increased inflammation may be one cause of increased endothelium-mediated contraction in prolonged diabetes; as COX-2 expression in vascular tissue is increased in late diabetes.

5.2.3 Possible Clinical Implication

The use of tail arteries, which has been reported similar to distal blood circulation of human limbs and digits in terms of the anatomy and reactivity profile, may suggest similarities to the microcirculation at the peripheral arteries in diabetes. From our study, impairment in endothelium-dependent relaxation with trend of increased contraction was seen early in diabetes indicating endothelium dysfunction occurs very early in diabetes. As the diabetes duration increases, impairment in endothelial function worsened with abolished response in relaxation observed together with augmentation in endothelium-dependent contractions. Therefore, early management of diabetes would be useful to prevent and ameliorate early endothelial dysfunction and its progression to clinical vasculopathy. In this study, endothelium-dependent relaxation was predominantly mediated by the vasodilator NO. Interventions that restore the endothelium relaxing factor NO such as life style modification which include exercising (Silva Aguiar *et al.*, 2018) should be encouraged. Findings from this study also showed that endothelium-dependent contraction is mainly mediated by COX products and TXA_2-receptor. Future studies exploring ways / interventions to reduce the vasoconstrictor effects of COX, and TXA_2 receptors without affecting other functions should be explored to improve imbalances between EDRF and EDCF in diabetes.

5.3 Study Limitations and Future Research

In this study, not all the prostanoids (PGD_2, PGF_2, and PGE2), oxidative stress and other vasoconstrictors such as endothelin and angiotensin were able to be studied due to the budget constraints and the enormous amount of work and animals involved. These could be performed in future studies to get a clearer picture on the contribution of each vasoconstrictor in mediating endothelial mediated contractions in small vessels of diabetes.

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APPENDIX A

(ANIMAL ETHICS APPROVAL)

USM
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UNIVERSITI SAINS MALAYSIA
DITERIMA
01 APR 2015
JABATAN FARMAKOLOGI
PUSAT PENGAJIAN SAINS PERUBATAN

Jawatankuasa Etika Haiwan USM (JEHUSM)
Animal Ethics Committee USM (AECUSM)

Kampus Induk:
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Pulau Pinang
Kampus Kesihatan:
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W : www.research.usm.my

Date : 30th March 2015

Prof Aida Hanum Ghulam Rasool,
Department of Pharmacology,
School of Medical Sciences,
Universiti Sains Malaysia, Health Campus,
16150 Kubang Kerian,
Kota Bharu, Kelantan.

Dear Prof.,

Animal Ethics Approval

Project title (632) : Understanding Development of Vasculopathy in Diabetic Microcirculation – Role of Endothelial Prostanoids, Prostacyclin and Nitric Oxide.

The Animal Ethics Committee USM held its meeting on the 17th February 2015 and has approved the above research project.

No. of Animal Ethics Approval: USM / Animal Ethics Approval / 2015 / (95) (632)

Title	: Understanding Development of Vasculopathy in Diabetic Microcirculation – Role of Endothelial Prostanoids, Prostacyclin and Nitric Oxide.
Research Centre	: 1. Animal Research and Service Centre (ARASC), USM (Health Campus) 2. Animal Room in Pharmacology Laboratory, PPSP
Duration	: April 2015 – June 2017
Number of Samples	: 104 Sprague Dawley Rats [Male]
Name of Principal Investigator	: Prof Aida Hanum Ghulam Rasool
Co-Investigator	: Dr Rapeah Suppian Seetha Munisamy Mariana Ibrahim

The following items (X) were received and reviewed in connection with the above study to be conducted by the investigator.

