EFFECTS OF VITAMIN D ON VASCULAR FUNCTION AND OXIDATIVE STRESS IN THE MICROCIRCULATION OF DIABETICS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACh	Acetylcholine
BH_4	Tetrahydrobiopterin
BK_{Ca}	Large conductance calcium-activated potassium channels
BMI	Body mass index
Ca^{2+}	Calcium ions
CaI	Calcium ionophore
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
cGMP	Cyclic guanosine monophosphate
COX	Cyclooxygenase
CVD	Cardiovascular diseases
DBP	Vitamin D-binding protein
DM	Diabetes mellitus
EC	Endothelial cells
EDCF	Endothelium-derived contracting factors
EDH	Endothelium-dependent hyperpolarization
EDHF	Endothelium-derived hyperpolarizing factor
EDRF	Endothelium-derived relaxing factors
ER	Endoplasmic reticulum
eNOS	Endothelial nitric oxide synthase
FAD	Flavin adenine dinucleotide
FBG	Fasting blood glucose
FMD	Flow-mediated dilation
FMN	Flavin mononucleotide
GDM	Gestational diabetes mellitus

GSH	Glutathione
GSH-Px	Glutathione peroxidase
GTP	Guanosine triphosphate
H_2O_2	Hydrogen peroxide
HbA_{1c}	Glycated haemoglobin
IDF	International Diabetes Federation
IMT	Intima-media thickness
iNOS	Inducible nitric oxide synthase
IP	Prostacyclin receptor
IP_3R	Inositol-1,4,5-triphosphate receptor
IRAG	IP_3R-associated cGMP kinase substrate
K^+	Potassium ions
KCl	Potassium chloride
LDL	Low-density lipoprotein
$_L$-NAME	L-NG-Nitroarginine methyl ester hydrochloride
MDA	Malondialdehyde
MLCK	Myosin light chain kinase
NADPH	Nicotinamide adenine dinucleotide phosphate
NHMS	National Health and Morbidity Survey
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
PE	Phenylephrine
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin
PGIS	Prostacyclin synthase
PKA	Protein kinase A

PKG	Protein kinase G
PTH	Parathyroid hormone
RIPA	Radioimmunoprecipitation assay
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SB	Salbutamol
SBP	Systolic blood pressure
SERCA	Sarcoplasmic/endoplasmic reticulum calcium ATPase
sGC	Soluble guanylyl cyclase
SNP	Sodium nitroprusside dehydrate
SOD	Superoxide dismutase
STZ	Streptozotocin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TBARS	Thiobarbituric acid reactive substances
TXA_2	Thromboxane A_2
VSMC	Vascular smooth muscle cells
VDR	Vitamin D receptors
WHO	World Health Organization
25(OH)D	25-hydroxyvitamin D
$1,25(OH)_2D$	1,25-dihydroxyvitamin D

KESAN VITAMIN D KE ATAS FUNGSI VASKULAR DAN TEKANAN OKSIDATIF SALUR DARAH KECIL DIABETES

ABSTRAK

Diabetes menyumbang kepada komplikasi salur darah besar dan kecil lalu menyebabkan penyakit kardiovaskular. Kekurangan vitamin D berhubung kait dengan pembentukan komplikasi kardiovaskular diabetes. Kajian ini dibahagikan kepada dua bahagian, iaitu kajian haiwan dan kajian manusia. Kajian haiwan ini bertujuan untuk mengkaji (a) peranan kekurangan vitamin D ke atas fungsi lapisan endotelium dan otot licin salur darah kecil tikus normal dan diabetes; (b) kekurangan vitamin D ke atas perubahan dalam ekspresi enzim eNOS dan parameter tekanan oksidatif dalam tisu salur darah kecil; (c) sama ada pembekalan tambahan calcitriol secara oral dapat membaik pulih gangguan fungsi salur darah kecil tikus yang kekurangan vitamin D. Kajian manusia ini bertujuan untuk mengkaji peranan kekurangan vitamin D ke atas tahap tekanan oksidatif dalam tisu arteri subkutin pesakit kencing manis. **Kajian haiwan:** (a) Tikus normal dibahagikan kepada tiga kumpulan dengan 10 ekor tikus setiap kumpulan: (i) tikus normal yang dibekalkan makanan biasa selama 10 minggu (Kumpulan NC), (ii) tikus normal yang dibekalkan makanan yang kekurangan vitamin D selama 10 minggu (Kumpulan ND), (iii) tikus normal yang dibekalkan makanan yang kekurangan vitamin D selama 10 minggu, tambahan pula dengan pembekalan tambahan calcitriol secara oral selama empat minggu, bermula dari minggu ketujuh (Kumpulan NDS). (b) Tikus diabetes aruhan streptozotosin dibahagikan kepada tiga kumpulan dengan 10 ekor tikus setiap kumpulan: (i) tikus diabetes yang dibekalkan makanan biasa selama 10 minggu (Kumpulan DC), (ii) tikus diabetes yang dibekalkan makanan yang kekurangan vitamin D selama 10 minggu (Kumpulan DD), (iii) tikus

diabetes yang dibekalkan makanan yang kekurangan vitamin D selama 10 minggu, tambahan pula dengan pembekalan tambahan calcitriol secara oral selama empat minggu, bermula dari minggu ketujuh sejak aruhan diabetes (Kumpulan DDS). Selepas 10 minggu, tikus dimatikan lalu arteri mesenterika diasingkan untuk menjalankan kajian fungsi vaskular dengan menggunakan miograf dawai. Ekspresi protein eNOS dalam tisu arteri mesenterika ditentukan dengan menjalankan pemblotan Barat. Imunohistokimia dijalankan untuk mengesan kehadiran dan penempatan enzim eNOS dalam arteri mesenterika tikus. Tahap penanda biologi MDA dan SOD dalam tisu arteri mesenterika, gula darah berpuasa (FBG), tahap vitamin D dan kalsium dalam darah turut diukur. **Kajian manusia*:*** Pesakit kencing manis dikategorikan kepada dua kumpulan mengikut tahap vitamin D: (i) pesakit kencing manis yang tidak mengalami kekurangan vitamin D (Kumpulan DNP, n = 10) dan (ii) pesakit kencing manis yang mengalami kekurangan vitamin D (Kumpulan DDP, n = 13). Tahap penanda biologi MDA dan SOD dalam tisu arteri subkutin pesakit diukur. **Hasil kajian haiwan:** (a) Tikus normal. Pengembangan berperantara-endotelium kepada acetilkolin (ACh) telah dilemahkan secara ketara dalam arteri mesenterika daripada Kumpulan ND. Pengurangan tahap penanda biologi SOD dan ekspresi protein eNOS turut dijumpai dalam Kumpulan ND. Namun begitu, pembekalan tambahan calcitriol tidak menunjukkan peningkatan yang ketara dalam parameter tersebut. Pengecutan berperantara-endotelium kepada kalsium ionofor (CaI) dipertingkatkan secara ketara dalam arteri mesenterika daripada Kumpulan NDS. Peningkatan tahap kalsium turut dijumpai dalam Kumpulan NDS. (b) Tikus diabetes. Pengembangan berperantara-endotelium aruhan-ACh telah dilemahkan secara ketara dalam arteri mesenterika daripada Kumpulan DD. Pengurangan tahap penanda biologi SOD dan ekspresi protein eNOS, dan peningkatan tahap penanda biologi MDA turut

dijumpai dalam Kumpulan DD. Kerosakan ini telah berjaya dibaik pulih oleh pembekalan tambahan calcitriol. Peningkatan pengecutan berperantara-endotelium aruhan-CaI dan pengurangan pengembangan tidak berperantara-endotelium kepada natrium nitroprusid (SNP) dijumpai dalam Kumpulan DD. Namun begitu, pembekalan tambahan calcitriol tidak dapat membaik pulih kerosakan tersebut. Pengembangan tidak berperantara-endotelium kepada salbutamol (SB) dan pengecutan kepada phenylephrine (PE) dan juga parameter umum seperti perubahan berat badan dan tahap FBG tidak menunjukkan perbezaan dalam semua kumpulan kajian yang melibatkan tikus normal dan diabetes. **Hasil kajian manusia:** Peningkatan tahap MDA yang ketara dijumpai dalam tisu arteri subkutin daripada Kumpulan DDP. Namun begitu, tahap penanda biologi SOD dalam Kumpulan DDP menunjukkan trend berkurangan (p = 0.072) berbanding dengan Kumpulan DNP. Kesimpulannya, kajian ini menunjukkan keadaan kekurangan vitamin D telah melemahkan fungsi lapisan endotelium salur darah kecil tikus biasa dan juga tikus diabetes. Kerosakan tersebut berkemungkinan disebabkan oleh pengurangan penglibatan nitrik oksida yang berhubung kait dengan kekurangan ekspresi protein eNOS dan peningkatan tekanan oksidatif. Kekurangan vitamin D pada tikus diabetes turut melemahkan fungsi lapisan otot licin salur darah kecil. Kajian ini juga menunjukkan pembekalan tambahan calcitriol kepada tikus diabetes yang kekurangan vitamin D membaik pulih pengembangan berperantara-endotelium dengan meningkatkan ekspresi protein eNOS dan menambah baik status tekanan oksidatif. Pembekalan tambahan calcitriol kepada tikus biasa yang kekurangan vitamin D meningkatkan tahap kalsium lalu menyebabkan peningkatan pengecutan berperantara-endotelium. Di samping itu, kekurangan vitamin D pada pesakit kencing manis turut menunjukkan peningkatan tahap tekanan oksidatif.

EFFECTS OF VITAMIN D ON VASCULAR FUNCTION AND OXIDATIVE STRESS IN THE MICROCIRCULATION OF DIABETICS

ABSTRACT

Diabetes mellitus contributes to macro- and microvascular complications, leading to adverse cardiovascular events. Vitamin D deficiency is associated with the development of diabetes-related cardiovascular complications. This study was divided into two parts: (i) animal study and (ii) human study. This animal study aims to determine the effects of vitamin D deficiency on (a) microvascular endothelial and smooth muscle functions in normal and diabetic rats; (b) the changes to endothelial nitric oxide synthase (eNOS) protein expression and oxidative stress parameters in mesenteric arterial tissue of normal and diabetic rats; (c) to study whether oral calcitriol supplementation is able to ameliorate microvascular dysfunction in vitamin D-deficient rats. This human study aims to evaluate the effects of vitamin D deficiency on oxidative stress status in subcutaneous arteries of diabetic patients. **Animal study:** (a) Male Sprague-Dawley (SD) rats were subdivided into three equal groups of 10 rats each: (i) rats receiving 10-weeks of normal diet (Group NC), (ii) rats receiving 10-weeks of vitamin D-deficient diet (Group ND) and (iii) rats receiving 10-weeks of vitamin D-deficient diet with four weeks of oral calcitriol supplementation, starting from week 7 (Groups NDS). (b) Streptozotocin-induced diabetic male SD rats were subdivided into three equal groups of 10 rats each: (i) diabetic rats receiving 10-weeks of normal diet (Group DC), (ii) diabetic rats receiving 10-weeks of vitamin D-deficient diet (Group DD) and (iii) diabetic rats receiving 10-weeks of vitamin D-deficient diet with four weeks of oral calcitriol supplementation, starting from week 7 of diabetes induction (Groups DDS). At the end of 10 weeks, all rats were sacrificed. Rats'

mesenteric arteries were isolated and dissected to undergo vascular function studies using wire myograph. Protein expression of eNOS in mesenteric arterial tissue was determined using Western blot. Immunohistochemistry was used to detect the presence and localization of eNOS in mesenteric arteries. Superoxide dismutase (SOD) and malondialdehyde (MDA) levels in mesenteric arterial tissue; fasting blood glucose (FBG), serum 25(OH)D and calcium levels in blood were also measured. **Human study:** Diabetic patients were categorised into two groups based on serum 25(OH)D levels: (i) vitamin D non-deficient diabetic patients (Group DNP, n = 10) and (ii) vitamin D-deficient diabetic patients (Group DDP, n = 13). The levels of SOD and MDA in subcutaneous arterial tissue were measured. **Results of animal study:** (a) Normal rats. Endothelium-dependent relaxation to acetylcholine (ACh) was significantly attenuated in mesenteric arteries of vitamin D-deficient rats. Reduced SOD levels and protein expression of eNOS were observed in vitamin D-deficient rats. However, calcitriol supplementation showed no significant improvement in these parameters. Endothelium-dependent contraction to calcium ionophore (CaI) was augmented in vitamin D-deficient rats receiving calcitriol supplementation. Increased calcium levels were also found in calcitriol-supplemented vitamin D-deficient rats. (b) Diabetic rats. ACh-induced endothelium-dependent relaxation was significantly impaired in mesenteric arteries of vitamin D-deficient diabetic rats. Reduced SOD levels and protein expression of eNOS and enhanced MDA levels were found in vitamin D-deficient diabetic rats. These impairments were successfully ameliorated by calcitriol supplementation. Augmented CaI-induced endothelium-dependent contraction and impaired sodium nitroprusside (SNP)-induced endothelium-independent relaxation occurred in vitamin D-deficient diabetic rats. However, calcitriol supplementation failed to show improvement in these vascular responses.

There were no significant differences in endothelium-independent relaxation to salbutamol (SB) and contraction to phenylephrine (PE) as well as in general parameters such as body weight changes and FBG levels between study groups in both normal and diabetic rats. **Results of human study:** Markedly augmented MDA levels were found in subcutaneous arterial tissues of vitamin D-deficient diabetic patients. However, SOD levels in vitamin D-deficient diabetic patients showed the reduced trend ($p = 0.072$) compared to vitamin D non-deficient diabetic patients. In conclusion, this study demonstrated that vitamin D deficiency attenuates microvascular endothelial function in both normal and diabetic rats. The impairment for endothelial function was likely due to the diminished nitric oxide contribution, associated with reduced eNOS protein expression and augmented oxidative stress. Vitamin D deficiency in diabetic rats also impairs vascular smooth muscle function. The study also showed that calcitriol supplementation to diabetic rats with vitamin D deficiency improves endothelium-mediated vasodilation, by upregulating eNOS expression and improving oxidative stress status. However, calcitriol supplementation to normal rats with vitamin D deficiency induces hypercalcaemia, leading to augmented endothelium-dependent contraction. Besides that, vitamin D deficiency in diabetic patients as well showed augmented oxidative stress.

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Diabetes mellitus (DM) is characterised by chronic hyperglycaemia resulting from defective insulin secretion and/or insulin action (Maritim *et al.*, 2003). In recent years, DM is a major threat gaining global concern for its epidemic proportion. The global prevalence of DM has increased tremendously for the past decade; from 285 million (6.6%) in year 2010 to 463 million (9.3%) in year 2019. This figure is predicted to exceed 700 million (10.6%) by year 2045 as predicted by International Diabetes Federation (IDF, 2019). The national prevalence of DM in Malaysia has increased from 15.2% in year 2011 to 18.3% (with 3.6 million reported cases of diabetes) in year 2019 (Ministry of Health Malaysia, 2011; Ministry of Health Malaysia, 2019). National Health and Morbidity Survey (NHMS) has predicted that diabetic patients in Malaysia would increase to 7 million (31.3%) by year 2025 (Ministry of Health Malaysia, 2019).

The chronic nature of DM requires long-term monitoring to minimise related secondary complications. Hence, the management of DM constitutes an ever-increasing proportion of global as well as Malaysia national healthcare budgets. In year 2019, the global annual diabetes treatment cost constituted USD 760 billion, which was equivalent to 10% of global health expenditure. The cost is expected to reach USD 845 billion by year 2045 (IDF, 2019). In Malaysia, the annual expenditure for diabetes treatment was reported to be RM20.9 billion in year 2015 (Ministry of

Health Malaysia, 2015). The economic burden brought by this pandemic disease could undermine the development and advancement of the nation worldwide.

In year 2017, World Health Organization reported that DM ranks seventh in the world top ten leading causes of death (WHO, 2017). IDF also indicated that DM constitutes 11.3% of global all-cause mortality, causing 4.2 million deaths globally in year 2019 (IDF, 2019). Cardiovascular diseases (CVD) were reported to be the major contributor to morbidity and mortality in diabetic patients (Bate and Jerums, 2003); approximately 65-75% of diabetic deaths were attributed to CVD (Moss *et al.*, 1991; Geiss *et al.*, 1995; Shi and Vanhoutte, 2009). The condition of elevated blood glucose levels over a prolonged period exposes diabetic individuals to a significant risk of secondary cardiovascular complications, leading to a two to four-fold increased risk of developing CVD. Hence, the prevention of these diabetes-related cardiovascular complications, occurring at the micro- and macrocirculation, will be effective means in the management of DM (Bate and Jerums, 2003).

Low vitamin D levels have been suggested as one of the risk factors in the development of DM and also adverse cardiovascular events. Low vitamin D levels decrease pancreatic insulin release, underscore insulin resistance, impair insulin sensitivity, deteriorate glucose tolerance, and accelerate coronary calcification, leading to DM and CVD (Zittermann *et al.*, 2007; Holick, 2007; Nemerovski *et al.*, 2009). Lower vitamin D levels were associated with a 40% higher risk of developing DM in women, whereas individuals that developed DM had lower levels of vitamin D compared to non-diabetics (Scragg *et al.*, 2004; Di Cesar *et al.*, 2006). Similarly, the incidences of CVD were doubled in individuals with vitamin D deficiency, indicating a 50% increased risk of developing CVD when compared to subjects with optimal

vitamin D levels (Wang *et al.*, 2008). Reduced vitamin D levels were also observed in subjects with CVD such as myocardial infarction, stroke, peripheral arterial disease and cerebrovascular deaths (Lee *et al.*, 2008; Kendrick *et al.*, 2009).

Since low vitamin D levels bring adverse impacts to human health, the widespread problem of vitamin D deficiency is now raising global attention (Holick, 2007; Lips, 2010). Approximately 40-50% of individuals in Western societies with seasonal changes have vitamin D insufficiency (Lips, 2010; Parva *et al.*, 2018). It is astonishing to discover that this public health issue is also common in tropical countries such as Singapore and Malaysia, where plentiful sunshine is available all year long. Literature reported that 30.0% to 48.0% of men in Singapore (Hawkins, 2013) and 15.5% to 32.7% of men in Malaysia (Chin *et al.*, 2014) were having vitamin D deficiency. The prevalence appears to be more pronounced in the diabetic population (Scragg *et al.*, 2004; Di Cesar *et al.*, 2006), which were reported to be 91.1% in India (Daga *et al.*, 2012), 73.6% in Saudi Arabia (Al-Othman *et al.*, 2012) and 43.0% in Malaysia (Munisamy *et al.*, 2016).

Vitamin D deficiency is closely associated with endothelial dysfunction. Some *in vivo* studies have discovered that normal and diabetic patients with vitamin D deficiency had lower flow-mediated dilation (FMD), which is the standard indicator of endothelial dysfunction (Yiu *et al*., 2013; Malik *et al.*, 2016). Endothelial dysfunction predisposes to a higher risk of diabetes-associated cardiovascular complications that leads to the development of CVD. Hence, endothelial dysfunction is an independent precursor of early atherosclerotic development and a prognosis of CVD (Park *et al.*, 2001; Taddei *et al.*, 2003). The presence of endothelial dysfunction

is not only limited to the macrocirculation, but also occurs in the microcirculation of a variety of vascular beds before the onset of atherosclerosis (Abularrage *et al.*, 2005).

Apart from that, low vitamin D levels have also been reported to induce oxidative stress in both normal and diabetic models (Tarcin *et al.,* 2009; Efterkhari *et al.*, 2014; Foroozanfard *et al.*, 2015). Chronic hyperglycaemia coupled with low vitamin D levels in diabetics predisposes to augmented vascular oxidative stress levels (Halliwell and Gutteridge, 2007). Enhanced oxidative stress levels are due to the disruption in the homeostasis between the generation of reactive oxygen species (ROS) and the effectiveness of antioxidant defence system to scavenge them. Consequently, augmented oxidative stress further propagates ROS generation that subsequently diminishes the antioxidant activities, leading to cardiovascular complications and CVD (Moussa, 2008).

Vitamin D deficiency is involved in the development of diabetes-related secondary complications; improving the suboptimal vitamin D levels by supplementation may have favourable effects on vascular and oxidative stress parameters. In randomised control trials, vitamin D supplementation showed improvement in brachial artery FMD in normal and diabetic patients with vitamin D deficiency (Sugden *et al.*, 2008; Harris *et al.*, 2011). Oxidative stress parameters in normal and diabetic subjects with vitamin D deficiency also improved by vitamin D supplementation (Tarcin *et al.*, 2009; Shab-Bidar *et al.*, 2015).

1.2 Rationale of the Study

The health issue of vitamin D insufficiency and deficiency is highly prevalent globally. Considering the adverse impacts of low vitamin D levels in the development

of cardiovascular complications, particularly in the diabetic population, there is an urgency to study the possible underlying pathology of low vitamin D levels in the development of diabetes-related cardiovascular complications.

Endothelial dysfunction is an important prognostic marker for cardiovascular complications; hence the assessment of endothelial function is a useful measure commonly used in vascular function study. To our best knowledge, the evidence on the effects of vitamin D deficiency is limited to the study performed on the diabetes macrocirculation, yet the effects in the microcirculation of diabetics remain poorly understood. Since microcirculation is the initial site where the early manifestation of vascular dysfunction can be noticed before macrovascular dysfunction, conducting a study on endothelial function in the microcirculation of vitamin D-deficient diabetics allows early assessment of vascular functional abnormalities.

Based on this rationale, the present study was designed to investigate the effects of vitamin D deficiency on microvascular function, which consisted of endothelial and smooth muscle responses, in normal and streptozotocin (STZ)-induced diabetic rats. This study also assessed whether the vascular functional abnormalities attributed to vitamin D deficiency involved alterations in the protein expression and localization of endothelial nitric oxide synthase (eNOS) in the microvascular tissue of rats with vitamin D deficiency. eNOS is the enzyme involved in the synthesis of nitric oxide (NO), the major endothelium-derived relaxing factor.

To date, there is no related information available on the effects of vitamin D deficiency on oxidative stress status in the microcirculation of diabetics as a possible mechanism accounting for microvascular dysfunction. Hence, this consideration leads to the assessment on the levels of oxidative stress parameters which include superoxide

dismutase (SOD, an antioxidant enzyme) and malondialdehyde (MDA, the product of lipid peroxidation) in the microvascular tissue of normal and diabetic rats with different vitamin D status. This study also investigated the potential of calcitriol supplementation to reverse or alleviate the adverse effects attributed to low vitamin D levels on microvascular function and microvascular tissue oxidative stress levels. Calcitriol is the active metabolite of vitamin D, which does not need to undergo hydroxylation processes in the liver and kidney for the conversion, plays its physiological roles as a hormone by binding to the receptors.

Unfortunately, insufficient human tissue samples and the difficulties faced in obtaining viable tissue samples do not allow the present study to look into the effects of vitamin D deficiency on vascular function in the microcirculation of diabetic patients. However, this study was able to investigate the effects of vitamin D deficiency on the levels of oxidative stress parameters in microvascular tissue of diabetic patients to assess if there are similarities in the findings of microvascular tissue oxidative stress parameters between animal and human diabetic models.

The findings could provide a better understanding of the effects of vitamin D deficiency on microvascular function and oxidative stress status in normal and diabetic models. This study could also suggest the possible contributing factors leading to microvascular dysfunction in vitamin D-deficient diabetic models. The potential of vitamin D supplementation in improving any impairment in microvascular function and oxidative stress status in vitamin D-deficient diabetic rats was also studied. These may help in combating microvascular complications that predispose to a significant cardiovascular risk in diabetics and facilitate the development of effective therapeutic strategies in diabetes management and treatment.

1.3 Objectives of the Study

General Objectives:

Animal Study: To investigate the effects of vitamin D deficiency on vascular function and tissue oxidative stress levels in the microcirculation of normal and diabetic rats.

Human Study: To examine the effects of vitamin D deficiency on tissue oxidative stress levels in the microcirculation of diabetic patients.

Specific Objectives:

1. To investigate the effects of vitamin D deficiency on microvascular endothelium-dependent and independent relaxing and contracting abilities in mesenteric arteries of normal and STZ-induced diabetic rats.

2. To examine the effects of vitamin D deficiency on oxidative stress parameters (MDA and SOD) in mesenteric arterial tissue of normal and STZ-induced diabetic rats.

3. To study the alterations in the protein expression and localization of eNOS in mesenteric arteries of vitamin D-deficient normal and diabetic rats.

4. To evaluate any reversibility in microvascular function and oxidative stress levels with vitamin D supplementation among vitamin D-deficient rats.

5. To determine the effects of vitamin D deficiency on oxidative stress parameters (MDA and SOD) in subcutaneous arterial tissue of diabetic patients.

CHAPTER 2

LITERATURE REVIEW

2.1 Diabetes Mellitus

2.1.1 Definition and Classification

Diabetes mellitus (DM) is a metabolic disorder manifested by elevated blood glucose levels. This metabolic disorder is due to the failure of the body to produce or respond to insulin (American Diabetes Association, 2008), resulting in abnormal metabolism of carbohydrate, protein and fat (Shi and Vanhoutte, 2009). There are three major types of DM according to different underlying aetiologies.

Type 1 diabetes mellitus (T1DM), which is also known as insulin-dependent DM, is a diabetic condition associated with absolute insulin deficiency. It is due to the autoimmune destruction of pancreatic β-cells that disrupts the ability of the pancreas for insulin synthesis, thus leading to insufficient insulin production for glucose metabolism. T1DM only accounts for about 5 to 10% of all DM cases, commonly diagnosed during childhood and adolescence (American Diabetes Association, 2008). Although the actual causes of T1DM remain unknown, the researcher suggested that genetic and environmental factors might be the possible causes (Aathira and Jain, 2014).

Type 2 diabetes mellitus (T2DM), which is also known as non-insulin dependent DM, is initially associated with insulin resistance, progressively leading to insulin deficiency. At the insulin resistance stage, the targeted cell is unable to produce an appropriate response, upon which it should act, to the insulin that binds correctly to

the receptor. Hence, more insulin to be produced to compensate for insulin resistance. Progressively, the amounts of insulin are deficient in meeting further cellular requirements, which is known as relative insulin deficiency, predisposing to the onset of hyperglycaemia. At last, the condition of hyperglycaemia further damages the pancreatic β-cells, resulting in absolute insulin deficiency (Pratley, 2013). T2DM constitutes nearly 90% of the diabetic population (American Diabetes Association, 2008).

Gestational diabetes mellitus (GDM) is a glucose intolerance disorder with first recognition during pregnancy. Insulin-blocking hormones produced by the placenta in pregnant women lead to high blood glucose levels in their body in variable severity. Women affected with gestational diabetes have a seven-fold increased risk of developing T2DM in the few years following initial diagnosis. Their children are also prone to develop T2DM early in life (Bellamy *et al.*, 2009; Rayanagoudar *et al.*, 2016).

2.1.2 Diagnosis, Symptoms and Medications

Glycated haemoglobin (HbA_{1c}) and fasting blood glucose (FBG) tests are among the most common tests used to diagnose DM. HbA_{1c} indicates the average blood glucose levels for the past three months while FBG indicates the blood glucose levels after overnight fasting. HbA_{1c} levels of 6.5% or higher and FBG levels of 7.0 mmol/L and above is diagnosed as DM (IDF, 2017).

Besides conducting a specific test for DM diagnosis, recognising noticeable diabetic symptoms is also essential to catch DM at an early stage. Frequent urination, excessive thirst, increased appetite, unusual fatigue, sudden weight loss, nausea, dizziness and slow in wound healing are among the most common signs to be spotted

with diagnosed DM. Undiagnosed and untreated DM could lead to the development of severe vascular complications and even premature death (IDF, 2017).

Oral medication and/or insulin therapy sometimes are required, especially when a strict diet and exercise plan is unable to keep the condition of hyperglycaemia in diabetics under control. Biguanides (metformin) is prescribed for T2DM as first-line treatment to lower glucose generation and improve the body's sensitivity to insulin. Sulfonylureas (gliclazide, glimepiride) and meglitinides (repaglinide, nateglinide) stimulate the body to secrete more insulin. Insulin therapy is normally the last resort if glycaemic goals in T2DM patients are not met (Ministry of Health Malaysia, 2011).

2.1.3 Diabetes-related Vascular Complications

The condition of chronic hyperglycaemia in DM gradually leads to the complications of diabetic vasculopathy that damage many organs in a vascular system. The complications of diabetic vasculopathy are commonly grouped into macrovascular and microvascular complications. Macrovascular complications include coronary vascular disease, cerebrovascular disease and peripheral vascular disease while microvascular complications comprise of diabetic nephropathy (kidney damage), diabetic neuropathy (nerve damage) and diabetic retinopathy (eye damage) (Forbes and Cooper, 2013).

The management of these complications is cost and time consuming, not only to the patients and families but also to the health authorities and the nation. Without proper management, these complications lead to the development of adverse

cardiovascular events. CVD contribute to morbidity and mortality in diabetics, leaving them with diminished life quality and reduced life expectancy (Szerafin *et al*., 2006).

2.2 Vascular System

The vascular system plays a prominent role in delivering vital nutrients, hormones and oxygen to body cells as well as removing metabolic wastes from all parts of the body to maintain cellular homeostasis. There are three major types of blood vessels in the vascular system; namely arteries, veins and capillaries. Arteries carry oxygenated blood away from the heart to other parts of the body, while veins return deoxygenated blood collected from other parts of the body to the heart. Capillaries are the interchange medium that facilitates the exchange of materials between blood and tissues (Pugsley and Tabrizchi, 2000).

Artery and vein are slightly different in specific characteristics to serve their particular functions as peripheral resistance and capacitance blood vessels respectively. An artery has a thicker wall to withstand a higher blood pressure whereas a vein has a bigger lumen to accommodate a higher blood volume. However, both vessels exhibit a similar general structure. Their vessel walls consist of three distinct layers; tunica adventitia, tunica media and tunica intima. The outermost layer, tunica adventitia consists of collagen fibres and connective tissues, interlaced with the vasa vasorum and nervi vasorum. Tunica media as the middle layer composes of vascular smooth muscle cells (VSMC) and circularly arranged elastic fibres. It is separated from tunica adventitia and tunica intima respectively by the external and internal elastic lamina, which is a group of thick elastic tissue. The innermost layer, tunica intima is a

single layer of endothelial cells (EC) surrounded by a thin layer of subendothelial connective tissue that lines the lumen (Figure 2.1).

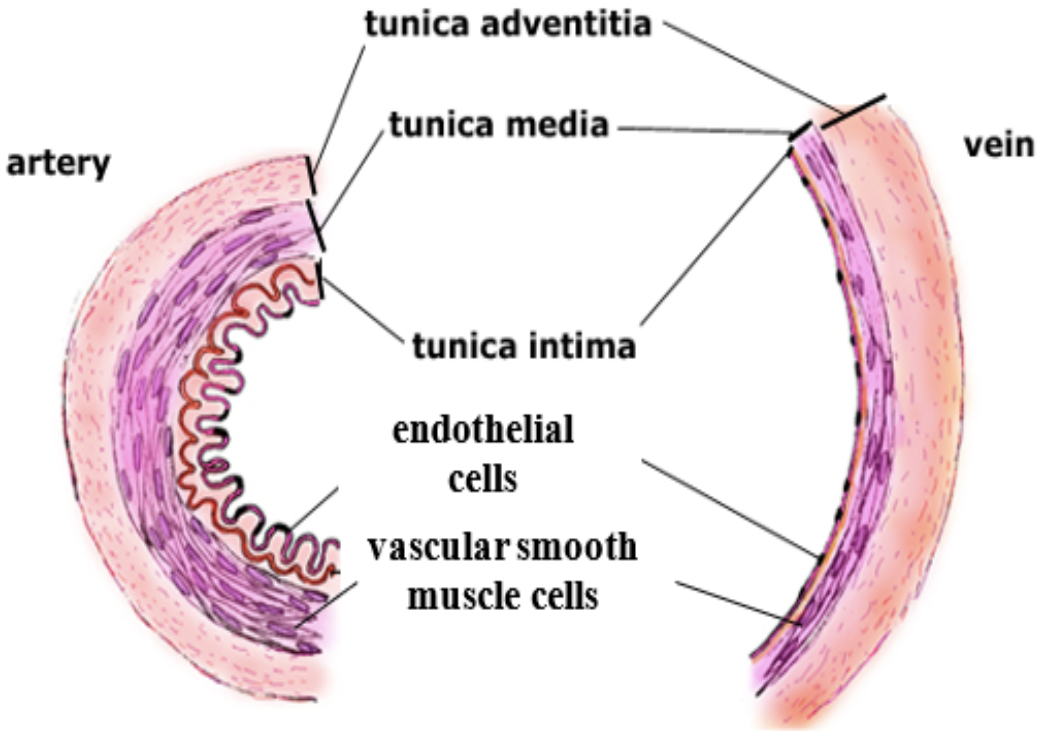


Figure 2.1 Structure of the blood vessel.

2.2.1 Microcirculation

The microcirculation refers to the blood circulation in the microvasculature present within the organ tissues. Microvasculature composes of a branching network of microvessels classified as arterioles (10-100 μm), capillaries (5-8 μm) and venules (10-200 μm). Arterioles carry oxygenated blood to the capillaries, and the blood flows out of the capillaries into the venules. The vital function of microcirculation is to supply adequate tissue perfusion with nutrients and oxygen in response to demand (Levy *et al.*, 2001; Sandoo *et al.*, 2010).

Small arteries in microcirculation are responsible for providing tissue perfusion to the peripheral organs according to their metabolic demand (Christensen and Mulvany, 2001; Gutterman *et al.*, 2016). Thus, small arteries adapt their diameter via vasoconstriction or vasodilation to regulate blood flow to the peripheral organ. This specific functional property makes small arteries important in the regulation of overall peripheral resistance to blood flow, which accounts for 70-90% of the systemic arterial pressure (DeLano *et al.*, 1991; Christensen and Mulvany, 2001; Levy *et al.*, 2001).

Since the microcirculation is an important site to investigate vascular health (Mulvany and Aalkjaer, 1990), assessing structural and functional changes in the microcirculation, particularly in small arteries, has become an area of interest for studies involving a variety of pathological states, such as DM, obesity and hypertension (Levy *et al.*, 2001; Dokken, 2008; Al-Tahami *et al.*, 2011). In the present study, small mesenteric arteries in normal and diabetic rats were used as the resistance artery to study microvascular function. This artery has been suggested as a potential representative artery to study microvascular function that may herald the development of CVD at an early stage (Rizzoni *et al.*, 2001; Ang *et al.*, 2002; Abularrage *et al.*, 2005; Georgescu *et al*., 2011; Yiu *et al.*, 2013).

2.3 Vascular Function

The vascular system plays an important function in regulating vascular tone to provide organ and tissue with the optimum perfusion of nutrient and oxygen for maintaining vascular health. EC and VSMC are the main components in playing this particular vascular function. EC produce and release diffusible vasoactive paracrine

factors for vasoconstriction and vasorelaxation. These factors diffuse to the underlying VSMC for an appropriate response (Furchgott and Zawadzki, 1980).

2.3.1 Endothelial Function

The endothelium is a single layer of EC lining the interior surface of blood vessels and cardiac valves in the entire vascular system. Besides acting as a physical barrier that separates the circulating blood in the lumen from the underlying VSMC in the vessel walls, EC also regulate the passage of materials into and away from the bloodstream (Verma and Anderson, 2002; Galley and Webster, 2004; Rajendran *et al.*, 2013). In addition, EC also involved in regulating cell growth and proliferation, modulating inflammatory responses and platelet activation, controlling leukocyte adhesion and thrombosis, modulating local vascular homeostasis, as well as exhibiting anti-thrombotic activities (Kuvin and Karas, 2003).

Healthy and intact EC produce and release endothelium-derived relaxing factors (EDRF) and endothelium-derived contracting factors (EDCF) to regulate vasodilation and vasoconstriction respectively. In general, EDRF comprise of nitric oxide, prostacyclin and endothelium-derived hyperpolarizing factor while EDCF consist of endothelin I, angiotensin II and thromboxane A_2 (Furchgott and Vanhoutte, 1989; Kuvin and Karas, 2003).

2.3.1(a) Endothelium-derived Relaxing Factors

2.3.1(a)(i) Nitric Oxide

Nitric oxide (NO) is gaining interest owing to its tremendous biological and medical importance, especially its recognised role as a potent endogenous vasodilator

responsible for the vascular tone (Furchgott and Zawadzki, 1980; Moncada and Higgs, 1993). NO is also an anti-atherogenic and anti-inflammatory molecule that suppresses key processes leading to atherosclerosis. NO inhibits platelet and leukocyte adhesion, VSMC proliferation and low-density lipoprotein (LDL) oxidation, as well as suppresses pro-inflammatory cytokines production and expression (Lloyd-Jones and Bloch, 1996; Rubbo and O' Donnell, 2005).

NO synthesis involves the enzymatic action of the nitric oxide synthase (NOS) isozymes (Tousoulis *et al.*, 2012). These isozymes vary in their structures and functions. They are identified as neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). nNOS is expressed primarily in neurons, iNOS is expressed in macrophages, neutrophils, platelets, and VSMC, as well as in non-vascular cells; and eNOS is expressed in endothelial cells (Boettger *et al.*, 2007). Since there is a large difference in the amount generated by these isozymes, nNOS and eNOS are more critical for normal physiology, whereas iNOS is associated with injury (Kaszkin *et al.,* 2004).

Chemical stimuli such as acetylcholine, bradykinin and thrombin or physical stimulus trigger the eNOS activity in an intact vascular endothelium. The stimulation invokes the increase in intracellular calcium ion (Ca^{2+}) concentrations, causes structural changes of calmodulin in the cell cytoplasm, which allows the binding of eNOS for activation (Sandoo *et al.*, 2010). eNOS catalyses the formation of NO (main product) and $_{L}$-citrulline (by-product) from amino acid $_{L}$-arginine, in the presence of co-substrates and cofactors. Co-substrates comprise of nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen (O_2) while cofactors consist of tetrahydrobiopterin (BH_4), heme, flavin mononucleotide (FMN) and flavin adenine

dinucleotide (FAD) (Sandoo *et al*., 2010). Synthesised NO diffuses across EC to the underlying VSMC. NO binds to soluble guanylyl cyclase to activate the conversion of guanosine triphosphate (GTP) to the second messenger, cyclic guanosine monophosphate (cGMP). Increased intracellular cGMP levels activate protein kinase G (PKG) that involved in several mechanisms for vasodilation.

Activated PKG stimulates myosin light chain kinase (MLCK) that dephosphorylates myosin, preventing its binding to actin for vasodilation. The activation of PKG also triggers the opening of large conductance calcium-activated potassium channels in VSMC (Nelson and Quayle, 1995), causing membrane hyperpolarization and the closure of voltage-dependent calcium channels, thus decreasing Ca^{2+} influx to VSMC (Fukao *et al.*, 1999). Besides that, activated PKG promotes vasorelaxation via the phosphorylation of inositol-1,4,5-trisphosphate receptor (IP_3R)-associated cGMP kinase substrate (IRAG) that inhibits the release of Ca^{2+} from the endoplasmic reticulum into the cytosol of VSMC (Schlossmann *et al.*, 2000). These mechanisms, commonly known as NO-mediated cGMP pathway, account for endothelium-dependent relaxation.

Besides the involvement in cGMP-dependent protein kinase activation, NO also directly stimulates the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) in VSMC to facilitate the restoration of cytosolic Ca^{2+} into sarcoplasmic reticulum and reduce Ca^{2+} release from the sarcoplasmic reticulum (Lincoln and Cornwell, 1991). Hence, this cGMP-independent signalling pathway reduces the intracellular Ca^{2+} concentration, leading to vasorelaxation (Cohen and Adachi, 2006).

2.3.1(a)(ii) Prostacyclin

Prostacyclin (PGI_2) is a major prostanoid produced by EC that contributes to vasorelaxation. In healthy individuals, prostacyclin acts similarly as NO in the prevention of platelet aggregation and regulation of vasodilation (de Nucci *et al.*, 1988; Mitchell *et al.*, 2008). However, PGI_2 may contribute to vasoconstriction instead of vasodilation under certain pathological conditions (Vanhoutte *et al.*, 2009).

Hormonal stimuli increase the intracellular Ca^{2+} concentrations that trigger the enzymatic action of phospholipase A_2 to release an intermediate arachidonic acid from the endothelial phospholipids (Mitchell and Warner, 1999). Then, the enzymes cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) catalyse arachidonic acid to form prostaglandin H_2 (PGH_2). PGH_2 acts as a precursor which leads to the production of other prostaglandins by their respective enzymes. PGI_2 produced by prostacyclin synthase (PGIS) in EC, diffuses to the underlying VSMC to bind with prostacyclin receptor (IP) on the cell membrane for subsequent vasodilation (Mitchell and Warner, 2006). Activated IP stimulates the synthesis of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP) in the presence of enzyme adenylyl cyclase. Increased cytosolic cAMP levels activate protein kinase A (PKA) that decreases the intracellular Ca^{2+} concentrations via several mechanisms, leading to vasorelaxation. Activated PKA similarly stimulates MLCK which prevents the binding of myosin to actin, thus promoting vasorelaxation (Somlyo and Somlyo, 2003).

Besides PGI_2, another prostanoid known as thromboxane (TXA_2) is also produced from PGH_2 via the COX pathway. PGI_2 and TXA_2 work as physiological

antagonists to maintain the homeostasis of vascular function. In contrast to PGI_2, TXA_2 promotes vasoconstriction and platelet aggregation. Under normal conditions, PGI_2 counteracts the biological effects of TXA_2 whereas their actions are reversed when blood vessels are severely damaged (Mitchell and Warner, 2006).

2.3.1(a)(iii) Endothelium-derived Hyperpolarizing Factor

Endothelium-dependent hyperpolarization (EDH)-type responses cause vasorelaxation through classical and non-classical pathways by NO- and PGI_2-independent mechanisms (Feletou and Vanhoutte, 2009; Edwards *et al.*, 2010). The classical EDH pathway requires the activation of endothelial small and intermediate conductance calcium-activated potassium channels and the hyperpolarization of the EC, subsequently leads to vasodilation. The non-classical EDH pathway requires the release of EDRF to activate potassium channels by increasing potassium ion (K^+) conductance, evoking the hyperpolarization of the underlying VSMC that leads to vasodilation (Edwards *et al.*, 2010). These factors, which are neither a COX derivative nor NO, have been generally termed as endothelium-derived hyperpolarizing factor (EDHF).

This study aimed to determine the effects of vitamin D deficiency and supplementation on vascular responses involved EC and VSMC in small mesenteric arteries of normal and diabetic rats. Hence, the present study assessed endothelium-dependent relaxation in general and endothelium-independent relaxation in both cGMP and cAMP pathways. Besides, this study also assessed endothelium-dependent contraction and endothelium-independent contraction.

Since NO is the primary vasodilator that contributes significantly to vasodilation over PGI_2 and EDHF (Palmer *et al.*, 1987; Furchgott and Vanhoutte, 1989), any alteration in NO production and its activity indicates that endothelial dysfunction might occur. Hence, quantification of protein expression and localization of eNOS was also conducted in this study via Western blot and immunohistochemistry respectively, to demonstrate the possible underlying mechanisms related to endothelial dysfunction attributed to the impairment in NO production and action.

2.4 Oxidative Stress

Oxidative stress reflects an imbalance between the production of highly reactive species and the effectiveness of antioxidant defence system in our body (Betteridge, 2000). Highly reactive species comprises of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which is subdivided into free radical or non-radical species. Free radicals are the chemical entities that contain unpaired electrons in an atomic orbital. In order to achieve stable electronic configuration, free radicals either act as an oxidant that tends to accept an electron from other molecules or a reductant that tends to donate an electron to other molecules (Cheeseman and Slater, 1993). Hence, they are relatively unstable and highly reactive with a short half-life (Ullah *et al*., 2015).

Superoxide anion ($\bullet O_2^-$), peroxyl ($\bullet RO_2$), hydroxyl radical ($\bullet OH$) and hydroperoxyl ($\bullet HRO_2^-$) are free radical ROS whereas non-radical ROS includes hydrochlorous acid (HOCl) and hydrogen peroxide (H_2O_2). Free radical RNS comprises of nitric oxide ($\bullet NO$) and nitrogen dioxide ($\bullet NO_2^-$) while nitrous oxide (HNO_2), peroxynitrite ($ONOO^-$), and alkyl peroxynitrates (RONOO) are non-radical

RNS (Turko *et al.*, 2001; Evans *et al.*, 2002). The classification of highly reactive species is illustrated in Figure 2.2.