( ) Copy of Proposal	(Date : -)
(X) Animal Ethics Committee Approval Application Form	(Date : Sept 2014)
(X) Reviewer's Comment Form	(Date : Feb 2015)
(X) Reply for Clarification Letter	(Date : March 2015)

UNIVERSITI SAINS MALAYSIA

Jawatankuasa Etika Haiwan USM (JEHUSM)
Animal Ethics Committee USM (AECUSM)

Kampus Induk:
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W : www.research.usm.my

Date : 7 January 2014

Prof Dr Aida Hanum Ghulam Rasool
School of Medical Sciences
16150 Kubang Kerian,
Kota Bharu, Kelantan.

Dear Prof.,

Animal Ethics Approval

Project title (498) : Role of Endothelial Contracting Factors in Microcirculatory Dysfunction of Streptozotocin induced Diabetic Rats

The Animal Ethics Committee USM held its 90th meeting on the 11th December 2013 and has approved the above research project.

No. of Animal Ethics Approval: USM / Animal Ethics Approval / 2013 / (90) (498)

Title : Role of Endothelial Contracting Factors in Microcirculatory Dysfunction of Streptozotocin induced Diabetic Rats

Research Center : Animal Research and Service Centre (ARASC), USM (Health Campus)

Duration : Jan 2014 – Dec 2016

Number of Samples : 40 Sprague Dawley Rats [Male]

Name of Principal Investigator : Prof Dr Aida Hanum Ghulam Rasool

Co-Investigator : Dr Rapeah Suppian
Seetha A/P Munisamy
Siti Safiah Bt Mokhtar

The following items (X) were received and reviewed in connection with the above study to be conducted by the investigator.

(X) Copy of Proposal	(Date : Oct 2013)
(X) Animal Ethics Committee Approval Application Form	(Date : Oct 2013)
(X) Reviewer's Comment Form	(Date : Dec 2013)
( ) Reply for Clarification Letter	(Date : -)

APPENDIX B

(PRESENTED ABSTRACT, PAPERS AND AWARD)

Methodology: HTM cells were divided into ten groups: Group 1 was cultured in Dulbecco's modified Eagle's medium (DMEM) only; group 2 in DMEM with dexamethasone 10^{-7} M dissolved in 0.1% DMSO. Groups 3 to 10 were co-treated with dexamethasone 10^{-7} M and losartan potassium or enalaprilat dehydrate in concentrations of 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} M for both drugs. All groups were incubated for 7 and 14 days. MTS assay was done to exclude cytotoxic effect, and immunocytochemistry was performed to visualize the extent of extracellular matrix (ECM) deposition. **Results:** None of the groups revealed reduction in cell viability. Dexamethasone significantly ($p<0.05$) increased deposition of FN (2.43 and 2.92 folds) and α-SMA (2.88 and 3.24 folds) compared to that by DMEM treated cells at both time points, respectively. Co-treatment with both the tested RAS inhibitors abolished the effects of dexamethasone on ECM deposition by decreasing production of FN and α-SMA by all four tested concentrations at both time points. The maximum reduction of FN and α-SMA production was 1.82 and 2.62 folds ($p<0.05$) in enalaprilat treated group and 1.93 and 1.95 folds ($p<0.05$) in the group treated with losartan, respectively. **Conclusion:** RAS inhibitors significantly decreased dexamethasone-induced deposition of both FN and α-SMA in all tested concentrations. The effect was more prominent on day 14 of co-treatment.

OA14

UNDERSTANDING DEVELOPMENT OF VASCULOPATHY IN DIABETIC MICROCIRCULATION – ROLE OF ENDOTHELIAL PROSTANOIDS, PROSTACYCLIN, EDHF AND NITRIC OXIDE.

Seetha Munisamy, Siti Safiah Mokhtar, Aida Hanum Ghulam Rasool
Department of Pharmacology, School of Medical Sciences, Universiti Sains Malaysia, 16150, Kota Bharu, Kelantan

Objective: To compare whether duration of diabetes affects the role of individual endothelial-derived relaxing factors (EDRF) and endothelial dependent contraction (EDCF) in microcirculation of streptozotocin – induced diabetic rats at 2 weeks and 10 weeks. **Methodology**: Thirty male Sprague-Dawley rats were induced diabetes using streptozotocin (60 mg/kg) intraperitoneally. They were equally divided into two groups: diabetic 2 weeks and diabetic 10 weeks. Rat tail arteries were collected two or ten weeks after the diabetes induction to study the relaxation and contractions of small resistance arteries in vitro using wire myography. The contributions of individual EDRF [nitric oxide, prostacyclin and endothelial derived hyperpolarizing factors (EDHF)] and the response of endothelial dependent contractions (EDCF) were evaluated. **Results**: ACh induced relaxations were significantly reduced in diabetic 10 weeks compared to diabetic 2 weeks rats [R_{max}; 58.84 ± 4.86 vs 73.49 ± 2.85 %, p=0.015]. Incubation with the combination of non-selective cyclooxygenase (COX) inhibitor (indomethacin) and potassium channel blockers (30 mMKCl ,TRAM 34 and UCL 1684) revealed that NO-mediated relaxation was diminished significantly in diabetic 10 weeks rats compared to diabetes 2 weeks [R_{max}; 31.31 ± 4.71% vs 53.67 ± 4.65, p=0.002]. Similarly, reduced prostacyclin-mediated relaxation was also observed in diabetic 10 weeks [R_{max}; 20.38 ± 4.64 % vs 35.10 ± 3.63 %, p=0.018] when the vessels were incubated with NO synthase inhibitor (LNAME) and potassium channel blockers (30 mM KCl ,TRAM 34 and UCL 1684). There was a trend of lower EDHF – mediated relaxation (artery incubated with indomethacin and LNAME) in diabetic 10 weeks compared to diabetic 2 weeks [R_{max}; 19.81 ± 4.72 % vs 30.12 ± 3.56 %, p= 0.092]. Significantly higher EDCF was seen in diabetic 10 weeks [E_{max}; 113.73 ± 13.25% vs 71.8 ± 11.88 %, p=0.026] compared to diabetic 2 weeks. **Conclusion**: ACh-induced relaxations were significantly lower in 10 weeks diabetic rats compared to 2 weeks diabetic rats. All the relaxations mediated by individual EDRF factors were lower with augmented response in EDCF were seen indicating prolonged diabetes impaired the endothelial functions.

OA15

NEUROPROTECTIVE EFFECT OF TAURINE ON ENDOTHELIN-1-INDUCED RETINAL GANGLION CELL [illegible]

CERTIFICATE

OF AWARD

This is to certify that

Seetha MuniSamy
Siti Safiah Mokhtar
Aida Hanum Ghulam Rasool

has won the

1ST PRIZE ORAL PRESENTATION

at

31st SCIENTIFIC MEETING OF
MALAYSIAN SOCIETY OF PHARMACOLOGY & PHYSIOLOGY (MSPP) 2017

Professor Dr. Aida Hanum Ghulam Rasool
Chairperson
31st Scientific Meeting of MSPP 2017

Associate Professor Dr. Rahimah Zakaria
Head of Scientific Committee
31st Scientific Meeting of MSPP 2017

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LIST OF PUBLICATIONS, PRESENTATIONS AND AWARD

Publications

1. **Munisamy S,** Mokhtar SS, Low JH, Rasool AH, Vitamin D, inflammation and atherosclerosis, Tropical Journal of Pharmaceutical Research (submitted and accepted for publication)(IF=0.569).

Presentation

1. Munisamy S, Mokhtar S.S & Rasool, A. H . "Understanding development of vasculapathy in diabetic microcirculation- role of endothelial prostanoids, prostacyclin, EDH and nitric oxide (oral presentation)" at the 31th Scientific Meeting of Malaysian Society of Pharmacology and Physiology 2017.

Awards

2. First prize oral presentation at the 31st Scientific Meeting of Malaysian Society of Pharmacology and Physiology 2017. Tittle: Understanding development of vasculapathy in diabetic microcirculation- role of endothelial prostanoids, prostacyclin, EDH and nitric oxide.