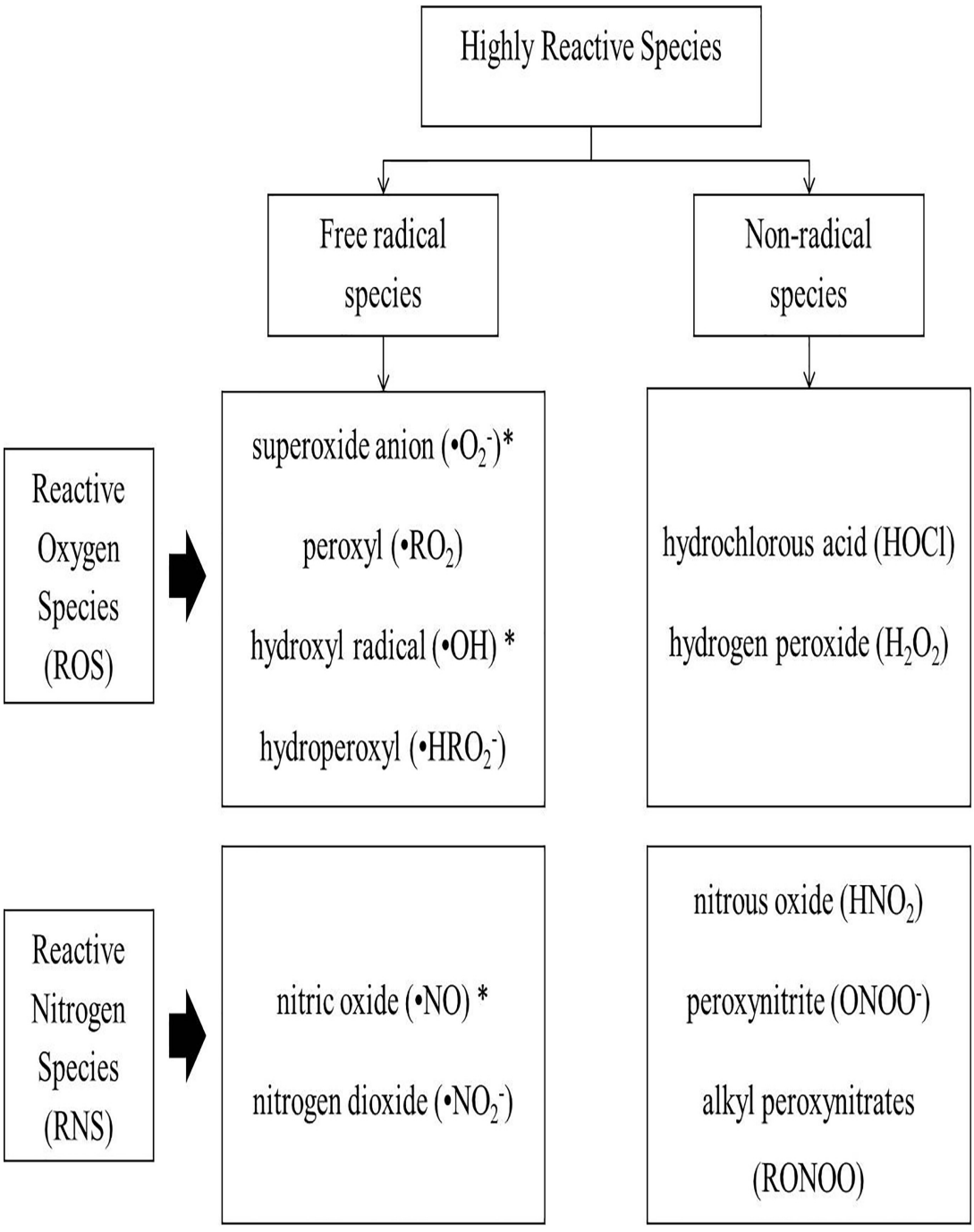


Figure 2.2 Classification of highly reactive species.

(Adapted from Lee *et al.*, 2018)

* indicates free radical species that involved in diabetes-induced cardiovascular complications

2.4.1 Reactive Oxygen Species

Reactive oxygen species (ROS) is generated by neutrophils and macrophages during the respiratory process to eliminate antigens. They are also generally produced as the by-product of aerobic metabolism (Miwa and Brand, 2003). A certain amount of ROS is necessary to exert a protective role in maintaining normal metabolic processes in our body. They serve as the mediator in signalling other physiological functions including vascular tone regulation, fibroblast proliferation, host defence, signal transduction and gene expression (Gomes *et al.*, 2012).

However, the uncontrolled generation of ROS stimulated by metabolic abnormalities is likely to bring deleterious effects to the human body. Thus, ROS needs to be sufficiently removed by the antioxidants to maintain cellular homeostasis (Valko *et al.*, 2007). The inability of the antioxidant defence system to effectively scavenge the excess ROS and RNS contributes to oxidative stress (Halliwell and Gutteridge, 2007; Pandey *et al.*, 2010).

Oxidative stress has been proposed as one of the underlying factors in the pathogenesis of DM (Ceriello and Motz, 2004). Pancreatic islets of Langerhans are the main structure to regulate glucose metabolism, at which ß-cells account for insulin synthesis and secretion. However, pancreatic ß-cells have the lowest intrinsic antioxidant defence levels. Hence, they are more susceptible to oxidative stress. Excess ROS initially induces insulin resistance, progressively causes glucose intolerance and subsequently the loss of pancreatic ß-cell function, leading to the occurrence of DM (Negre-Salvayre *et al.*, 2009).

Besides the involvement in the pathogenesis of DM, ROS is also implicated in diabetes-related secondary complications (Lei and Marko, 2011). Chronic hyperglycaemia in DM induces mitochondrial overproduction of ROS, particularly in the endothelium of both large and small blood vessels (Wolff, 1993). Free radical ROS alters the antioxidant defence system by reducing the levels of enzymatic antioxidants and diminishing the activities of antioxidant enzymes, subsequently exacerbating the imbalance condition of oxidative stress (Maritim *et al.*, 2003). This impairment makes the vascular cell and tissue more prone to oxidative damage, ultimately contributes to the risks of secondary vascular complications and hence adverse cardiovascular events (Giacco and Brownlee, 2010).

"ROS" and "free radicals" are the terms frequently used interchangeably in the context of oxidative stress. Among these highly reactive species, $\bullet O_2^-$, •OH and •NO have been widely addressed and actively involved in diabetes-induced cardiovascular complications (Johansen *et al*., 2005). $\bullet O_2^-$ which is produced by an electron reduction of molecular oxygen (O_2) during oxygen metabolism (in both enzymatic and non-enzymatic pathways), initiates the free radical chain reactions. It pathologically modifies •NO (produced from L-arginine by NOS) into cytotoxic $ONOO^-$ (Guzik *et al.*, 2002), causing the reduction in NO bioavailability. Subsequently, the role of NO in mediating vasorelaxation and its property of anti-proliferation is altered, leading to endothelial dysfunction (Turko *et al.*, 2001). Besides, $\bullet O_2^-$ also stimulates LDL oxidation. Oxidised LDL is subsequently taken up by the scavenger receptors of macrophages, leading to the formation of foam cell and atherosclerotic plaque in the progression of atherosclerosis (Johansen *et al.*, 2005).

During SOD-catalysed dismutation, $\bullet O_2^-$ is converted into H_2O_2 and O_2. However, H_2O_2 is converted into •OH in the presence of transition elements such as copper and iron, further propagating the free radical chain reactions (Evans *et al.*, 2003). •OH is the most potent oxidant that attacks important macromolecules in the body, leading to homeostatic disruption and cellular oxidative damage (Lobo *et al.*, 2010). Oxidative damage comprises of cellular protein nitration, membrane lipid peroxidation and the damage to nucleic acids (McCord, 2000). The production and propagation of free radicals and its adverse impacts are illustrated in Figure 2.3.

2.4.2 Antioxidant Defence System

Antioxidant defence system involves endogenous and exogenous antioxidants, is important in scavenging the elevated amount of ROS and neutralising the toxicity arising from excess free radicals (Pham-Huy *et al.*, 2008). Enzymatic endogenous antioxidants include superoxide dismutase, catalase and glutathione (peroxidase and reductase) while exogenous antioxidants can be acquired from diet and supplements.

2.4.2(a) Enzymatic Endogenous Antioxidants

$\bullet O_2^-$ and H_2O_2 are the potent ROS that initiate and propagate the free radical chain reactions, building up vascular oxidative stress that brings detrimental effects to our body system. Thus, ROS needs to be removed or converted into harmless molecules by antioxidants.

2.4.2(a)(i) Catalase

Catalase (CAT) is one of the major regulators in H_2O_2 metabolism that

metabolises reactive H_2O_2 into molecular oxygen and water in lysosome (Johansen *et al.*, 2005). Chronic hyperglycaemia in DM induces the excess generation of H_2O_2 and downregulates the gene expression of CAT. In turn, reduced CAT gene expression decreases enzyme production. The inability of reduced CAT levels to effectively combat with excess H_2O_2 production leads to the accumulation of H_2O_2 for subsequent free radical chain reactions that cause cellular injury. Reduced CAT levels have been reported in diabetic models in both human and animal studies (Hwang *et al.*, 2012; Patel *et al.*, 2013; Wang and Zhang, 2017).

2.4.2(a)(ii) Glutathione (Peroxidase and Reductase)

Glutathione exists in reduced (GSH) and oxidised (GSSG) states. In healthy cells and tissue, GSH constitutes more than 90% of the total glutathione. An increased ratio of GSSG to GSH is an indicative measure of cellular oxidative stress (Lu, 2013).

Glutathione peroxidase (GSH-Px) is another antioxidant enzyme that involved in H_2O_2 metabolism in mitochondria (Johansen *et al.*, 2005). GSH-Px metabolises reactive H_2O_2 into molecular oxygen and water, and in turn converts GSH to GSSG. The oxidised state is converted back to the reduced state in the presence of glutathione reductase. Glutathione reductase is critical in maintaining the reducing environment of the cells in view that reduced glutathione is important in the regulation of the intracellular redox state of vascular cells (Deponte, 2013). Both enzymes act as the major defence in resisting vascular oxidative stress. Reduced GSH-Px expression and activity have been reported in diabetic human and animal models (Hamden *et al.*, 2009; George *et al.*, 2012; Shab-Bidar *et al.*, 2015).

2.4.2(a)(iii) Superoxide Dismutase

Superoxide dismutase (SOD) is an important antioxidant enzyme that regulates oxidative stress in the pathogenesis of vascular complications (Wang *et al.*, 2008). It acts as the first-line defender against free radicals to encounter oxidative stress and subsequently reduces cellular injury (Tiwari *et al.*, 2013). The different forms of SOD, mitochondrial manganese superoxide dismutase (Mn-SOD) and cytosolic copper superoxide dismutase (Cu-SOD) catalyse the dismutation of $\bullet O_2^-$ into O_2 and H_2O_2 (Evans *et al.*, 2003). The antioxidant defence mechanism is illustrated in Figure 2.3.

Kim (2013) reported that diabetic skin tissue expressed relatively low levels of extracellular SOD, which might be due to the alterations in its metabolic state that elevate ROS production (Kim, 2013). In the same year, Lucchesi and colleagues also reported that diabetes-induced oxidative stress diminished the activity of SOD and other enzymatic antioxidants in the liver tissue of diabetic rats (Lucchesi *et al.*, 2013). A similar outcome of diminished activity and reduced levels of SOD attributed to the altered metabolic state of DM also observed in diabetic organs and blood samples (Shukla *et al.*, 2012; Kayama *et al.*, 2015).

In view that enzyme SOD can be the significant parameter that demonstrates the effectiveness of enzymatic antioxidants in combating diabetes-induced ROS and free radicals, SOD levels have been chosen as one of the parameters to assess oxidative stress status. SOD concentrations in the mesenteric arterial tissue of rats and subcutaneous arterial tissue of diabetic patients were measured using a commercial assay kit.

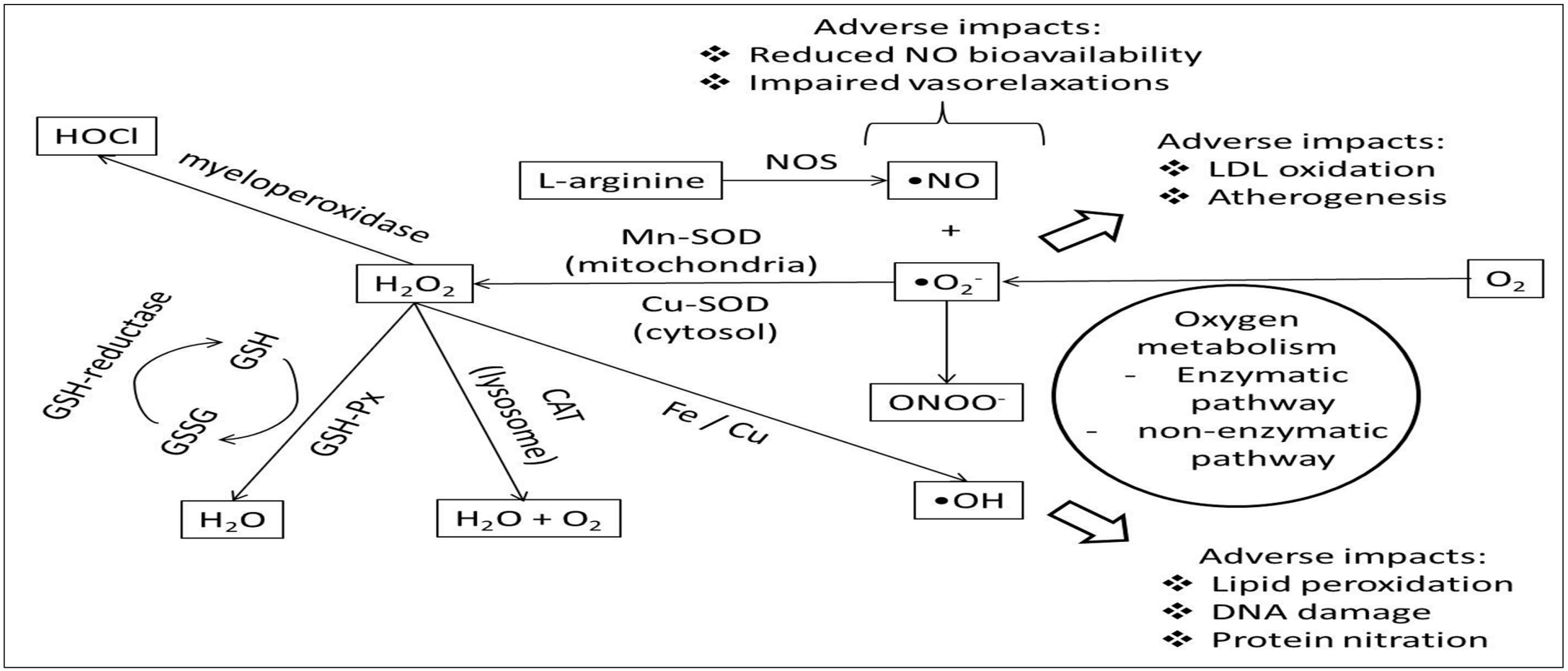


Figure 2.3 Free radicals' production and antioxidant defence mechanism.

[SOD: superoxide dismutase; CAT: catalase; GSH: reduced glutathione; GSSG: oxidised glutathione; GSH-Px: glutathione peroxidase, NOS: nitric oxide synthase; O_2: oxygen; $\bullet O_2^-$: superoxide anion; •OH: hydroxyl radical; HOCL: hydrochlorous acid; H_2O_2: hydrogen peroxide; •NO: nitric oxide; $ONOO^-$: peroxynitrite; H_2O: water; LDL: low-density lipoprotein; DNA: deoxyribonucleic acid; Mn: manganese; Cu: copper; Fe: iron]

2.4.2(b) Exogenous Antioxidants

Among non-enzymatic exogenous antioxidants, vitamin C and vitamin E are the classic antioxidant vitamins against oxidative stress to prevent diabetes-related vascular complications. These antioxidant vitamins either neutralise free radicals directly or interact in recycling processes to regenerate reduced forms of vitamins for further antioxidant actions in combating oxidative stress (Ayeleso *et al.*, 2016).

Vitamin E protects the vascular structural and functional components from oxidative damage by discontinuing a potentially destructive series of oxidative chain reactions (Ashor *et al.*, 2015). Vitamin E prevents glucose-induced LDL oxidation by downregulating the protein expression of scavenger receptors. Hence, the uptake of oxidised LDL by macrophages is reduced, subsequently reducing foam cell and atherosclerotic plaque formed (Ricciarelli *et al.*, 2000). The supplementation of vitamin E (alone or in combination with vitamin C) showed a significant improvement in plasma total antioxidant capacity and enzymatic antioxidant concentrations (Balbi *et al.*, 2018).

Vitamin C has the molecular structure to act as a reducing agent in free radical-mediated oxidative processes. Vitamin C donates electrons to stabilise free radicals, efficiently preventing them from initiating a series of oxidative events (Zerin *et al.*, 2010). Apart from that, vitamin C neutralises the radical forms of other antioxidants to regain their antioxidant ability. Duarte and Lunec (2005) reported that the intervention using vitamin C did not show an impactful outcome as those using vitamin E (Duarte and Lunec, 2005). However, intervention using both vitamin C and E showed enhanced GSH and SOD activity (Rafighi *et al.*, 2013; Balbi *et al.*, 2018).

2.4.3 Oxidative Stress Biomarkers

It is a major problem to perform direct measurement on oxidative stress levels as the reactions between free radicals and antioxidants occur almost instantaneously (Betteridge, 2000). Thus, the indirect way to evaluate oxidative stress levels can be performed through the measurement of observable biomarkers (Matough *et al.*, 2012). Excess production of ROS can be determined by measuring the levels of products produced due to free radical-mediated oxidative damage. The effectiveness of the antioxidant defence system in combating elevated ROS can be determined by measuring the levels of enzymatic antioxidants.

Among the oxidative damages, augmented lipid peroxidation is closely associated with high glycaemic and oxidative stress levels in DM (Bandeira *et al.*, 2012). The natural presence of multiple bonds in polyunsaturated fatty acids in the cell membrane makes them more susceptible to lipid peroxidation by free radicals. During lipid peroxidation, $\bullet RO_2$ alters the cellular lipid profile by removing hydrogen from lipids to produce $\bullet HRO_2^-$, further propagating the free radical pathway (Lobo *et al.*, 2010). $\bullet HRO_2^-$ can either exert toxicity directly on the cells, or it can also be degraded to •OH, or to react with transition metals such as iron or copper to form a stable aldehyde, which consequently damages cell membranes (Halliwell and Chirico, 1993; Giacco and Brownlee, 2010).

Malondialdehyde (MDA) is a stable aldehyde, typically studied with thiobarbituric acid reactive substances (TBARS) assay to measure the degree of lipid peroxidation in diabetic models (Lefevre *et al.*, 1997; Shodehinde and Oboh, 2013). Elevated TBARS and MDA levels reported in plasma, serum and other tissues in

diabetics indicated that peroxidative injury has involved in the development of diabetes complications (Moussa, 2008; Bandeira *et al.*, 2012).

In view that MDA is the primary biomarker to evaluate lipid peroxidation in diabetics, MDA levels have been chosen as another parameter to assess oxidative stress status in this study. MDA concentrations in the mesenteric arterial tissue of rats and subcutaneous arterial tissue of diabetic patients were measured using a commercial assay kit.

2.5 Vitamin D

Vitamin D is well known for its classical role in promoting calcium and phosphorus absorption and suppressing parathyroid hormone (PTH) for mineral homeostasis and bone metabolism (Heaney, 2008). Vitamin D as well a potent steroidal hormone involved in multiple biological actions. Vitamin D binds to its widely distributed vitamin D receptors (VDR) to exert various physiological effects. VDR are abundantly discovered on various body cells and organs, including those that do not account for calcaemic action. Vitamin D has been reported to play an important role in maintaining vascular health such as regulation of vascular tone and inflammatory mediators, induction of cell differentiation, inhibition of cell growth and proliferation, and immunomodulation (Holick, 2003; Holick, 2007; Wacker and Holick, 2013), as well as express its antioxidant action (Wiseman, 1993; Lin *et al.*, 2005; Javanbakht *et al.*, 2010; Nikooyeh *et al.*, 2014; Shab-Bidar *et al.*, 2015).

2.5.1 Synthesis and Metabolism

Vitamin D is well known as the sunshine vitamin. Cutaneous production under sunlight exposure contributes more than 90% of vitamin D whereas approximately 10-

20% is obtained through dietary intake. The consumption of food rich in vitamin D especially from animal sources, such as fortified dairy products and animal fats, and also the ingestion of vitamin D supplements, are the alternatives to supply sufficient vitamin D to the human body (Holick, 2003).

Ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3) are the two primary forms of vitamin D. They are chemically different from each other only in their side chains, at which ergocalciferol has a double bond formed between carbon 22 and 23, and a methyl group at carbon 24. Ergocalciferol is synthesised by ultraviolet irradiation of plant sterols (ergosterol) and invertebrates. Cholecalciferol is produced endogenously when solar ultraviolet B radiation (280-320 nm) strikes human epidermis and stimulates the non-enzymatic photolytic conversion of pro-vitamin D (7-dehydrocholesterol) to pre-vitamin D, which then undergoes thermal isomerisation into vitamin D_3 (Holick, 2003). Just like other lipid-soluble vitamins, vitamin D absorbed in the small intestines is conveyed into the blood circulation via the lacteal and the lymphatic system (Holick, 2007).

Vitamin D is biologically inert and needs to be biologically activated via two hydroxylation processes in the body. In blood circulation, vitamin D binds to vitamin D-binding protein (DBP) and is then transported to the liver for the first hydroxylation process (Juzeniene *et al.*, 2011). In the liver, hepatic cells transform vitamin D into the inactive precursor, 25-hydroxyvitamin D (25(OH)D or termed as calcidiol) in the presence of vitamin D 25-hydroxylase. 25(OH)D is then transported to the kidney via blood circulation for the second hydroxylation process. In the kidney, it is metabolised either intrarenally by 25(OH)D-1α-hydroxylase or intracellularly in a variety of cells and tissue at extra-renal sites to the physiologically active metabolite of vitamin D,

1,25-dihydroxyvitamin D ($1,25(OH)_2D$ or termed as calcitriol) (Holick, 2007; Christakos *et al.*, 2012). Calcitriol enters the bloodstream and circulates in the body, which is then bound to VDR of targeted cells, exerting genomic and/or rapid non-genomic responses for many physiological actions (Geiss *et al*., 1995; Bate and Jerums, 2003). The chemical structures of calcidiol (25(OH)D) and calcitriol ($1,25(OH)_2D$) are shown in Figure 2.4 while the process of vitamin D synthesis and metabolism is illustrated in Figure 2.5.

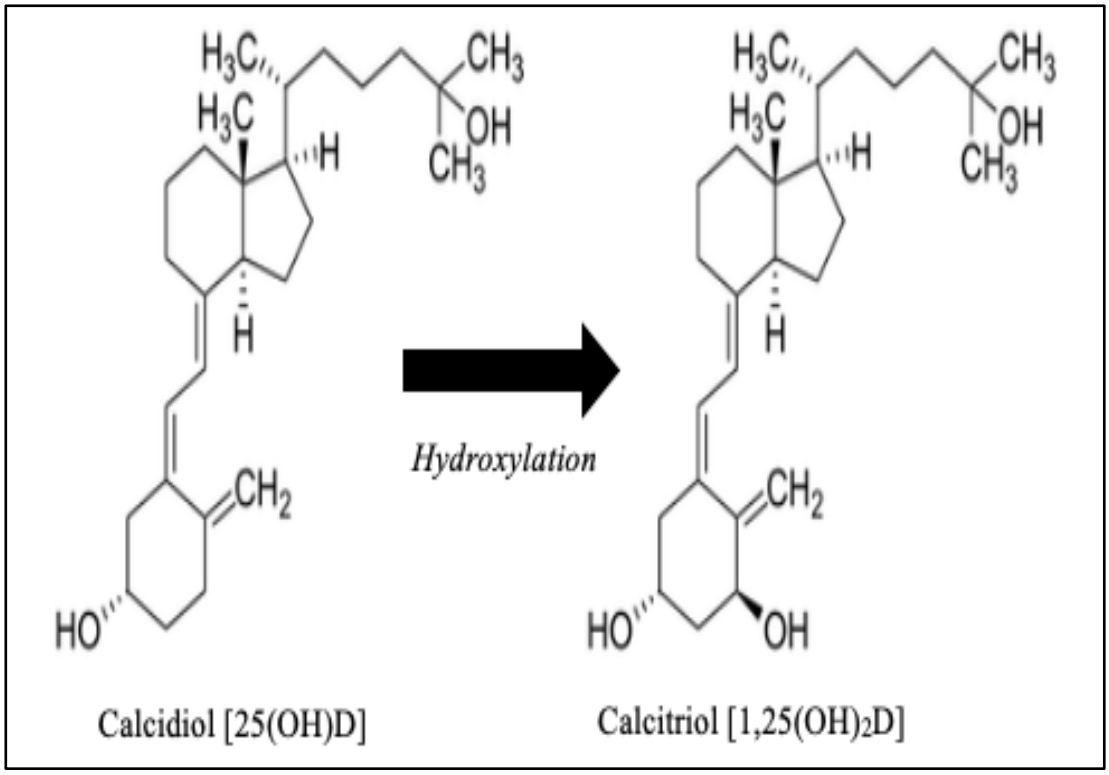


Figure 2.4 Chemical structures of calcidiol and calcitriol.

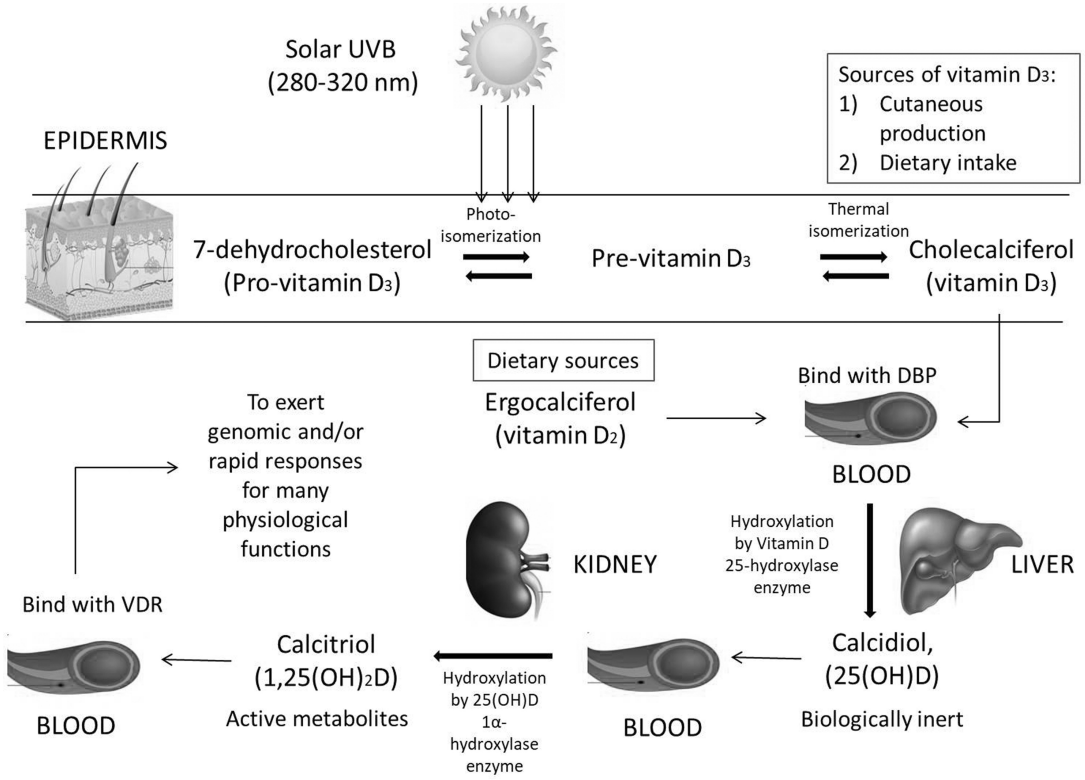


Figure 2.5 Vitamin D synthesis and metabolism.

(Adapted from Lee *et al.*, 2018)

[UVB: ultraviolet B radiation; DBP: vitamin D-binding protein; 25(OH)D: 25-hydroxyvitamin D; $1,25(OH)_2D$: 1,25-dihydroxyvitamin D; VDR: vitamin D receptor]

2.5.2 Measurement of Vitamin D Levels

Although $1,25(OH)_2D$ is the biologically active metabolite of vitamin D, it does not serve as an ideal indicator to determine vitamin D levels. Indeed, the measurement of 25(OH)D levels is the most commonly used indicator to determine vitamin D levels in the body (Alshahrani and Aljohani, 2013). 25(OH)D has a longer circulating half-life of 15 days compared to 15 hours that $1,25(OH)_2D$ has (Jones, 2008). Hence, the concentrations of 25(OH)D are more stable and less fluctuate for measurement. Besides that, 25(OH)D concentrations reflect the precise storage

amount of vitamin D (both by solar-activated cutaneous production and long period dietary intake) in human body as 25(OH)D is the primary circulating form of vitamin D in our body (Heaney, 2008).

The levels of $1,25(OH)_2D$ are regulated by parathyroid hormone depending on the calcium needs of the body (Zerwekh, 2008). Unless the condition of vitamin D deficiency is severe, $1,25(OH)_2D$ concentrations are typically held constant through homeostatic regulation or may show elevation due to secondary hyperparathyroidism, in the condition of vitamin D deficiency (Holick, 2007). Hence, $1,25(OH)_2D$ concentrations do not reliably show the actual vitamin D levels in the body at that particular time. Also, vitamin D supplementation more effectively raises the concentrations of 25(OH)D compared to $1,25(OH)_2D$ (Trang *et al.*, 1998).

2.5.3 Classification of Vitamin D Status

In general, vitamin D status in the body can be defined as vitamin D deficiency, insufficiency and sufficiency based on the measurement of serum 25(OH)D levels. The Endocrine Society of USA defines vitamin D deficiency as serum 25(OH)D concentrations of less than 50 nmol/L while circulating serum 25(OH)D levels between 50–74 nmol/L is defined as vitamin D insufficiency (Holick *et al.*, 2011). Vitamin D sufficiency or commonly known as optimal vitamin D levels is accepted as serum 25(OH)D of at least 75 nmol/L as this level is needed to optimise intestinal calcium absorption and to cover all physiological functions of vitamin D (Bischoff-Ferrari, 2007). Vitamin D deficiency and insufficiency, at which serum 25(OH)D concentrations of less than 75 nmol/L are considered as low or suboptimal vitamin D levels in this context.

2.5.4 Hypovitaminosis and Hypervitaminosis D

Hypovitaminosis D, or commonly known as vitamin D deficiency, is characterised as nutritional inadequacy or depletion in the body, causing disturbances in body functions. Hypovitaminosis D may be accompanied by severe clinical conditions such as hyperthyroidism, hyperparathyroidism, calcium malabsorption, rickets, osteomalacia and myopathy (Vieth *et al.*, 2001).

Although sunlight exposure can supply 10,000 IU vitamin D for covering human's biological needs (Holick, 2007), the cutaneous production of vitamin D is still insufficient. The insufficient vitamin D production might due to aging, skin pigmentation, culture and clothing, application of sunscreen, sedentary lifestyle and other environmental factors that lead to limited sunlight exposure such as geographic aspects and seasonal changes. Inadequate dietary intake of vitamin D, lack of vitamin D supplementation and medical conditions that attenuated vitamin D conversion, also lead to hypovitaminosis D (Holick, 2007).

Hypervitaminosis D, or commonly known as vitamin D toxicity, occurs when there is excess vitamin D intake that translates to a vast increase in circulating 25(OH)D concentrations. Excessive sunlight exposure does not lead to hypervitaminosis D as pre-vitamin D_3 will undergo photo-degradation to inactive sterols in the skin (Holick, 2003). However, excessive oral supplementation of vitamin D without close monitoring of 25(OH)D concentrations can lead to hypervitaminosis D as 25(OH)D concentrations are beyond DBP binding capacity (Holick, 2003). The main clinical consequence of hypervitaminosis D is hypercalcaemia, at which intestinal calcium absorption is elevated, and net bone resorption is increased

(Zittermann, 2003). Hypervitaminosis D may also be accompanied by other clinical symptoms including hypercalciuria, ectopic calcifications, hyperphosphatemia, kidney stones, polyuria and polydipsia, hypertension, anorexia, nausea, vomiting and constipation (Alshahrani and Aljohani, 2013).

Although it is uncommon, hypervitaminosis D has been reported in multiple age groups and from multiple causes, including errors in milk fortification (Blank *et al.*, 1995), errors in manufacture (Kara *et al.*, 2014), megadose ingestion of vitamin D supplements (Nimesh *et al.*, 2015) and incorrect dosing from liquid preparations (Lee *et al.*, 2018). Nimesh *et al.* (2015) reported that a one-year-old child developed acute hypertension and severe hypercalcaemia due to vitamin D toxicity after high doses of oral calcitriol supplementation (Nimesh *et al.*, 2015). Gallagher (2016) also showed that the administration of annual bolus doses of vitamin D at concentrations of 300,000 IU or 500,000 IU resulted in an increased risk of falls and fractures among elderly individuals (Gallagher, 2016).

Previously, only exceptionally high serum 25(OH)D levels of more than 220 nmol/L (Rizzoli *et al.*, 1994) and more than 250 nmol/L (Vieth *et al.*, 2001; Zittermann *et al.*, 2005) were reported to cause vitamin D toxicity. Nevertheless, recent findings recommend a much lower upper limit for the levels of 25(OH)D. The upper limit of 125 nmol/L is suggested for elderly individuals (Gallagher, 2016), infant (Lee *et al.*, 2013) and healthy young adult (Diab and Watts, 2018) to prevent adverse health impacts due to hypervitaminosis D.

2.5.5 Vitamin D Supplementation

Since there are studies reported an increase in cancer incidence rates and mortality risks at both low and high levels of serum 25(OH)D (Abnet *et al.*, 2007; Melamed *et al.*, 2008), vitamin D levels should keep at the optimal range to ensure no disturbance to the normal physiological functions. Vitamin D supplementation appears as a promising approach in enhancing vitamin D levels when there is insufficient cutaneous production or dietary intake. However, the effectiveness of vitamin D supplementation in correcting vitamin D status depends on the types of vitamin D analogues employed for treatment. Different vitamin D analogues have a different binding capacity to DBP and undergo different vitamin D metabolism (Aronov *et al.*, 2008).

Vitamin D_3 is 87% more effective in raising serum 25(OH)D levels and is converted 500% faster to calcitriol compared to vitamin D_2 (Houghton and Vieth, 2006). Vitamin D_3 is also the natural form of vitamin D that produced by our body; hence it is more potent in raising vitamin D concentrations and maintaining it in the circulatory system for a longer period, providing two- to three-fold greater storage of vitamin D in the body. Vitamin D_3 also binds with higher affinity to DBP in plasma, offering approximately three times more effective in its action (Holick, 2007).

Among the vitamin D_3 analogue, calcitriol is preferable compared to calcidiol and cholecalciferol. Calcitriol is the active metabolite of vitamin D that can act immediately as a hormone to exert its physiological effects more effectively. In contrast, inactive analogues need to undergo hydroxylation processes in the liver and kidney to convert to its active metabolite. This might be a problem due to the

diminished ability of kidney in patients with kidney dysfunction, which is common in the diabetic population (Williams *et al.*, 2009).

Since calcitriol has been used to treat several medical conditions in both human and animal diabetic models (Hamden *et al.*, 2009; Eftekhari *et al.*, 2014), vitamin D supplementation in this project was conducted by providing oral calcitriol to the vitamin D-deficient rats, in both normal and diabetic models.

2.6 Vitamin D Status and Vascular Function

Numerous human studies have reported on the effects of vitamin D insufficiency or deficiency on macrovascular function in normal population and also in a variety of disease models. Brachial artery FMD was significantly lower in vitamin D-deficient subjects than controls (Tarcin *et al.*, 2009; Malik *et al.*, 2016). Lower FMD has also been demonstrated in vitamin D-deficient subjects with early Parkinson disease (Yoon *et al.,* 2015) and T2DM (Yiu *et al.*, 2013). Vitamin D supplementation did enhance vascular function in term of improving FMD in normal population and T2DM patients with vitamin D deficiency (Sugden *et al.*, 2008; Harris *et al.*, 2011).

Endothelial dysfunction, which is due to the imbalance in the production and action of EDRF and EDCF, is the precursor of early atherosclerotic development. Vitamin D exerts direct vasoprotective effects against endothelial dysfunction by enhancing endothelium-dependent vasorelaxation and inhibiting vasoconstriction (Vanhoutte *et al.*, 2009; Sitia *et al.*, 2010). Vitamin D improves the bioavailability of endothelial NO, the potent vasorelaxing factor and inhibitor of platelet and leukocyte aggregation and adhesion. This occurs via direct enhancement of transcriptional regulator of eNOS (Andrukhova *et al.*, 2014) and/or affecting phosphatidylinositol 3

kinase in EC, which activates eNOS to catalyse the NO production (Wang *et al.*, 2008; Menezes *et al.*, 2014). By inducing NO production, vitamin D also promotes EC proliferation and migration and inhibits apoptosis (Xiang *et al.*, 2011). Vitamin D also induces the production of PGI_2 in VSMC through the COX pathway, which prevents thrombus formation, cell adhesion and VSMC proliferation (Wakasugi *et al.*, 1991; Chen *et al.*, 2010).

2.7 Vitamin D Status and Oxidative Stress

Hyperglycaemia coupled with vitamin D deficiency leads to the overproduction of ROS and free radicals in diabetics, provoking oxidative stress that exacerbates diabetes-related cardiovascular complications (Wang *et al.*, 2008; Dong *et al.,* 2012). For the past decades, considerable efforts have been made in studying the antioxidant action of vitamin D in the pathogenesis of DM and its related secondary complications.

Wiseman (1993) demonstrated that vitamin D acts as a direct membrane antioxidant to inhibit iron-induced lipid peroxidation of brain liposomes. Its antioxidant ability may be due to the homologous structure to cholesterol, allowing vitamin D to stabilise membrane from lipid peroxidation through molecular relations with the hydrophobic parts (Wiseman, 1993). Vitamin D helps to diminish the formation of ROS by suppressing the gene expression of NADPH oxidase, which is one of the main resources of ROS (Labudzynskyi *et al.*, 2015). Also, vitamin D inhibits the augmented mitochondrial production of ROS via regulating the autophagy process to maintain redox homeostasis (Wong *et al.*, 2008).

Vitamin D supplementation has been reported to improve the activities of antioxidant enzymes such as SOD, CAT, GSH-Px and reduce lipid peroxidation in

combating oxidative stress (Hamden *et al.*, 2009; George *et al.*, 2012; Nikooyeh *et al.*, 2014; Shab-Bidar *et al.*, 2015). Vitamin D involved in GSH metabolism through upregulating glutamate-cysteine ligase (a key enzyme in GSH synthesis) and glutathione reductase genes expression (Kanikarla-Marie and Jain, 2016). Hence, the normal reducing environment within the cell can be maintained to efficiently remove ROS, thereby oxidative stress can be attenuated. Vitamin D may possess antioxidant properties as potent as, or even better than the classical antioxidant vitamin E (Wiseman, 1993, Sardar *et al.*, 1995, Lin *et al.*, 2005; Javanbakht *et al.*, 2010).

In animal studies, there were significantly higher levels of serum TBARS found in vitamin D-deficient diabetic rats, which were significantly reduced nearly to control values after vitamin D_3 supplementation (George *et al.*, 2012). A study has reported on the potential of vitamin D_3 supplementation either as a preventive measure or therapeutic strategy in diabetic rats (Hamden *et al.*, 2009). In that study, the rats received vitamin D_3 supplementation (5000 IU/kg body weight daily) by gastric gavage for two months before and after alloxan-induction respectively to serve as preventive and therapeutic groups of diabetics. The results showed that administration of vitamin D_3 in both groups (preventive and therapeutic groups) enhanced hepatic and renal activity of SOD, CAT and GSH-Px, as well as reduced lipid peroxidation as indicated by decreased TBARS levels compared to untreated diabetic rats. Vitamin D might be better in regulating oxidative stress before the development of diabetes as shown by a better significant improvement in the preventive group compared to the therapeutic group of diabetic rats (Hamden *et al.*, 2009).

In human studies, serum TBARS levels of asymptomatic vitamin D-deficient subjects receiving vitamin D_3 supplementation (300,000 IU monthly for three months)

were significantly reduced nearly to basal TBARS levels as in control group (Tarcin *et al.*, 2009). In subjects with T2DM, treatment with vitamin D_3-fortified doogh (a Persian yogurt drink) (500 IU twice a week for 12 weeks) showed a reduction in MDA and increased GSH levels (Shab-Bidar *et al.*, 2015). In hypertensive, hyperlipidaemia and obesity patients, calcitriol supplementation also improved the activities of antioxidant enzymes and attenuated ROS production (Dong *et al.*, 2012).

However, treatment with vitamin D_3 capsules (50,000 IU, given twice throughout six-weeks study duration) did not affect the biomarker of oxidative stress (MDA and GSH levels) in women with GDM (Asemi *et al.*, 2013). Similarly, Eftekhari and colleagues (2014) reported that MDA levels in T2DM subjects after receiving calcitriol supplementation (0.5 μg/day for 12 weeks) showed no significant difference (Eftekhari *et al.*, 2014). Another study in vitamin D-deficient T2DM patients reported that vitamin D_3 treatment (5,000 IU/day for 12 weeks) did not significantly affect SOD levels (Yiu *et al.*, 2013).

2.8 Vitamin D Status and Atherosclerosis

Epidemiological studies demonstrated that low vitamin D levels are a risk factor for the development of atherosclerosis (Kassi *et al.*, 2013). Vitamin D deficiency correlates with endothelial dysfunction, VSMC proliferation and migration, enhanced oxidative stress, increased intima-media thickness (IMT) and augmented systemic inflammation, leading to atherosclerosis (Zittermann *et al.*, 2007; Wang *et al.*, 2008; Wang *et al.*, 2010; Liu *et al.*, 2012).

IMT, which is a reflection of atherosclerotic burden, was negatively related to serum 25(OH)D concentrations (Liu *et al.*, 2012). There was a marked increase in

carotid artery IMT in T2DM patients with low vitamin D levels compared to those with higher vitamin D levels. T2DM patients who developed carotid plaque has significantly lower 25(OH)D concentrations compared to T2DM patients without carotid plaque (Wang and Zhang, 2017). Vitamin D analogues suppress the mechanisms that lead to increased IMT and vascular calcification by inhibiting the over-expression of multiple adhesive molecules on EC (Zittermann *et al.*, 2007) and the accumulation of plaque lipid in VSMC (Willerson and Ridker, 2004).

Vitamin D also possesses anti-inflammatory properties. It may suppress the production and release of several pro-inflammatory cytokines (Zittermann *et al.*, 2007) and increase the production of anti-inflammatory cytokines (Tukaj *et al.*, 2012). Vitamin D downregulates the inflammatory process by limiting the major role of T-helper 1 in pro-atherogenic response and shifting the T-cell response to T-helper 2 to limit the pro-atherogenic response (Hewison, 2010). Vitamin D displays anti-atherogenic properties through endoplasmic reticulum (ER) stress-dependent mechanism. ER stress acts as a major regulator in modulating macrophage differentiation that may have a role in atherosclerotic plaque regression in diabetics (Oh *et al.*, 2012). Vitamin D acts as an ER stress reliever to prevent foam cell formation during macrophage differentiation, reduce macrophage infiltration and migration and stimulate an anti-atherogenic macrophage phenotype, thus reducing vascular inflammation and complications in T2DM patients. Vitamin D also reverses atherogenic cholesterol metabolism deposition by preventing the progression of macrophage cholesterol uptake and promoting cholesterol efflux in macrophages from T2DM patients (Riek *et al.*, 2013).

CHAPTER 3

METHODOLOGY

3.1 Animal Study

3.1.1 Ethical Approval

The ethical approval for animal study was obtained from the Animal Ethics Committee, Universiti Sains Malaysia (USM), Health Campus, Kelantan [No. USM/Animal Ethics Approval/2016/(98)(711)] (Appendix A).

3.1.2 Animals

A total of 60 male Sprague-Dawley rats aged between 8- to 9-week-old with body weight ranged between 200-300 g were purchased from the Animal Research and Service Centre, USM, Health Campus, Kelantan. Sprague-Dawley rat is commonly used as an animal diabetic model and has contributed significantly in diabetes-related research studies (Shi *et al.*, 2007; Leo *et al.*, 2011). Male rats were used in this study considering that female rats are more physiologically influenced by sexual hormones, that might interfere with the study outcome.

Rats were housed in polypropylene cage in a well-ventilated animal room with room temperature of 25±2°C and the exposure to 12-hours light-dark cycle. The rats had unrestricted access to tap water *ad libitum* including during fasting. Food pellets provided to the rats were maintained at 25±5 g daily unless they were fasted for a specific experimental procedure. Bedding was changed regularly to minimise infections or stress to the rats. The rats were acclimatised to the surrounding conditions

for one to two weeks before the experiment. The experiment started when the rats achieved 10-week-old.

3.1.3 Preparation of Working Solutions

i. Sodium citrate buffer (0.1 M, pH 4.5)

To prepare 0.1 M sodium citrate buffer, 2.94 g of trisodium citrate dehydrate (Sigma Aldrich, USA) was fully dissolved in 100 mL of double distilled water (ddH_2O). The solution was adjusted to pH 4.5. This solution was freshly prepared prior to usage. Sodium citrate buffer was used as the diluent of streptozotocin (STZ) for diabetes induction and also as the vehicle for normal rats.

ii. Lysis buffer

To prepare lysis buffer, a tablet of cOmplete^{TM} Protease Inhibitor Cocktail (Roche Diagnostic, Germany) were fully dissolved in 10 mL of radioimmunoprecipitation assay (RIPA) lysis buffer (Sigma Aldrich, USA), mixed well and stored at 4°C.

3.1.4 Experimental Design

3.1.4(a) Diabetes Induction

At 10-week-old, rats were randomly divided into two groups of 30 rats each: normal and diabetic groups. Following an overnight fast, diabetic group was intraperitoneally injected with STZ dissolved in sodium citrate buffer, at the dosage of 50 mg/kg body weight (Nishigaki *et al.*, 1989). Normal group was intraperitoneally administered with an equivalent volume of sodium citrate buffer. Administration of

STZ destroys the Langerhans islet ß-cells of the rats within two to four days (Weiss, 1982). STZ be administered to fasted rats is to minimise the competition between STZ and glucose for the uptake into ß-cells via low-affinity glucose transporter (GLUT-2), thus improving the efficacy of STZ in inducing experimental hyperglycaemia (Chaudhry *et al*., 2013).

After 72 hours, blood samples of the rats were drawn from tail tips by needle pricking to measure baseline fasting blood glucose (FBG) concentrations using a portable one-touch glucometer (Accu-check, Roche Diagnostics, USA), following an overnight fast. Rats with FBG concentrations of more than 16.6 mmol/L, which indicate the complete and irreversible degeneration in Langerhans islet ß-cells (Shi *et al.*, 2007), were regarded as the successfully induced diabetic group.

FBG concentrations and body weight measured on the day of successful diabetes induction (defined as week one, W_1) were recorded and used as baseline value. FBG concentrations and body weight were monitored weekly throughout the study period to ensure the rats remained under normal or diabetic conditions in respective study groups. At the end of 10-weeks study duration, FBG concentrations and body weight measured on the day of sacrifice (defined as week ten, W_{10}) were recorded and used as final value.

3.1.4(b) Diet and Supplementation

After the confirmation of normal and diabetes status, both normal and diabetic groups were respectively subdivided into three study groups of 10 rats each: (i) rats receiving control diet for 10 weeks, (ii) rats receiving vitamin D-deficient diet for 10 weeks, and (iii) rats receiving vitamin D-deficient diet for 10 weeks, with calcitriol

supplementation for four consecutive weeks, starting from the beginning of week seven (W_7) to the end of week ten (W_{10}). The group classification of normal and diabetic rats is illustrated in Table 3.1 and Table 3.2 respectively.

Table 3.1 Group classification of normal rats.

Groups	Number of Rats	Diet/Supplementation
NC	10	Control diet for 10 weeks (W_1 to W_{10})
ND	10	Vitamin D-deficient diet for 10 weeks (W_1 to W_{10})
NDS	10	Vitamin D-deficient diet for 10 weeks (W_1 to W_{10}) + calcitriol supplementation for four weeks (W_7 to W_{10})

[W_1: week one; W_7: week seven; W_{10}: week ten]

Table 3.2 Group classification of diabetic rats.

Groups	Number of Rats	Diet/Supplementation
DC	10	Control diet for 10 weeks (W_1 to W_{10})
DD	10	Vitamin D-deficient diet for 10 weeks (W_1 to W_{10})
DDS	10	Vitamin D-deficient diet for 10 weeks (W_1 to W_{10}) + calcitriol supplementation for four weeks (W_7 to W_{10})

[W_1: week one; W_7: week seven; W_{10}: week ten]

Control diet is a standard rodent diet (AIN93G, Specialty Feeds, AUS) containing 1000 IU/kg of vitamin D_3, 0.70% of calcium and 0.35% of phosphorus.

Vitamin D-deficient diet is a modified AIN93G rodent diet with no added vitamin D (SF03-009, Specialty Feeds, AUS) containing 0.70% of calcium and 0.35% of phosphorus. All diets were provided to the rats throughout the study duration of 10 weeks (from W_1 to W_{10}).

In this study, calcitriol, the physiologically active metabolite of vitamin D, is the vitamin D analogue used for supplementation. Calcitriol (Rocaltrol, Roche, Switzerland) dissolved in extra virgin organic coconut oil (Country Farm Organics, Philippines) was given by oral gavage feeding to the rats with the dosage of 0.15 µg/kg body weight (Mancuso *et al.*, 2008; Dong *et al.*, 2012; Lee *et al.*, 2014). Calcitriol supplementation was given five days per week for four consecutive weeks (Lee *et al.,* 2014; Huda *et al.*, 2014), starting from the beginning of week seven until the end of the 10-weeks study duration (from W_7 to W_{10}). Based on a previous study conducted on the aorta of hypertensive rats, vitamin D deficiency causes endothelial dysfunction in six-week duration (Dong *et al.*, 2012). Hyperglycaemia in diabetes has also been reported in leading to microvascular endothelial dysfunction in a six weeks' study (Mokhtar *et al.*, 2016). Giving a four-weeks calcitriol supplementation shall be sufficient in correcting vascular functional abnormality and also preventing vasculopathy in diabetes microcirculation attributed to vitamin D deficiency. At the end of week ten after successful diabetes induction (Palmer *et al.*, 1998), the rats were fasted overnight, anaesthetized with an intraperitoneal injection of sodium pentobarbitone (70 mg/kg body weight) and sacrificed (Shi and Vanhoutte, 2008).

3.1.4(c) Blood Sample Collection and Serum Preparation

During deep anaesthetization, blood samples (approximately 5 mL) were drawn from the left ventricle of the rats via cardiac puncture and were collected into a plain Vacutainer tube. The blood samples were allowed to clot at 4ºC before being centrifuged using Eppendorf Centrifuge (5810R, Kubota, Japan) at 4000 x *g* at 4ºC for 10 minutes. The yielded serum was collected and outsourced to a local certified laboratory (BP Healthcare, Kota Bharu, Malaysia) for the measurement of serum calcium concentrations. The balance of the serum was kept at -80ºC for subsequent measurement of 25(OH)D concentrations using a commercially available assay kit.

3.1.4(d) Dissection and Isolation of Mesenteric Arteries

The rats were dissected to isolate mesenteric vascular bed after blood sample collection. The isolated mesenteric vascular bed was immediately placed in a petri dish filled with pre-chilled, oxygenated physiological saline solution of the following composition (mM): NaCl 118.0, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2, glucose 5.5 (details please refer to Table 3.3), ready for microscopy dissection of mesenteric artery under a dissecting microscope (Motic, China). During artery dissection, several distinctive physical characteristics were used to differentiate artery from vein. Artery has a smooth adventitial surface, a thicker wall in proportion to a smaller lumen, a visible distinction between media and lumen, a V-shaped branch tip, is relatively rigid in vessel shape, and brighter in blood colour. After artery identification, first branch order of superior mesenteric arteries (luminal diameter of 150-200 μm) (Figure 3.1) was harvested as a small resistance artery for microvascular functional study (Pannirselvam *et al.*, 2005). The microvascular functional study

conducted in this study included the assessments of endothelium-dependent relaxation and contraction, as well as endothelium-independent relaxation and contraction.

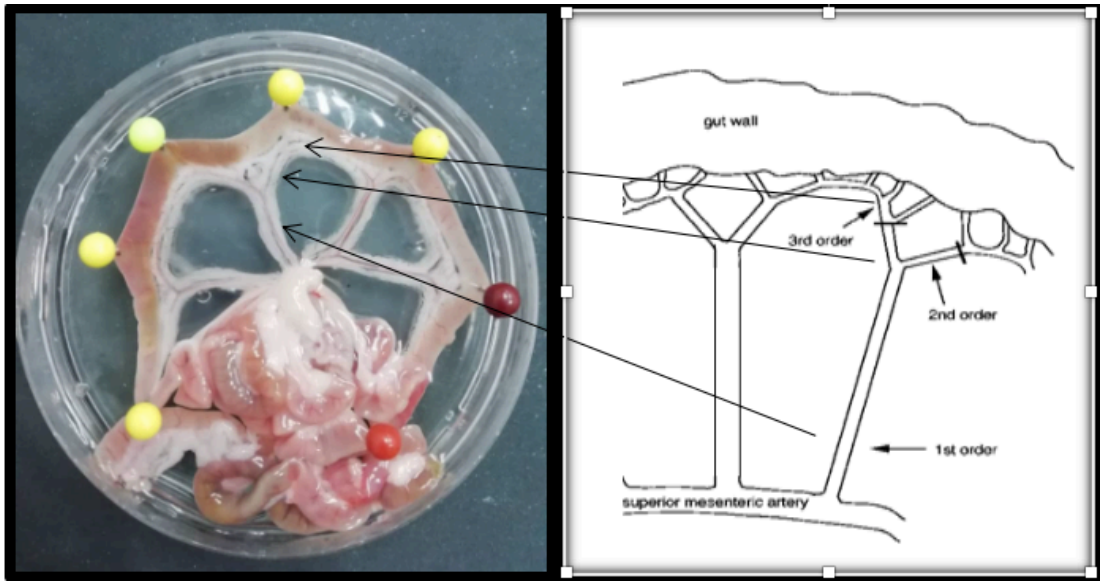


Figure 3.1 Identification of branch order of mesenteric artery from an isolated mesenteric vascular bed of Sprague-Dawley rat.

[Schematic diagram was adapted from Fabrice Pourageaud, 1997]

The remaining mesenteric arteries were collected to prepare vascular tissue lysate for subsequent oxidative stress analysis and also to conduct Western blot. Oxidative stress analysis on mesenteric arterial tissue was performed by measuring the concentrations of oxidative stress parameters such as superoxide dismutase (SOD), an antioxidant enzyme and malondialdehyde (MDA), the biomarker of oxidative injury. Western blot was conducted to quantify the protein expression of endothelial nitric oxide synthase (eNOS) in the vascular tissue samples. An intact mesenteric artery was harvested to conduct immunohistochemistry for the detection of the presence and localization of protein eNOS. Artery dissecting procedures were handled with gentle care to prevent damage to the endothelial cells (EC) and vascular smooth muscle cells (VSMC) for vessel's viability. The detailed flow chart of the experimental design is illustrated in Figure 3.2.

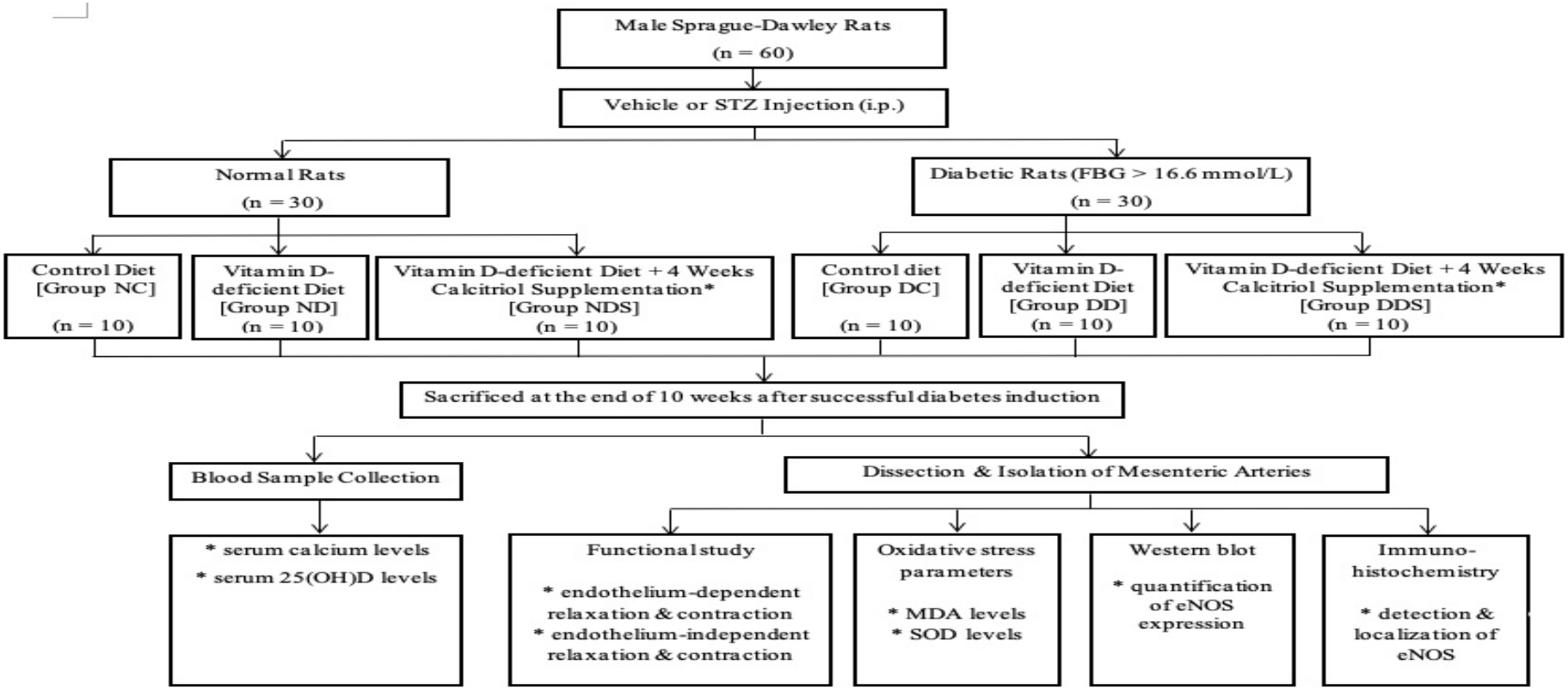


Figure 3.2 Detailed flow chart of experimental design for animal study.

* Four-weeks calcitriol supplementation was starting from the beginning of week 7 to the end of 10-weeks study duration (W_7 to W_{10}).

[STZ: streptozotocin; i.p.: intraperitoneal; FBG: fasting blood glucose; 25(OH)D: 25-hydroxy vitamin D; eNOS: endothelial nitric oxide synthase; MDA: malondialdehyde; SOD: superoxide dismutase]

3.1.4(e) Tissue Lysate Preparation

Mesenteric artery cleaned of excess fat and connective tissues were rinsed three times with physiological saline solution to remove excess blood that might interfere with the study outcome. The artery was then finely chopped in a microcentrifuge tube filled with 100 μL of pre-chilled lysis buffer on ice using dissecting scissors. The tissue lysate was incubated at 4ºC for 10 minutes, followed by centrifugation at 12,000 x *g* at 4ºC for five minutes. The yielded supernatant was kept at -80°C and ready to be used for total protein quantification, oxidative stress analysis and Western blot.

3.2 Vascular Functional Study

3.2.1 Wire Myography

Myography is a technique developed by Mulvany and Halpern (1976) for the *in vitro* investigation of active and passive properties of small isolated vessels (with an internal diameter of 100 to 1000 μm). The properties of small vessels included their functional responses and vascular reactivity to vasoactive substances are evaluated under isometric conditions in myography (Mulvany and Halpern, 1976; Mulvany and Aalkjaer, 1990). The standard experimental protocols of myography consist of preparation and mounting of vessel rings, normalization procedures and vascular functional responses towards specific pharmacological agents.

This technique has been widely used to determine the functional and mechanical properties of isolated arteries from a variety of vascular bed in various pathological states (Spiers and Padmanabhan, 2005). In this study, dual chamber wire myograph (410A, DanishMyo Technology, Denmark) (Figure 3.3) was used to

evaluate the microvascular functional properties in the mesenteric arteries of normal and diabetic rats with different vitamin D status.

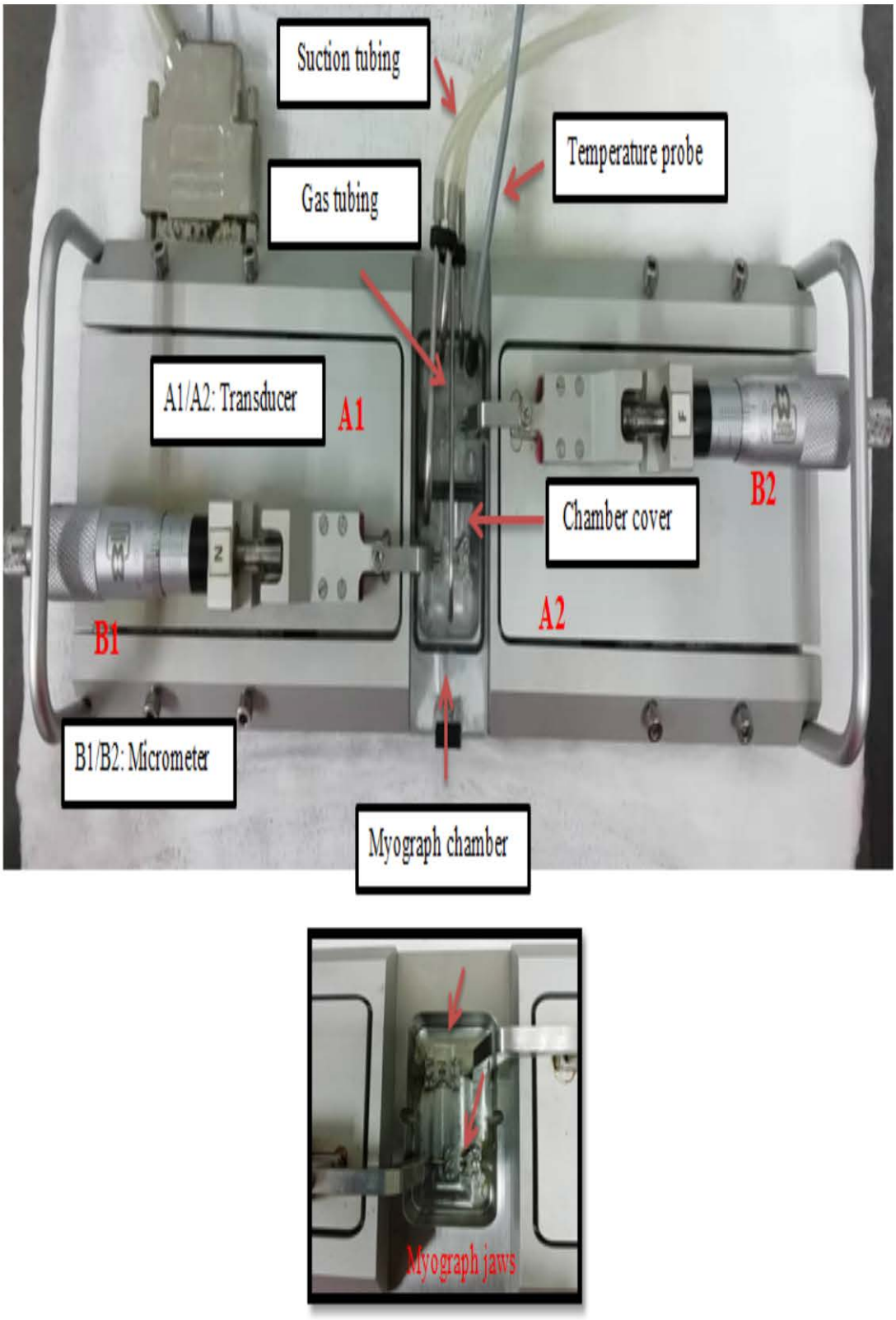


Figure 3.3 Dual chamber wire myograph with labelled components.

3.2.2 Physiological Saline Solution

Physiological saline solution (PSS) used in this study is a modified Krebs buffer with specific salt concentrations, aeration, pH and temperature to maintain the similar physiological conditions of the vessel during wire myography as the vessel exists in the living body. To prepare PSS, the chemicals purchased from Merck KGaA, Darmstadt, Germany as listed in Table 3.3 were fully dissolved in 1 L of double distilled water according to the desired mass and mixed well. The solution was slowly aerated with carbogen gas (95% oxygen + 5% carbon dioxide) for approximately 30 minutes until the solution reached pH 7.4 (Mokhtar *et al.*, 2016). PSS was freshly prepared in view that the glucose levels of the solution might deteriorate over time.

Table 3.3 Chemicals used for the preparation of physiological saline solution.

Chemicals	Chemical Formula	Molecular Weight (g/mol)	Concentration (mmol/L)	Concentration (g/L)
Sodium chloride	NaCl	58.44	118.99	6.95
Potassium chloride	KCl	74.55	4.69	0.35
Magnesium sulphate heptahydrate	$MgSO_4.6H_2O$	246.48	1.17	0.29
Potassium dihydrogen phosphate	KH_2PO_4	136.08	1.18	0.16
Sodium hydrogen carbonate	$NaHCO_3$	84.01	25.00	2.10
D(+)-glucose monohydrate	$C_6H_{12}O_6.H_2O$	198.17	5.50	1.09
Calcium chloride dehydrate	$CaCl_2.2H_2O$	147.02	2.50	0.37

3.2.3 Pharmacological Agents for Vascular Functional Responses

Vascular functional responses of the vessel towards particular pharmacological agents were assessed in this study. All stock solutions of pharmacological agents were freshly prepared to prevent the deterioration in their pharmacological potency over time. To prepare pharmacological agents for myography, the chemicals were fully dissolved in respective diluents and were serially diluted to the appropriate concentrations. The final concentrations of each chemical loaded in the myograph chamber were determined based on preliminary studies or previous study in the literature (Mokhtar *et al.*, 2016). The pharmacological agents used in vascular functional study are listed in Table 3.4.

Table 3.4 Pharmacological agents used in vascular functional study.

Pharmacological Agent (Abbreviation)	Molecular Weight (g/mol)	Diluent	Manufacturer, Country of Origin
Acetylcholine chloride (ACh)	181.66	ddH_2O	Sigma Aldrich, USA
Calcium ionophore A23187 (CaI)	523.62	DMSO	Sigma Aldrich, USA
L-NG-Nitroarginine methyl ester hydrochloride ($_L$-NAME)	269.70	ddH_2O	Cayman Chemicals, USA
Phenylephrine (PE)	203.67	ddH_2O	Sigma Aldrich, USA
Salbutamol hemisulfate (SB)	576.70	ddH_2O	Tocris Bioscience, UK
Sodium nitroprusside dehydrate (SNP)	297.95	ddH_2O	Sigma Aldrich, USA

[DMSO: dimethyl sulfoxide; ddH_2O: double distilled water]

3.2.4 Preparation and Mounting of Vessel Rings

The myograph chambers were pre-washed with ice-cold PSS for three times to remove any dust or dirt. Then, the chambers were filled up with PSS with the chamber cover on to prevent evaporation. A temperature probe was inserted, and the internal heating system set at 37ºC was turned on. Gas tubing with clamps was manually adjusted to start the aeration of carbogen gas (95% oxygen + 5% carbon dioxide) in the chambers.

The isolated first branch order of mesenteric artery which had been cleaned from fat and connective tissues was then cut into vessel rings with 2-mm-length. For endothelium-dependent response study, blood vessel with intact endothelium was used. For endothelium-independent response study, the endothelium of blood vessel was removed by gently scrubbing the lumen of the vessel ring with a human hair during dissection (Kirkby *et al.*, 2012).

The lumen of the vessel ring was gently cannulated with first tungsten wire (40 μm in diameter) held by forceps in a smooth motion with the aid of the dissecting microscope, ready for mounting in myograph chambers. Once sufficient aeration and an optimum temperature of 37ºC were achieved, the chamber cover was removed, and the chamber was moved under the microscope. The cannulated vessel ring was then transferred to the middle of the chamber.

The myograph jaws were brought near to each other by adjusting the micrometer to hold the wire in place, followed by securing both free ends of the wire tightly underneath the screws of the jaw attached to a micrometer. Once the first wire was mounted, the jaws were moved slightly apart following by threading the second

tungsten wire into the lumen along the top of the first wire. Cannulation was done in a smooth motion to avoid endothelial injury or creating trauma to the vessel.

Again, the jaws were moved to be in contact to secure the wire end underneath the screws of the jaw attached to an isometric force transducer. The jaws were then moved slightly apart to adjust the wires to be parallel on the same plane, thereby allowing the radial forces generated in the vessel segment during vascular responses towards pharmacological agents to be measured (Mulvany and Halpern, 1976). The chamber cover was then put on. The mounting protocol of vessel rings in myograph chamber is illustrated schematically in Figure 3.4.

Vessel ring was suspended in the myograph chamber filled with PSS of pH 7.4, with a maintained temperature of 37°C and continuous carbogen gas aeration throughout the experimental procedures. Once the vessel ring was mounted in the chamber, the ring was washed three times with PSS to remove excess blood. The vessel ring was then left to equilibrate in PSS for 30 minutes before performing normalization.

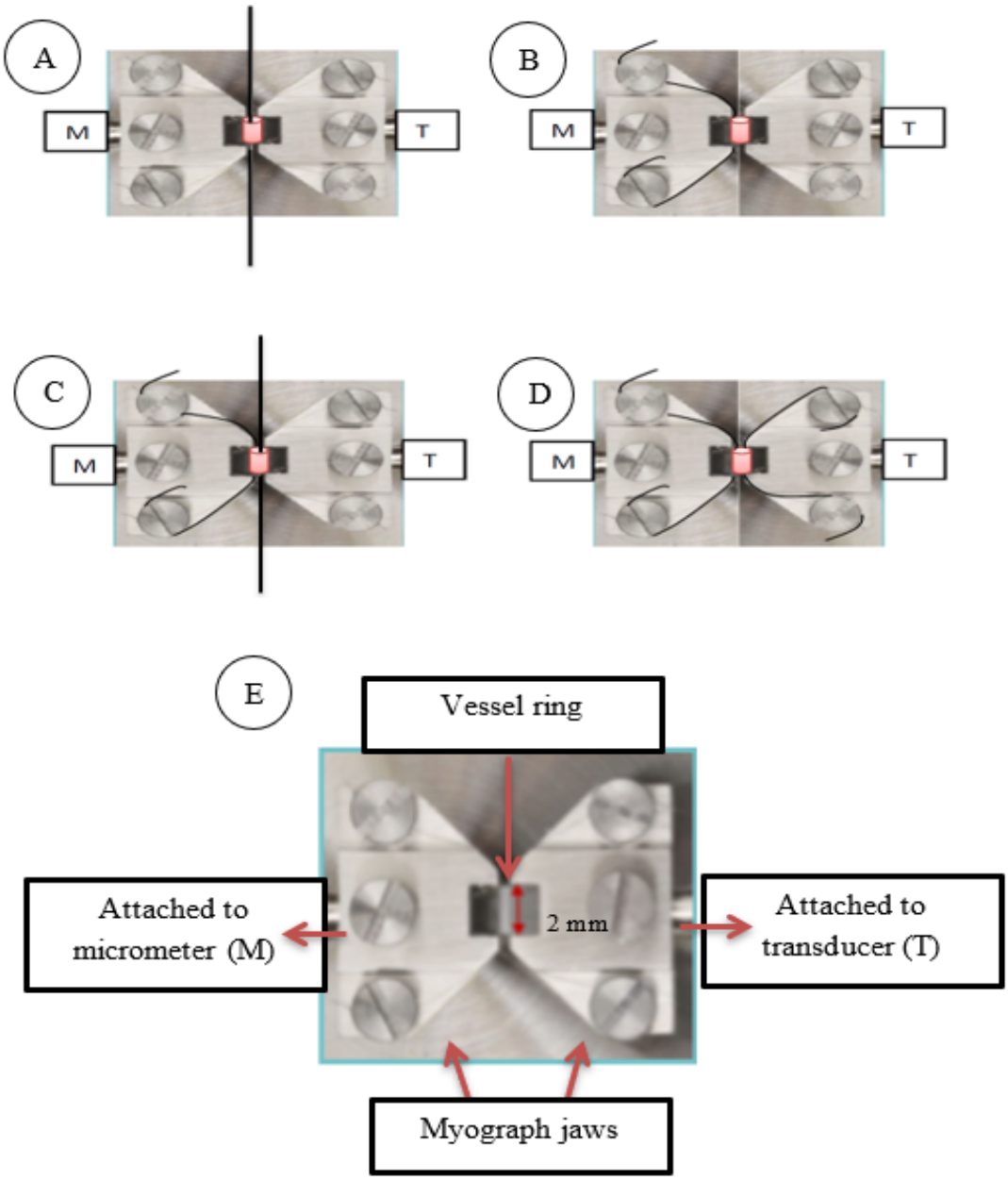


Figure 3.4 Schematic diagram of the mounting protocol of vessel rings in myograph chamber.

A. Arterial ring cannulated with first wire placed in the middle of the jaws.
B. The free ends of the wire were then secured tightly under the screws of the myograph jaw attached to a micrometer (M).
C. Second wire was inserted on top of the first wire.
D. The free ends of second wire were then secured tightly under the screws of the myograph jaw attached to a transducer (T).
E. Labelled diagram of myograph chamber with vessel ring mounted.

(Modified from https://www.dmt.dk/dual_wire_myograph_420a_520a.html - online Myograph User Guide)

3.2.5 Normalization

The purpose of normalization is to obtain the optimum degree of stretch for optimum vascular responses. The active response and sensitivity of the vessel to pharmacological stimulation depends on the degree of stretch. Less stretch renders the vessel in a less sensitive condition whereas excessive tension causes damage to the delicate EC and VSMC.

The normalization procedure was performed in accordance to the Normalization Module of the Chart Software (AD Instruments, UK). A series of stepwise passive stretches were performed on the vessel by adjusting the micrometer at small increments. Passive tension developed across the vessel wall was obtained by using a chart recorder. The stretch was continued at 8-minutes "stress-relaxation" intervals, until the internal circumference of the vessel was pre-determined at a given transmural pressure of 100 mmHg. Then, the vessel was stretched to achieve an internal circumference equivalent to 90% of pre-determined internal circumference. The passive tension-internal circumference of the vessel at its standard initial condition for optimal contraction was then determined (Mulvany and Halpern, 1976). The internal circumference was remained constant throughout the experimental protocols, so that vascular responses can be examined under isometric conditions.

3.2.6 Smooth Muscle Viability Test

The vessels were equilibrated for an hour after normalization. Following that, smooth muscle viability test was conducted before subsequent isometric tension measurement. The VSMC of vessel ring were tested for the contractile function by exposing the vessel ring to high concentrations of potassium ions. High exposure to potassium ions depolarizes the membrane of VSMC and activates the opening of

voltage-dependent calcium channels, resulting in an influx of extracellular calcium ions (Ca^{2+}). The influx of Ca^{2+} raises the intracellular Ca^{2+} levels in VSMC, thus inducing arterial contraction (Vanhoutte *et al.*, 2017).

In this procedure, the vessel in the myograph chambers was exposed to 60 mM KCl solution for 10 minutes or until the contraction was stable. Allowing at least 10-minutes interval alleviates the possibility that the vessels might spontaneously relax to baseline. The vessel is maximally contracted with KCl to ensure that the technique of artery isolation and mounting did not have any adverse effect on the viability of VSMC. VSMC was considered intact and experience no damage if a substantial contraction of at least 4 N in response to KCl achieved. Vessel segment that failed to substantially contract in response to KCl was discarded.

In addition, this procedure is also necessary to provide a reference contraction for subsequent vascular response study. This is in view that the exposure of vessel to KCl produces a regular and sustained contraction, enabling greater reproducibility when analysing contraction and relaxation subsequently. The solution in the myograph chamber was then removed by using a hand vacuum and replaced with fresh PSS. Vessel segment was allowed to relax to the baseline for 10 minutes. The application of KCl solution was then repeated for a second time.

3.2.7 Endothelial Function Test

This test is used to examine the integrity of the endothelium of vessel ring. This test is necessary in view that the delicate endothelium can be easily damaged during dissecting or mounting procedure. This test is able to give confirmation that the endothelium is intact and viable for the study evaluating endothelium-dependent

responses and also to confirm whether the endothelium is successfully removed for the study evaluating endothelium-independent responses.

The vessel ring was pre-constricted with a standard vasoconstrictor, PE (10^{-4} M) for 10 minutes or until a stable contraction is achieved (Mokhtar *et al.*, 2016). This procedure is to observe the contractile function of the vessel ring. PE induces vasoconstriction by acting on the α_1-adrenergic receptor located on VSMC. The contraction of the vessel to PE must reach at least 80% of KCl reference contraction, to be included in endothelium-dependent response study.

Once the PE contraction remained plateau, the vessel ring was then exposed to an endothelium-dependent vasodilator, ACh (10^{-4} M) to observe the relaxing ability of EC. ACh activates the muscarinic receptor located on EC, which stimulates the release of NO from the functioning endothelium; thus mediating endothelium-dependent relaxation. A normal functioning vessel that showed a substantial ACh-induced relaxation (greater than 60% of the pre-contracted tone) confirmed the endothelium as functionally intact and can be used for endothelium-dependent response study (Ang *et al.*, 2002). For endothelium-independent response study, a partial (less than 30% of the pre-contracted tone) or no relaxation in respond to ACh would be observed to verify successful removal of the endothelium (Prieto *et al.*, 2010).

Once the vessels reached maximum relaxation, the vessels were washed and incubated with fresh PSS. The washing procedures were repeated for at least three times to alleviate the problem of remaining pharmacological agents in the chamber that may cause spontaneous contraction or relaxation to the vessel. The vessel was then allowed to relax to baseline before conducting subsequent experimental protocols.

3.2.8 Vascular Responses Study

The protocol for vascular functional study in rats' mesenteric arteries is summarised in Table 3.5.

3.2.8(a) Endothelium-dependent Relaxation

Endothelium-dependent relaxation was investigated in a pre-constricted mesenteric artery with intact endothelium. In brief, vessel ring with intact endothelium was pre-constricted with the α_1-adrenergic receptor agonist, PE (10^{-4} M) to produce a sustained maximal contraction of the response curve. Then, the vessel ring was exposed to cumulative concentrations (10^{-8} to 10^{-4} M) of the muscarinic receptor agonist, ACh for relaxation (Wang *et al.*, 2000).

3.2.8(b) Endothelium-dependent Contraction

Endothelium-dependent contraction was studied in a quiescent mesenteric artery with intact endothelium. In brief, vessel ring with intact endothelium was incubated with $_L$-NAME (10^{-4} M) for 30 minutes, then exposed to cumulative concentrations of calcium ionophore (CaI) (10^{-8} to 10^{-4} M). $_L$-NAME is the inhibitor of eNOS; incubation with $_L$-NAME hinders the production of NO, eradicating NO-induced relaxation (Georgescu *et al.*, 2011). The increase in intracellular Ca^{2+} levels induced by CaI in turn evokes the release of cyclooxygenase products such as thromboxane A_2 and prostaglandin E_2 from EC, thus inducing vasoconstriction (Shi *et al.*, 2007).

3.2.8(c) Endothelium-independent Relaxation

Endothelium-independent relaxation was determined in a pre-constricted mesenteric artery with removed endothelium. In brief, vessel ring without endothelium was pre-constricted with PE (10^{-4} M), thereafter exposed to cumulative concentrations of the following relaxing agents:

a) Sodium nitroprusside, SNP (10^{-8} to 10^{-4} M)

SNP is an exogenous NO donor that induces vasorelaxation through cGMP pathway reaction.

b) Salbutamol hemisulfate, SB (10^{-8} to 10^{-4} M)

SB is an agonist of the β_2-adrenergic receptor located on VSMC that induces vasorelaxation through cAMP pathway reaction.

3.2.8(d) Endothelium-independent Contraction

Endothelium-independent contraction was studied in a quiescent mesenteric artery with removed endothelium. In brief, vessel ring without endothelium was subjected to cumulative concentrations of PE (10^{-8} to 10^{-4} M), an agonist of the α_1-adrenergic receptor located on VSMC (Rizzoni *et al*., 2001).

Table 3.5 Protocol for vascular functional study in rats' mesenteric arteries.

Study	**Endothelium-dependent relaxation**	**Endothelium-dependent contraction**	**Endothelium-independent relaxation**	**Endothelium-independent relaxation**	**Endothelium-independent contraction**
Endothelium	Intact	Intact	Removed	Removed	Removed
Standard procedures	Equilibration (30 minutes); Normalization; Equilibration (60 minutes) Smooth muscle viability test (60 mM potassium chloride, twice) Endothelial function test (10^{-4} M PE; then 10^{-4} M acetylcholine)				
Specific incubation	N/A	$_{L}$-NAME (30 minutes)	N/A	N/A	N/A
Pre-contraction	PE (10^{-4} M)	N/A	PE (10^{-4} M)	PE (10^{-4} M)	N/A
Cumulative concentrations	Acetylcholine (10^{-8} to 10^{-4} M)	Calcium ionophore (10^{-8} to 10^{-4} M)	Sodium nitroprusside (10^{-8} to 10^{-4} M)	Salbutamol (10^{-8} to 10^{-4} M)	Phenylephrine (10^{-8} to 10^{-4} M)
Roles of pharmacological agents	Agonist of the muscarinic receptor located on EC (NO inducer)	Transport Ca^{2+} across cell membrane of EC, increases intracellular Ca^{2+} levels in EC	Exogenous NO donor (cGMP pathway)	Agonist of the β_2-adrenergic receptor located on VSMC (cAMP pathway)	Agonist of the α_1-adrenergic receptor located on VSMC

[$_{L}$-NAME: L-NG-Nitroarginine methyl ester hydrochloride; PE: Phenylephrine; NO: nitric oxide; Ca^{2+}: Calcium ions]

3.2.9 Experimental Parameters

From the raw myograph tracing obtained during vascular responses study, the percentage of relaxation with reference to the contraction induced by PE (10^{-4} M) and the percentage of contraction with reference to the contraction induced by KCl (60 mM) for each concentration was calculated using Microsoft Excel template. The value obtained was used to generate the individual dose-response curve of each agonist using Prism Version 7 (GraphPad Software, La Jolla, California, USA). Maximal relaxation (R_{max}) or maximal contraction (E_{max}) were calculated from dose-response curves.

3.3 Total Protein Quantification

Total protein quantification was used to determine total protein concentrations in vascular tissue samples. It is reliably used to normalise the readings obtained from oxidative stress analysis, and also as the indicator to ensure equal loading of vascular tissue samples during Western blot. In this study, Protein Determination Kit (704002, Cayman Chemical, USA) was used for rapid total protein quantification based on the well-known Bradford method.

In brief, the principle of this assay is based on the binding of the Coomassie dye to the proteins in the samples in an acidic medium. This protein complex showed visible colour changes from brown to blue immediately. Absorbance at the wavelength of 595 nm as per manufacturer's suggestion was measured using Varioskan^{TM} Flash Multimode Reader (Thermo Fisher Scientific, USA).

3.3.1 Preparation of Working Solutions

i. Diluted assay reagent

To prepare diluted assay reagent (dye reagent), 7.5 mL of concentrated assay reagent was diluted to 50 mL with ddH_2O. The diluted assay reagent was stored at 4°C in an amber bottle prior to usage. The diluted assay reagent was brought to room temperature and was gently mixed before used.

ii. Bovine serum albumin standards

To prepare bovine serum albumin **(**BSA) standard stock solution (40 µg/mL), 4 µL of BSA stock solution (10 mg/mL in a 0.9% saline solution with 0.05% sodium azide) was diluted to 1 L with ddH_2O. This stock solution was then serially diluted with ddH_2O to prepare BSA standards (Standard A to H) with ascending concentrations according to the manufacturer's suggestion.

3.3.2 Preparation of Tissue Samples

The stored supernatant of mesenteric arterial tissue lysate of rats was further diluted with ddH_2O in pre-labelled microcentrifuge tube in the dilution ratio of 1:200 (based on preliminary study) to prepare animal tissue samples for assay.

3.3.3 Assay Procedures

Briefly, 100 µL of each BSA standard (Standard A to H) and diluted tissue sample was added in the designated well on a 96-wells plate, followed by the addition of 100 µL of diluted assay reagent to each well. The plate was incubated at room temperature for five minutes, followed by measuring the absorbance at the wavelength

of 595 nm using Varioskan™ Flash Multimode Reader (Thermo Fisher Scientific, USA).

3.3.4 Calculations

The corrected absorbance value was calculated by subtracting the absorbance value of Standard A (blank) from the absorbance value of other standards and samples. BSA standard curve was constructed by plotting BSA concentrations against the corrected absorbance values of the standards as a second-order polynomial curve fit. The equation obtained from the curve fit was used to determine the total protein concentrations (mg protein/mL) of each sample, by inserting the corrected absorbance value of each sample to the x-value of the equation, and then multiplied by the respective dilution factor.

3.4 Oxidative Stress Analysis

Oxidative stress levels were determined by measuring SOD and MDA concentrations in vascular tissue samples using commercially available assay kits according to the manufacturer's instructions.

3.4.1 Superoxide Dismutase Levels

Superoxide dismutase (SOD) acts as an important antioxidant defence enzyme against oxidative damage by catalysing the dismutation of superoxide anion into molecular oxygen and hydrogen peroxide. In this study, SOD concentrations in the tissue samples were quantitatively determined by using EnzyChrom™ Superoxide Dismutase Assay Kit (ESOD-100, BioAssay Systems, USA) to assess whether the effectiveness of antioxidant defence system was affected in vitamin D-deficient rats.

This study also aimed to explore whether calcitriol supplementation could ameliorate SOD enzyme activity in rats with vitamin D deficiency.

Briefly, the principle of SOD assay is based on the reaction between superoxide anion (generated by xanthine oxidase (XO) catalysed reaction) and tetrazolium salt (WST-1), to form a stable formazan dye. The increase in SOD levels scavenges superoxide anions that available for the chromogenic reaction, reducing the formation of formazan dye. Thus, lower optical density (OD) at the wavelength of 440 nm was obtained.

3.4.1(a) Preparation of Working Solutions

i. Working reagent

To prepare working reagent for each standard and tissue sample, Assay Buffer, Xanthine and WST-1 in the ratio of 160:5:5 were mixed well and kept in amber bottle prior to usage.

ii. Diluted XO enzyme

To prepare diluted XO enzyme, concentrated XO enzyme was diluted in Diluent (50 mM potassium phosphate, pH 7.4, supplied ready-to-use) in the dilution ratio of 1:20.

iii. SOD standards

SOD standard stock solution (3 U/mL) was prepared by mixing 8 µL of SOD enzyme with 392 µL of Diluent. This stock solution was serially diluted with Diluent to prepare SOD standards (S1 to S8) with descending SOD concentrations according to the manufacturer's suggestion.

3.4.1(b) Preparation of Tissue Samples

The stored supernatant of the mesenteric arterial tissue lysate of rats was further diluted with ice-cold lysis buffer in the dilution ratio of 1:20 (based on preliminary study) to prepare animal tissue samples for assay.

3.4.1(c) Assay Procedures

Briefly, 20 µL of SOD standards and tissue samples were added to the designated well of a clear flat-bottom 96-well plate. Then, 160 µL of working reagent was transferred into each well and mixed well by tapping the plate gently to avoid spillage. Immediately, 20 µL of diluted XO enzyme was added in and mixed well. Colorimetric quantification was performed by using Varioskan^{TM} Flash Multimode Reader (Thermo Fisher Scientific, USA) to obtain OD at 440 nm (initial read). The plate was covered with aluminium foil and incubated at room temperature for an hour on a shaker, followed by reading OD at 440 nm (final read).

3.4.1(d) Calculations

Net absorbance (ΔOD) of each standard and sample was obtained by subtracting initial read from final read. The corrected absorbance value (ΔΔOD) was calculated by subtracting ΔOD of other standards (S1 to S7) and samples from ΔOD of S8 (blank). SOD standard curve was constructed by plotting the ΔΔOD values of the standards against their SOD working standard concentrations. The following equation was used to determine SOD concentrations of each sample, at which the slope and y-intercept were obtained from the standard curve.

$$\text{SOD (U/mL)} = \left[\frac{(\Delta\Delta\text{OD})\text{-(y-intercept)}}{\text{slope}}\right] \text{x dilution factor}$$

One unit of SOD is defined as the amount of SOD needed to exhibit 50% dismutation of superoxide anion. The SOD concentrations (U/mL) for each sample were normalised to the total protein concentrations (mg protein/mL) of the same sample, expressed in U/mg protein.

3.4.2 Malondialdehyde Levels

Malondialdehyde (MDA) is the product of lipid peroxidation. It is one of the most commonly used markers to evaluate the oxidative degradation of lipids. In this study, MDA concentrations in vascular tissue samples were measured using NWLSS^{TM} Malondialdehyde Assay (NWK-MDA01, Northwest Life Science Specialty, USA), to study whether vitamin D deficiency in rats enhances the degree of lipid peroxidation. This study also aimed to assess whether oxidative degradation of lipids improved by calcitriol supplementation to vitamin D-deficient rats.

Briefly, the principle of MDA assay is based on the reaction between MDA (generated from the lipid peroxidation process) and two thiobarbituric acids (TBA), forming an MDA-TBA_2 complex that has the highest absorbance at the wavelength of 532 nm. The increase in the availability of MDA increases the formation of the complexes, subsequently showing higher absorbance at the wavelength of 532 nm.

3.4.2(a) Preparation of Working Solutions

i. TBA reagent

To reconstitute TBA reagent, TBA powder in the bottle was fully dissolved with 10.5 mL of ddH_2O, mixed well and kept at room temperature prior to usage.

ii. Ready-to-use reagents

Acid reagent (1M phosphoric acid), butylated hydroxytoluene (BHT) reagent, calibrators (0-4 μM MDA equivalent) and assay buffer (phosphate buffer, pH 7.0) were supplied ready-to-use.

3.4.2(b) Preparation of Tissue Samples

The stored supernatant of the mesenteric arterial tissue lysate of rats was further diluted with assay buffer in the dilution ratio of 1:200 (based on preliminary study) to prepare animal tissue samples for assay.

3.4.2(c) Assay Procedures

Briefly, 10 μL of BHT reagent, 250 μL of each calibrator (C0 to C4) or tissue sample, 250 μL of acid reagent and 250 μL of TBA reagent was added in sequence to a pre-labelled microcentrifuge tube. The tubes were recapped and vortexed using vortex mixer (IKA, Germany) at five counts to allow complete mixing. The mixtures were incubated in a water bath set at 60°C for an hour. Following incubation, the mixtures were centrifuged at 10,000 x *g* for three minutes. The yielded supernatant from each mixture was loaded into a quartz semi-micro cuvette. The absorbance at the wavelength of 532 nm and 572 nm (for maximum and minimum absorbance respectively) was measured by using UV spectrophotometer (Shimadzu Corp., Japan). Absorbance at 572 nm removed most of the undesired background to yield more accurate absorbance value according to the manufacturer's suggestion.

3.4.2(d) Calculations

Net absorbance value of each sample was obtained by subtracting the absorbance value at 572 nm from the absorbance value at 532 nm. The corrected absorbance value was calculated by subtracting the net absorbance value of C0 (blank) from the net absorbance value of other calibrators and samples. MDA standard curve was constructed by plotting the corrected absorbance value of the standards against their MDA working standard concentrations. The following equation was used to determine MDA concentrations of each sample, at which the slope and y-intercept were obtained from the standard curve.

$$\text{MDA } (\mu\text{M}) = \left[\frac{(\text{Corrected absorbance})\text{-}(\text{y-intercept})}{\text{slope}}\right] \text{ x dilution factor}$$

MDA concentrations (μM) were normalised to the total protein concentrations (mg protein/mL) of the same sample, expressed in μmol/mg protein.

3.5 Western Blot

Western blot is used in this study to assess the relative protein expression of eNOS, which is responsible for NO production. Reduced NO bioavailability may lead to functional abnormalities occurring in the microcirculation of diabetics. Hence, this study aimed to determine whether the effects of vitamin D deficiency on microvascular function in rats are related to the changes in protein expression of eNOS. In addition, this study also assessed whether calcitriol supplementation to vitamin D-deficient rats is able to improve the protein expression of eNOS.

3.5.1 Chemicals

Chemicals used in Western blot are listed in Table 3.6.

Table 3.6 Chemicals used in Western blot.

Chemicals	**Manufacturer, Country of Origin**
Ammonium persulfate	Bio-Rad Laboratories, USA
Tris(hydroxymethyl)-aminomethane (Tris-base)	Merck, Germany
Sodium dodecyl sulfate (SDS)	Bio-Rad Laboratories, USA
Glycine	Bio-Rad Laboratories, USA
Methanol	HmbG Chemicals, Germany
Ponceau-S	Sigma Aldrich, USA
Isopropanol	Sigma Aldrich, USA
Coomassie Brilliant Blue	Sigma Aldrich, USA
Acetic acid	Sigma Aldrich, USA
Sodium chloride	Merck, Germany

Tween-20	Sigma Aldrich, USA
Skimmed milk (Anlene)	Fonterra, NZ
Bovine serum albumin (BSA)	Agros Organic, USA
30% Acrylamide/bis-acrylamide solution (19:1)	Bio-Rad Laboratories, USA
TEMED	Sigma Aldrich, USA

3.5.2 Preparation of Working Solutions

i. Ammonium persulfate solution (10%)

To prepare 10% ammonium persulfate solution, 0.01 g of ammonium persulfate was fully dissolved in 100 µL of ddH_2O. This solution was freshly prepared and kept in a microcentrifuge tube wrapped with aluminium foil.

ii. Resolving buffer (pH 8.8)

To prepare resolving buffer, 18.16 g of Tris-base and 0.40 g of SDS were fully dissolved in 70 mL of ddH_2O. The solution was adjusted to pH 8.8. The buffer was then topped up with ddH_2O to make it 100 mL and stored at 4°C.

iii. Stacking buffer (pH 6.8)

To prepare stacking buffer, 6.05 g of Tris-base and 0.40 g of SDS were fully dissolved in 70 mL of ddH_2O. The solution was adjusted to pH 6.8. The buffer was then topped up with ddH_2O to make it 100 mL and stored at 4°C.

iv. Reducing loading buffer (1X)

Blue Loading Buffer Pack (#7722) was purchased from Cell Signaling Tech, USA. To reconstitute reducing loading buffer (1X), 0.1 mL of DTT reducing agent (30X) was added into 1.0 mL of blue loading buffer (3X), then topped up with ddH_2O to 2.0 mL. The buffer was freshly prepared prior to usage. Reducing loading buffer is used to lyse cells and reduce protein disulphide bonds for Western blotting analysis.

v. Running buffer (pH 8.3)

To prepare running buffer, 3.03 g of Tris-base, 14.40 g of glycine and 1.00 g of SDS were fully dissolved in 800 mL of ddH_2O. The solution was adjusted to pH 8.3. The buffer was then topped up with ddH_2O to make it 1 L and stored at 4°C.

vi. Transfer buffer (pH 8.3)

To prepare transfer buffer, 3.03 g of Tris-base and 14.40 g of glycine were fully dissolved in 800 mL of ddH_2O. The buffer was then topped up to 1 L with methanol. The solution was adjusted to pH 8.3 and stored at 4°C.

vii. Ponceau-S solution

To prepare Ponceau-S solution, 0.5 g of Ponceau-S powder was fully dissolved in 1 mL of isopropanol, then topped up to 100 mL with ddH_2O.

viii. Coomassie blue solution (stainer)

To prepare Coomassie blue solution, 1.0 g of Coomassie Brilliant Blue powder was fully dissolved in 125 mL of isopropanol, topped up to 450 mL with ddH_2O.

ix. Acid solution (de-stainer)

To prepare acid solution, 100 mL of acetic acid was added to 100 mL of isopropanol, then topped up to 1 L with ddH_2O.

x. Tris-buffered saline (TBS) (pH 7.4)

To prepare 1 L of TBS, 2.4 g of Tris-base and 8.8 g of sodium chloride were fully dissolved in 800 mL of ddH_2O. The solution was adjusted to pH 7.4. The buffer was then topped up to 1 L with ddH_2O.

xi. Tris-buffered saline + Tween-20 (0.1%) (TBS-T)

To prepare TBS-T, 1 mL of Tween-20 was added into 1 L of TBS, mixed well and stored at room temperature. TBS-T was used as wash buffer.

xii. Blocking buffer

To prepare blocking buffer (2% skimmed milk in TBS-T), 2.0 g of skimmed milk powder was fully dissolved in 100 mL of TBS-T. This solution was freshly prepared prior to usage. Blocking buffer was also used as the diluent of secondary antibody.

xiii. Bovine serum albumin (BSA) (3%)

To prepare 3% BSA, 0.3 g of BSA powder was fully dissolved in 10 mL of TBS-T. This solution was freshly prepared. BSA was used as the diluent of primary antibody.

3.5.3 Apparatus Set-up

The cassette was assembled by placing a small sterile glass plate in front of a large sterile glass plate, inserting them into a casting clamp and clamped together. The bottom of these two plates was well aligned before assembling the cassette onto the casting stand. Sterile water was applied between two glass plates and allowed to stand for 30 minutes to check whether the set-up was tightly sealed with no leakage. At last, water was removed and dried completely.

3.5.4 Preparation of SDS-polyacrylamide Gel

Preparation of SDS-polyacrylamide gel was performed according to the method developed by Laemmli (1970). Resolving and stacking gels were prepared according to the composition of chemicals as listed in Table 3.7.

Table 3.7 Preparation of stacking and resolving gels.

Chemicals	**Resolving Gel (10%)**	**Stacking Gel (4%)**
	Volume (μL)	Volume (μL)
30% acrylamide/bis-acrylamide	2500	650
Resolving buffer	1875	-
Stacking buffer	-	1250
Double distilled water	3125	3150
Ammonium persulfate (10%)	50	25
TEMED	12	8

(Gel with 1.0 mm thickness)

To prepare resolving gel, all desired chemicals were mixed well, with ammonium persulfate and TEMED added at last. The mixture was then immediately loaded into the space between the glass plates to prevent undesired polymerisation. The mixture was loaded until the marked level, followed by relaying the top layer with sterile water. Water was relayed slowly as rushing may cause irregular shapes formed on the top surface of the resolving gel, that might incur unnecessary resistance. The resolving gel was left for 45 minutes to polymerise. The topping water was removed completely once the gel was set.

Following that, the mixture to prepare stacking gel was loaded on the top surface of the resolving gel. A preparatory comb was immediately inserted, and the stacking gel was left for 45 minutes to polymerise. Once the gel was set, the comb was gently removed, and the wells were cleaned with water to ensure the complete removal of acrylamide residue. Water and bubbles in the wells were drained completely.

3.5.5 Preparation of Tissue Samples

Total protein mass of tissue samples to be loaded was fixed at 30 µg (based on preliminary study) to ensure equal loading of each sample into the well. Total protein concentrations of tissue samples were quantified as described in Section 3.3. The loading volume of the stored supernatant of mesenteric arterial tissue lysate of rats was determined from the following formula:

$$\text{Loading volume } (\mu L) = \left[\frac{\text{total protein mass (30 } \mu g)}{\text{total protein concentrations (mg/mL)}}\right]$$

An equal amount of reducing loading buffer was mixed well with the tissue samples in the ratio of 1:1. The mixture was vortex mixed for two minutes, followed by heating to 95ºC for five minutes and was centrifuged at 12,000 *x* g for five minutes.

3.5.6 SDS-polyacrylamide Gel Electrophoresis

The glass plates containing set gel were removed from the casting clamp and placed into an electrode assembly with small plate facing inward and balance it with a plastic buffer dam on the other side (with the grove surface facing outward). The plates were clamped together and put in Mini-PROTEAN® Tetra Cell (Bio-Rad Laboratories, USA) according to the correct polarities. Running buffer was slowly poured into the small chamber to test for leakage, then into the big chamber to cover the plates up to the line marked 2-gels. Excess bubbles formation should be avoided as it might create more resistance during electrophoresis.

First well of the gel was loaded with 5 µL of 3-colour broad range (3.5 kDa to 245 kDa) protein marker (PM 2700, SMOBIO, USA) as protein ladder. The tissue samples (excluding pellet) were loaded to the remaining wells. The tank was covered taking note of the polarity, and the gel electrophoresis was performed at 100 volts for approximately 1.5 hours or until the dye front reached the bottom of the glass plates.

3.5.7 Protein Transfer

When electrophoresis was completed, the cassettes were removed and dissembled. A plastic blade was used to separate the glass plates. The stacking gel was gently removed and discarded, while the resolving gel was ready for blot 'sandwich' preparation.

Immobilon-P Polyvinylidene Difluoride (PVDF) membrane (Merck, Germany) was cut according to the size of blotting filter paper. PVDF membrane was pre-treated with methanol to activate the membrane for better protein adsorption. PVDF membrane and blotting filter papers were then soaked in separate container

filled with pre-chilled transfer buffer and placed on a shaker for five minutes (Scotsman Ice Systems, USA).

The flat surface of Trans-blot® SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, USA) was wet evenly with transfer buffer. Blot 'sandwich' was stacked up at the centre in the sequence of blotting filter paper at the bottom, followed by PVDF membrane, resolving gel and another blotting filter paper covered on the top. Plastic roller was used to roll on the blot 'sandwich' to remove bubbles trapped between the layers. Tissue paper was used to wipe off excess buffer surrounding the blot 'sandwich'. The apparatus was covered and connected to the terminals of PowerPac^{TM} Basic Power Supply (Bio-Rad Laboratories, USA) to transfer the proteins from gel to PVDF membrane at 15 volts for approximately 1.5 hours.

3.5.8 Staining of Membrane and Gel

Once the process of protein transfer was completed, PVDF membrane was removed and stained with Ponceau-S solution for approximately a minute, and then de-stained with sterile water slowly until the bands are visible. This step is important as it helps to identify the location of the desired protein according to the protein marker. A line was drawn horizontally just below the red colour protein band, and the membrane was cut into two parts horizontally along the line. The membrane was then de-stained thoroughly to remove Ponceau-S residue. The top part consisted of red colour protein band (protein size of 75 kDa) and the bottom part consisted of green colour protein band (protein size of 25 kDa) were ready for subsequent antibody incubation to identify the desired protein with different protein size.

The gel was stained with Coomassie blue solution for an hour on a shaker. Coomassie blue solution was then removed, followed by incubation with acid solution

for an hour to de-stain the gel. The acid solution was removed and replaced for few times until the background turned clear. This step is to ensure the protein transfer process is completed without protein leftover.

3.5.9 Blocking and Antibodies Incubation

The cut PVDF membrane was incubated in blocking buffer at room temperature for an hour on a shaker. Blocking process helps to block the remaining membrane surface from non-specific binding of any detection antibodies during incubation. The membrane was washed with TBS-T for five minutes three times.

Primary antibody was diluted in BSA with the dilution of 1:1000 for mouse monoclonal to eNOS (AB 76198, Abcam, UK) and 1:5000 for mouse monoclonal to ß-actin (AB 8226, Abcam, UK) (Mokhtar *et al.*, 2016). The top part of the membrane was incubated with anti-eNOS antibody to identify eNOS with protein size of 133 kDa, while bottom part was incubated with anti-beta actin antibody to identify ß-actin with protein size of 42 kDa. Beta actin was used as the loading control to normalise for the amount of protein. The membrane was incubated at 4ºC for 16 hours on a shaker, followed by washing with TBS-T for five minutes three times.

Secondary antibody was diluted in blocking buffer with the dilution of 1:1000 for eNOS and 1:2000 for ß-actin (Mokhtar *et. al.*, 2016). The membrane was incubated with horseradish peroxidase (HRP)-conjugated goat polyclonal secondary antibody at room temperature for an hour on a shaker. The membrane was washed with TBS-T five minutes three times and ready for subsequent visualization.

3.5.10 Visualization of Protein Expression

Chemiluminescent HRP substrate (Nacalai Tesque, Japan) was used for protein band visualization. Substrate A and B were mixed in the ratio of 1:1 before transferring the mixture onto the membrane placed on a transparent sheet. The sheet was slightly agitated to ensure the whole surface of the membrane was soaked with the mixture. Excess solution was drained, followed by covering the sheet and with all visible bubbles removed.

Chemiluminescent exposure to the membrane for 10 seconds was performed using Fusion Molecular Imager (FX-7.826.WL, Vilber Lourmat, France) to obtain the protein bands with visible intensity. A single band of eNOS with protein size of approximately 133 kDa and another single band of ß-actin with protein size of approximately 42 kDa were visible. The intensity of the protein bands was measured with Image J software version 1.8.0_112. The intensity of eNOS protein band represents the amounts of eNOS in the sample while the intensity of ß-actin protein band indicates the amount of total proteins in the same sample (Sigumura *et al.*, 2010). Relative protein expression of eNOS normalised to the amount of total proteins was calculated according to the following equation.

$$\text{Relative protein expression of eNOS} = \left[\frac{\text{Amount of eNOS}}{\text{Amount of total proteins}}\right]$$

3.6 Immunohistochemistry

Immunohistochemistry is conducted in this study to demonstrate the presence and localization of a specific protein in vascular tissue sections. This method is accomplished with a highly specific antibody that binds only with targeted antigens/proteins. The antibody-antigen relationship is then visualized using chromogenic detection. The outcomes from immunohistochemistry coupled with Western blot provide a better picture to explain the underlying mechanisms related to the effects of vitamin D deficiency on microvascular functional properties in rats, particularly regarded to the presence and localization of eNOS. Besides that, whether calcitriol supplementation improves the presence and localization of eNOS after a period of vitamin D deficiency was also examined.

3.6.1 Chemicals

Chemicals used in immunohistochemistry are listed in Table 3.8.

Table 3.8 Chemicals used in immunohistochemistry.

Chemicals	Manufacturer, Country of Origin
Formaldehyde solution	Mallinckrodt Chemicals, USA
Xylene	Merck, Germany
Absolute ethanol	HmbG Chemicals, Germany
Trisodium citrate dehydrate	Sigma Aldrich, USA
Phosphate buffered saline (PBS) tablets	Sigma Aldrich, USA
3,3'-Diaminobenzidine (DAB)	Invitrogen, USA
Harris hematoxylin solution	Labchem, Malaysia
Dibutyl phthalate (DPX) mountant	VWR International, UK

3.6.2 Preparation of Working Solutions

i. Formalin solution (10%, pH 7.4)

To prepare 100 mL of 10% formalin solution, 10 mL of formaldehyde solution was diluted to 100 mL with ddH_2O and mixed well. The solution was adjusted to pH 7.4.

ii. Ethanol solution (95%)

To prepare 100 mL of 95% ethanol solution, 95 mL of absolute ethanol (100%) was mixed well with 5 mL of ddH_2O.

iii. Ethanol solution (80%)

To prepare 100 mL of 80% ethanol solution, 80 mL of absolute ethanol (100%) was mixed well with 20 mL of ddH_2O.

iv. Ethanol solution (70%)

To prepare 100 mL of 70% ethanol solution, 70 mL of absolute ethanol (100%) was mixed well with 30 mL of ddH_2O.

v. Ethanol solution (50%)

To prepare 100 mL of 50% ethanol solution, 50 mL of absolute ethanol (100%) was mixed well with 50 mL of ddH_2O.

vi. Phosphate buffered saline (pH 7.4)

To prepare 1 L of phosphate buffered saline **(**PBS), 5 tablets of PBS was fully dissolved in 1 L of ddH_2O. The solution was adjusted to pH 7.4.

vii. Sodium citrate buffer (0.01 M, pH 6.0) + Tween-20 (0.05%)

To prepare 0.01 M sodium citrate buffer, 2.94 g of trisodium citrate dehydrate was fully dissolved in 1 L of ddH_2O. The solution was adjusted to pH 6.0. 500 µL of Tween-20 was added into the solution, mixed well and stored at room temperature.

viii. Tris-buffered saline + Tween-20 (0.1%) (TBS-T)

To prepare 1 L of TBS-T, 2.4 g of Tris-base and 8.8 g of sodium chloride were fully dissolved in 1 L of ddH_2O. The solution was adjusted to pH 7.6. 1 mL of Tween-20 was added into the solution and mixed well. TBS-T was used as wash buffer and also in the preparation of blocking buffer.

ix. Blocking buffer

To prepare 5% blocking buffer (5% skimmed milk in TBS-T), 5.0 g of skimmed milk powder was fully dissolved in 100 mL of TBS-T. This solution was freshly prepared prior to usage. Blocking buffer was used as the diluent for antibodies.

3.6.3 Experimental Protocols

3.6.3(a) Tissue Fixation, Embedding and Sectioning

The harvested mesenteric arteries of the rats which had been cleaned of excessive fat and connective tissues were rinsed with ice-cold PSS to remove blood residue prior to tissue fixation. This precaution step is to prevent the detection of haematological antigens that might interfere with the detection of the targeted antigens. The arteries were fixed in 10% formalin solution at room temperature for 16 hours. The fixed arterial tissues were then cut into a 5-mm-thick section, wrapped with gauze

and enclosed in a pre-labelled tissue processing cassette, ready to be processed in an automatic tissue processor (TP 1020, Leica, Germany).

The processed tissues were subsequently embedded in paraffin wax on the tissue embedding centre (Thermo Fisher Scientific, USA), left to solidify on a cold plate (Thermo Fisher Scientific, USA) to yield the paraffin-embedded tissue blocks. The blocks were pre-chilled, followed by sectioning into a 4-µm-thick tissue section using a rotary microtome (DM 2235, Leica, Germany). The sectioned tissues were flattened on a tissue floatation bath (HI 1210, Leica, Germany) set at 40ºC and subsequently mounted onto the surface of a clean pre-labelled poly-L-lysine slide. The slides were placed on a slide warmer (Thermo Fisher Scientific, USA) set at 50°C for an hour, followed by an overnight air-drying at room temperature and kept for subsequent immunohistochemical staining.

3.6.3(b) Heat Antigen Retrieval

The tissue slides were heated on a slide warmer set at 60°C for an hour to increase tissue adherence to the slides. The slides were arranged in a glass rack, and the paraffin-embedded tissue sections underwent deparaffinization in xylene and serial rehydration in descending grades of alcohol, ready for heat antigen retrieval. Heat antigen retrieval is used to reverse the loss of antigenicity of the tissues during formalin fixation and paraffin block embedding.

In brief, a glass beaker with sodium citrate buffer was heated in a water bath (Memmert, Germany) set at 95°C. The slides were arranged surrounding the inner side of the beaker and incubated for 30 minutes. The beaker was left to cool in a basin filled with running tap water for 30 minutes. The slides were placed back in the glass

rack, immersed in ddH_2O for five minutes and in TBS-T for 10 minutes three times, ready for primary antibody incubation.

3.6.3(c) Antibodies Incubation

Primary antibody against eNOS was diluted in blocking buffer in the dilution ratio of 1:100. Tissue slides arranged on a staining tray were incubated with primary antibody at room temperature for two hours on a slow agitating shaker. The slides were then placed back in the glass rack, immersed in ddH_2O for five minutes and in TBS-T for 10 minutes three times, ready for secondary antibody incubation.

HRP-conjugated secondary antibody was diluted in blocking buffer in the dilution ratio of 1:200. Tissue slides arranged back on a staining tray were incubated with secondary antibody at room temperature for an hour on a slow agitating shaker. The slides were then placed back in the glass rack, immersed in ddH_2O for five minutes and in TBS-T for 10 minutes three times, ready for tissue staining.

3.6.3(d) Tissue Staining

Tissue slides arranged back on a staining tray were incubated with DAB substrate for five minutes. The presence of a reddish brown-coloured product on the stained tissues is the result of the reaction between HRP and DAB substrate, for the detection and visualization of protein. The slides were then placed back in the glass rack, immersed in ddH_2O, TBS-T and ddH_2O for three minutes each, proceeding to serial dehydration in a series of ascending grades of alcohol solutions and finally cleared in xylene.

3.6.3(e) Slides Mounting and Visualization

The stained tissue sections were then mounted under a coverslip using DPX mountant and ready for microscopy visualization. The stained tissue sections were examined under a light microscope equipped with an image analyser and digital camera (Olympus Corp, Japan) to capture photomicrographs. The visual intensity of reddish-brown colour was used to qualitatively demonstrate the presence and localization of the targeted protein on the tissue samples. A brief summary of general steps for immunohistochemical staining is illustrated in Figure 3.5.

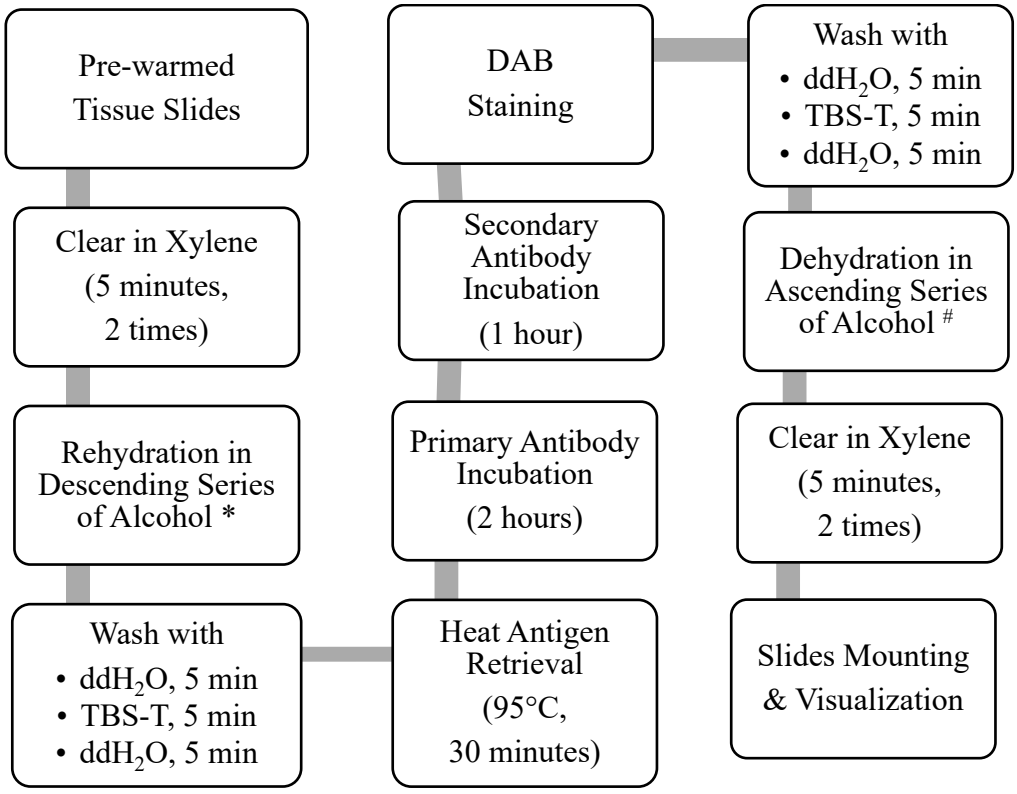


Figure 3.5 General steps for immunohistochemical staining.

*Descending series of alcohol: 100% (2 minutes, 2 times), 95% (2 minutes), 80% (2 minutes), 70% (2 minutes), 50% (2 minutes)

#Ascending series of alcohol: 50% (2 minutes), 70% (2 minutes), 80% (2 minutes), 95% (2 minutes), 100% (2 minutes, 2 times)

[ddH_2O: double distilled water; TBS-T: Tris-buffered saline + Tween-20, DAB: 3'3-diaminobenzidine].

3.7 Measurement of Serum 25(OH) D Levels

Serum concentrations of 25(OH)D is considered as the most reliable measure of overall vitamin D status. Serum 25(OH)D concentrations of rats and patients were measured using 25-Hydroxy Vitamin D^{s} Enzyme Immunoassay Assay Kit (AC-57SF1, IDS, UK) as per manufacturer's protocol.

The principle of this kit is based on the selective binding of HRP-labelled avidin to 25(OH)D in acidic medium, followed by detection with a chromogenic substrate. The absorbance at the wavelength of 450 nm (with reference to 650 nm) was obtained to develop a four-parameter logistic (4PL) curve fit using online MyAssay software (https://www.myassays.com).

3.7.1 Preparation of Working Solutions

i. 25(OH)D-biotin solution

All reagents were brought to room temperature prior to usage. Buffer (3 mL) was added to the lyophilised 25(OH)D labelled with biotin. The solution was mixed well to dissociate 25(OH)D from binding proteins. The reconstituted biotin concentrate was added back to the remaining buffer to make 25(OH)D-biotin solution. The solution was mixed well and stored at 4ºC in an amber bottle.

ii. Ready-to-use reagents

Calibrators and Controls (serum containing 25(OH)D and 0.09% sodium azide), Assay Buffer (proprietary reagent for dissociating 25(OH)D from binding proteins), Enzyme Conjugate (avidin linked to HRP), Acid Solution (0.5 M

hydrochloric acid) and TMB Substrate (aqueous tetramethylbenzidine and hydrogen peroxide) were supplied ready-to-use.

iii. Wash buffer

To prepare 1L of wash buffer (1X), 50 mL of wash concentrate (phosphate buffered saline containing Tween, 20X) was diluted with ddH_2O, mixed well and stored at room temperature.

3.7.2 Preparation of Serum Samples

Blood serum obtained from rats was further diluted with Assay Buffer in the dilution ratio of 1:5 (based on preliminary study) to prepare serum sample for assay.

3.7.3 Assay Procedures

Briefly, 25 µL of each calibrator, control and serum sample was added to a pre-labelled microcentrifuge tube, followed by the addition of 1 mL of 25(OH)D-biotin solution, and vortexed thoroughly for 10 seconds. Then, 200 µL of calibrators (C1 to C6), controls (Ctrl 1 and Ctrl 2) and serum samples were loaded into the designated well of the microplate coated with 25(OH)D sheep polyclonal antibody. The plate was covered with an adhesive plate sealer and incubated at room temperature for two hours on a shaker. After incubation, the wash step was performed for three times; inverting the plate sharply to decant the contents, followed by dispensing 250 µL of wash buffer to all wells. The inverted plate was tapped firmly on an absorbent tissue to remove excess wash buffer.

A multichannel pipette was used to add 200 µL of Enzyme Conjugate to all wells. Then, the plate was covered with adhesive plate sealer and incubated at room

temperature for 30 minutes on a shaker. Wash step as mentioned previously was done for three cycles. Following that, 200 µL of TMB Substrate was added to all wells using a multichannel pipette to avoid lapses in reaction time. Then, the plate was covered with adhesive plate sealer and incubated for another 30 minutes on a shaker. When the colour of the contents in the wells was visible, the reaction was stopped immediately by adding 100 µL of Acid Reagent to all wells. As soon, the absorbance of the mixtures was measured using Varioskan™ Flash Multimode Reader (Thermo Fisher Scientific, USA) set at the wavelength of 450 nm (with the reference absorbance at 650 nm).

3.7.4 Calculations

The net absorbance value of each sample was obtained by subtracting the absorbance value at 650 nm from the absorbance value at 450 nm. The corrected absorbance value was calculated by subtracting the net absorbance value of C0 (blank) from the net absorbance value of other calibrators, controls and samples. A 4PL curve fit was developed by constructing the corrected absorbance value of the standards against their respective 25(OH)D concentrations. The curve-fit equation generated was used to calculate the serum concentration of each sample based on their respective corrected absorbance values, expressed in SI units of nmol/L.

3.8 Sample Size Calculations

The sample size for vascular response study and oxidative stress analysis of animal study was shown in Table 3.9. Calculation of sample size for animal study was performed using two means statistical formula based on the method developed by Florey (1993).

$$n = 2 (Z_\alpha + Z_\beta)^2 s^2/d^2$$

$Z_\alpha = 1.96$ for the constant of α at 5% level of significance (two-tailed)

$Z_\beta = 1.28$ for the constant of β at 90% of study power.

[n: sample size for each group; s: standard deviation of the measured variable; d: difference of interest]

Table 3.9 Sample size calculations for vascular responses study and oxidative stress analysis of animal study.

Study	Difference of Interest	Standard Deviation	References	Sample Size
Vascular responses	14.24%	9.27%	Mokhtar *et al.*, 2016	8.89 ≈ 9
Details: Difference of interest was the R_{max} mean difference in ACh-induced endothelium-dependent relaxation between diabetic and control rats. Standard deviation for ACh-induced R_{max} was taken from diabetic rats. (*p* value = 0.001)				
Oxidative Stress (MDA Levels)	40µmol/g protein	22µmol/g protein	Coskun *et al.*, 2005	6.35 ≈ 7
Details: Tissue MDA levels. Difference of interest was the mean difference in tissue MDA concentrations between diabetic and control rats. Standard deviation for tissue MDA concentrations was taken from diabetic rats. (*p* value < 0.05)				
Oxidative Stress (SOD Levels)	0.51U/mg protein	0.34U/mg protein	Aydin and Celik, 2012	9.34 ≈ 10
Details: Tissue SOD levels. Difference of interest was the mean difference in tissue SOD concentrations between diabetic and control rats. Standard deviation for tissue SOD concentrations was taken from control rats. (*p* value < 0.005)				

3.9 Statistical Analysis and Data Presentation

Statistical analysis was performed using the IBM Statistical Package for the Social Sciences (SPSS) software version 24.0 (Armonk, NY, USA). The normality of data distribution was determined using both statistical test and descriptive methods involving visual inspection of the normality of data. Shapiro-Wilk test was preferred in this study compared to Kolmogorov-Smirnov test as Shapiro-Wilk test yielded higher study power and is an appropriate significance test for the normality of data involving a smaller sample size (Thode, 2002). The frequency distribution (histogram), stem-and-leaf plot, boxplot, P-P plot (probability-probability plot) and Q-Q plot (quantile-quantile plot) were the descriptive methods used to perform visual inspection of the normality of data (Field, 2009). Normally distributed data were presented as mean±standard deviation (SD) in the table and text. The data were presented as mean±standard error of mean (SEM) in the figure. Non-normally distributed data were expressed as median±interquartile range (IQR). In all cases, the *p* value of less than 0.05 was considered as statistically significant.

Levene's test was used to determine the homogeneity of variance to ensure the variance is equal across groups before performing parametric or non-parametric test. If the assumption of the homogeneity of variance is fulfilled, the differences of the mean between the groups in normal and diabetic rats respectively were analysed using one-way analysis of variance (ANOVA). If the test is significant, Bonferroni's post-hoc test was performed to determine the mean differences between each pair. If the assumption is violated, non-parametric test is more appropriate to be conducted. The differences of the median between the groups were analysed using Kruskal-Wallis test.

If the test is significant, Dunn's post hoc test was performed to compare the median differences between each pair.

In animal study, the outcomes of all parameters were presented and analysed into two parts; (i) in the microcirculation of normal rats and (ii) in the microcirculation of diabetic rats. In the microcirculation of normal rats, the effects of vitamin D deficiency were studied by comparing between groups NC and ND. Groups ND and NDS were compared to determine if there was any improvement in the same parameter by calcitriol supplementation. Groups NC and NDS were also compared to study whether vitamin D supplementation is able to normalise the values of the parameters studied.

Similarly, groups DC and DD were compared to determine the effects of vitamin D deficiency in diabetic rats, while DD and DDS groups were compared to study if there was any reversibility in the same parameter in the microcirculation of vitamin D-deficient diabetic rats by calcitriol supplementation. Groups DC and DDS were also compared to determine whether vitamin D supplementation normalised the values. There was no direct comparison made between normal and diabetic rats with different vitamin D status. However, any valuable finding on the pattern of studied parameters showed by normal and diabetic rats was discussed briefly.

3.10 Human Study

3.10.1 Ethical Approval

The ethical approval for human study was obtained from the Human Research Ethics Committee, USM, Health Campus, Kelantan (No. USM/JEPeM/15100337) (Appendix B). This cross-sectional study was conducted according to the provisions of the Declaration of Helsinki.

3.10.2 Patients

The study subjects were recruited from diabetic patients who needed to undergo lower limb surgical procedures by Orthopaedic Department, Hospital Universiti Sains Malaysia (HUSM), Kelantan.

3.10.2(a) General Information and Physical Evaluation

After recruitment, general information of the subjects such as age, gender, disease duration, previous medical conditions and medication history, current medical illness and on-going medication list were obtained and recorded in a case report form (Appendix D). Physical evaluation included measurements of weight and height for body mass index (BMI) determination, measurements of systolic (SBP) and diastolic (DBP) blood pressures were also conducted and recorded for all subjects.

3.10.2(b) Inclusion and Exclusion Criteria

All subjects needed to fulfil inclusion and exclusion criteria as listed in Table 3.10 to be accepted into this study. The accepted participants were required to give written informed consent after the research details have been explained to them prior to surgical procedures.

Table 3.10 Inclusion and exclusion criteria for diabetic patients' recruitment.

Inclusion Criteria	Exclusion Criteria
Aged between 18 to 65 years old.	Had uncontrolled hypertension (brachial blood pressure > 160/90 mmHg).
Diagnosed with diabetes (HbA_{1c} > 6.5%) for at least 10 years.	Had medical history of myocardial infarction and coronary heart disease.
Needed to undergo lower limb orthopaedic surgical procedures.	Had renal or hepatic failure.
Agreed to sign the informed consent form.	Underwent surgery for tumour tissue removal.
	Had taken or taking vitamin D and/or calcium as supplements within a year

At the beginning, a total of 50 diabetic patients were recruited to participate. Of these, only 23 patients who fulfilled the inclusion and exclusion criteria and agreed to sign the informed consent form (Appendix C) were included in this study.

3.10.3 Experimental Design

3.10.3(a) Blood Sample Collection

A day before the surgery, venous blood samples were collected from the subjects into a plain Vacutainer tube for the assay of serum 25(OH)D levels. The blood samples were allowed to clot at 4ºC before being centrifuged at 4000 x *g* at 4ºC for 10 minutes. The serum yielded was collected and stored at -80ºC before subsequent measurement of 25(OH)D concentrations using an assay kit.

For the assessment of other parameters such as FBG, glycated haemoglobin (HbA_{1c}) levels, calcium and total cholesterol levels, latest results (not longer than three

months) were extracted from their medical records prior to surgery. If the latest result is not available for the patient, the blood samples collected from the subjects were aliquoted and sent to HUSM laboratory for the measurements of these parameters.

3.10.3(b) Specimen Collection and Subcutaneous Artery Isolation

During lower limb surgical procedures such as below knee amputation, above knee amputation, ray amputation, wound debridement and skin grafting, subcutaneous fat tissue sample was collected from the removed parts of the subjects by the orthopaedic surgical team from Orthopaedic Department, HUSM, Kelantan. The amount of tissue sample collected varies depending on the types of surgical procedures conducted. A piece of normal and healthy tissue sample, away from the infected area and with intact blood vessels, with the dimension of approximately 2 cm (length) x 2 cm (width) x 2 cm (thickness), was preferred in this study. However, there were cases of less tissue could be taken as the area of surgery was limited, particularly during wound debridement and ray amputation. In that case, relatively smaller pieces of tissue samples were also acceptable. The non-infected tissue sample harvested was confirmed by the visualization of normal and healthy appearance (absence of signs of gross infection such as pus or slough), while the presence of blood vessels was identified by good bleeding from the tissue.

The specimen was immediately placed in a plastic container filled with ice-cold, oxygenated PSS and transferred to Pharmacology Vascular Laboratory for microscopy dissection. Subcutaneous arteries with luminal diameter of less than 200 μm were isolated with gentle care to ensure the viability of the vessels for subsequent measurement of oxidative stress parameters (SOD and MDA levels). The isolated subcutaneous artery was further dissected to remove excess fat and connective tissues.

3.10.3(c) Tissue Lysate and Supernatant Preparation

Isolated subcutaneous artery which had been cleaned of excess fat and connective tissues was rinsed with PSS for three times to remove excess blood that might interfere with the study outcome. The artery was then finely chopped in a microcentrifuge tube filled with 100 μL of pre-chilled lysis buffer on ice using dissecting scissors to prepare subcutaneous arterial tissue lysate. The tissue lysate was incubated at 4ºC for 10 minutes, followed by centrifugation at 12,000 x *g* at 4 ºC for five minutes. The yield supernatant was kept at -80°C for subsequent experimental protocols of total protein quantification and oxidative stress analysis.

3.10.3(d) Group Division

After sufficient collection of samples from study subjects, the measurement of serum 25(OH)D levels was performed. Based on the levels of serum 25(OH)D, the study subjects were categorised into two groups: vitamin D non-deficient diabetic patients (DNP) and vitamin D-deficient diabetic patients (DDP). As defined by the Endocrine Society of USA, subjects with serum 25(OH) D levels of < 50 nmol/L were considered as vitamin D-deficient (Holick *et al.*, 2011). Subjects with serum 25(OH)D concentrations of ≥ 50 nmol/L were regarded as vitamin D non-deficient. In this study, 10 subjects who had serum 25(OH)D levels of ≥ 50 nmol/L were categorised into group DNP; while 13 subjects who had serum 25(OH)D levels of < 50 nmol/L were categorised into group DDP. After group division, the measurements of oxidative stress parameters (MDA and SOD levels) were then conducted. The detailed flow chart of the human study is illustrated in Figure 3.6.

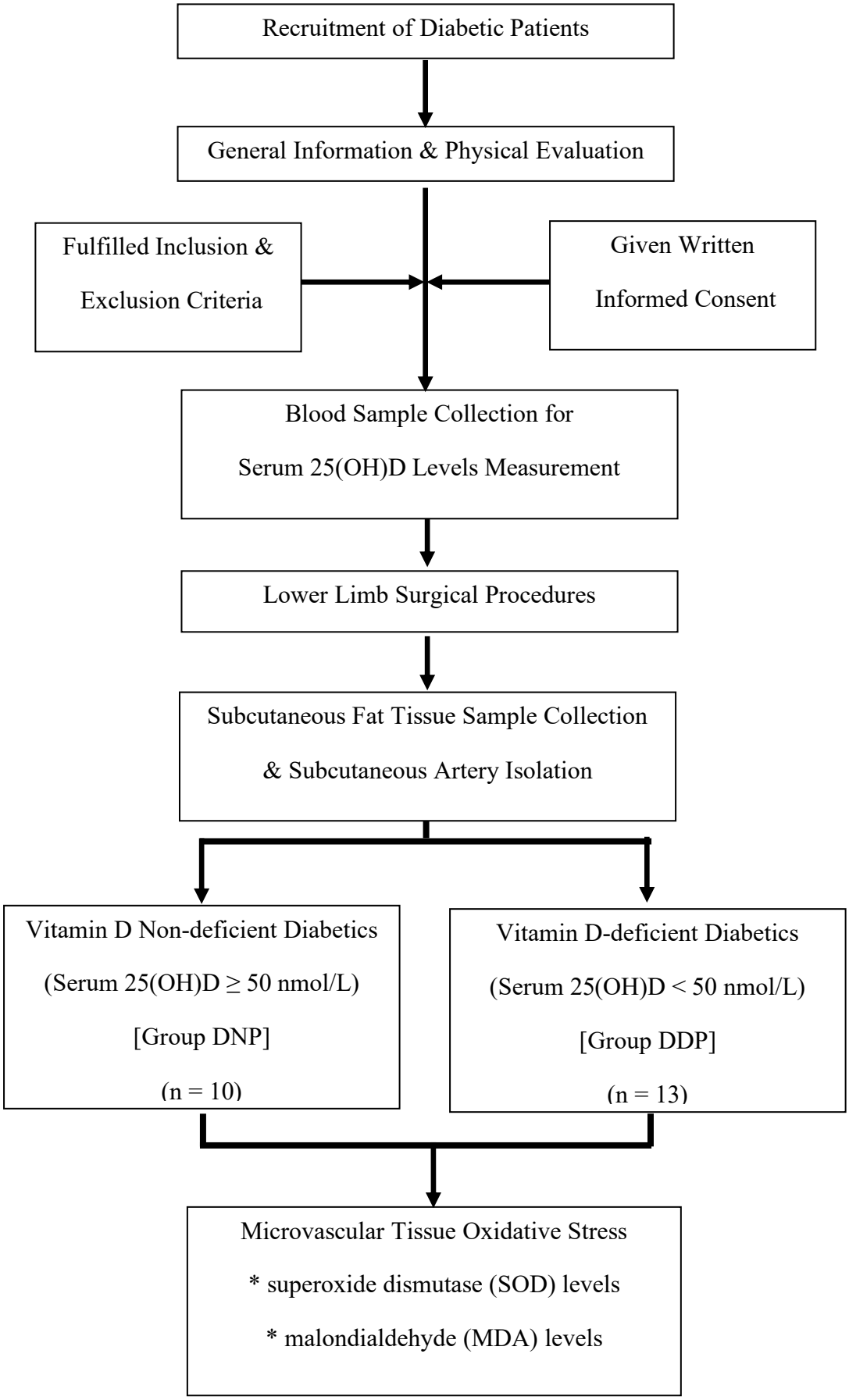


Figure 3.6 Flow chart for the human study.

3.10.4 Measurement of Serum 25(OH)D Levels

The stored blood serum was further diluted with Assay Buffer in the dilution ratio of 1:2 (based on preliminary study) to prepare human blood serum samples for assay. The assay was then performed as described in Section 3.7 to measure serum 25(OH)D concentrations.

3.10.5 Total Protein Quantification

The stored supernatant of subcutaneous arterial tissue lysate was further diluted with ddH_2O in the dilution ratio of 1:100 (based on preliminary study) to prepare human tissue samples for assay. The assay was performed as described in Section 3.3 to quantify total protein concentrations.

3.10.6 Oxidative Stress Analysis

3.10.6(a) Superoxide Dismutase Levels

The stored supernatant of the subcutaneous arterial tissue lysate was further diluted with ice-cold lysis buffer in the dilution ratio of 1:10 (based on preliminary study) to prepare human tissue samples for assay. The assay was performed as described in Section 3.4.1 to measure SOD concentrations.

3.10.6(b) Malondialdehyde Levels

The stored supernatant of the subcutaneous arterial tissue lysate was further diluted with assay buffer in the dilution ratio of 1:100 (based on preliminary study) to prepare human tissue samples for assay. The assay was performed as described in Section 3.4.2 to measure MDA concentrations.

3.10.7 Sample Size Calculations

Sample size calculation for oxidative stress analysis of human samples was performed using the following two means statistical formula (Florey, 1993). The sample size for oxidative stress analysis of human study was shown in Table 3.11.

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

$Z_\alpha = 1.96$, the constant of α at 5% level of significance (two-tailed)

$Z_\beta = 1.28$, the constant of β at 90% of study power.

[n: sample size for each group; s: standard deviation of the measured variable; d: difference of interest]

Table 3.11 Sample size calculations for oxidative stress analysis of human study.

Study	Difference of Interest	Standard Deviation	References	Sample Size
Oxidative Stress (SOD Levels)	54.79 U/mL	32.52 U/mL	Hisalkar *et al.*, 2012	7.40 ≈ 8
Details: Serum SOD levels. Difference of interest was the mean difference in serum SOD concentrations between diabetic patients and controls. Standard deviation for tissue SOD concentrations was taken from diabetic patients. (p value < 0.05)				
Oxidative Stress (MDA Levels)	3.18 nmol/mL	2.03 nmol/mL	Kumawat *et al.*, 2013	8.56 ≈ 9
Details: Plasma MDA levels. Difference of interest was the mean difference in plasma MDA concentrations between diabetic patients and controls. Standard deviation for plasma MDA concentrations was taken from diabetic patients. (p value < 0.05)				

3.10.8 Statistical Analysis and Data Presentation

Statistical analysis was performed using the IBM Statistical Package for the Social Sciences (SPSS) software version 24.0 (Armonk, NY, USA). The methods to determine the normality of data were as described in Section 3.9. Normally distributed data were presented as mean±standard deviation (SD) in the table and text. The data were presented as mean±standard error of mean (SEM) in the figure. Non-normally distributed data were expressed as median±interquartile range (IQR). In all cases, the *p* value of less than 0.05 was considered as statistically significant.

The differences for numerical variables between vitamin D-deficient and vitamin D non-deficient diabetic patients were determined by using independent t-test (for normally distributed data) or Mann-Whitney test (for non-normally distributed data) as appropriate. Pearson Chi-Square test was used to determine the differences for categorical variables between two study groups. When a significant difference for a particular parameter between vitamin D-deficient and vitamin D non-deficient diabetic patients was found, or the particular parameter has been suggested in previous study as the potential covariate, the parameter was subsequently tested in the analysis of covariance (ANCOVA).

CHAPTER 4

RESULTS

4.1 Animal Study

Normally distributed data were presented as mean±standard deviation (SD) while non-normally distributed data were expressed as median±interquartile range (IQR) in Tables. The data were presented as mean±standard error of mean (SEM) in Figures. In all cases, the *p* value of less than 0.05 was considered as statistically significant.

If the assumption of the homogeneity of variance is fulfilled, one-way analysis of variance (ANOVA) followed by a Bonferroni's post-hoc test was performed. If the assumption is violated, Kruskal-Wallis test followed by a Dunn's post hoc test was performed. The changes of a parameter over time within the same group were tested using paired t-test.

4.1.1 Body Weight

Baseline body weight (BW) was measured on the day of successful diabetes induction (defined as week one, W_1). At the end of 10-weeks study duration, final body weight was measured on the day of sacrifice (defined as week ten, W_{10}). Body weight changes throughout the 10-weeks study period were calculated based on the following formula:

$$\text{BW Changes (\%)} = \frac{\text{Final BW} - \text{Baseline BW}}{\text{Baseline BW}} \text{ x } 100$$

Normal Rats

Baseline and final body weight of normal rats with corresponding body weight changes were listed in Table 4.1. Baseline body weight of all study groups in normal rats was similar at the beginning of the study (W_1). Throughout the 10-weeks study duration, all groups in normal rats gained significant weight over time ($p < 0.001$). At the end of 10-weeks study period (W_{10}), all groups in normal rats had comparable final body weight.

In normal rats, there was no significant difference in body weight changes between rats receiving control diet (group NC) and rats receiving vitamin D-deficient diet (group ND) at the end of study duration (W_{10}) ($p = 0.480$). However, calcitriol supplementation to vitamin D-deficient rats (group NDS) showed the trend of reduced body weight changes compared to group ND ($p = 0.086$) (Figure 4.1).

Table 4.1 Body weight (BW) of normal rats at baseline and after ten-weeks study duration with corresponding body weight changes.

Groups	Baseline BW at W_1 (g)	Final BW at W_{10} (g)	BW Changes (%)
NC	250.70±28.39	442.60±74.15 *	77.80±30.15
ND	245.60±32.15	476.40±71.40 *	96.50±35.65
NDS	261.00±25.02	431.00±14.67 *	66.60±18.28
One-way ANOVA	$p = 0.483$	$p = 0.232$	$p = 0.084$

(n = 10 rats per group)

* $p < 0.001$ when compared to baseline body weight.

There was no significant difference in baseline body weight, final body weight and body weight changes between study groups in normal rats.

[NC: normal rats with control diet; ND: normal rats with vitamin D-deficient diet; NDS: normal rats with vitamin D-deficient diet + calcitriol supplementation.]

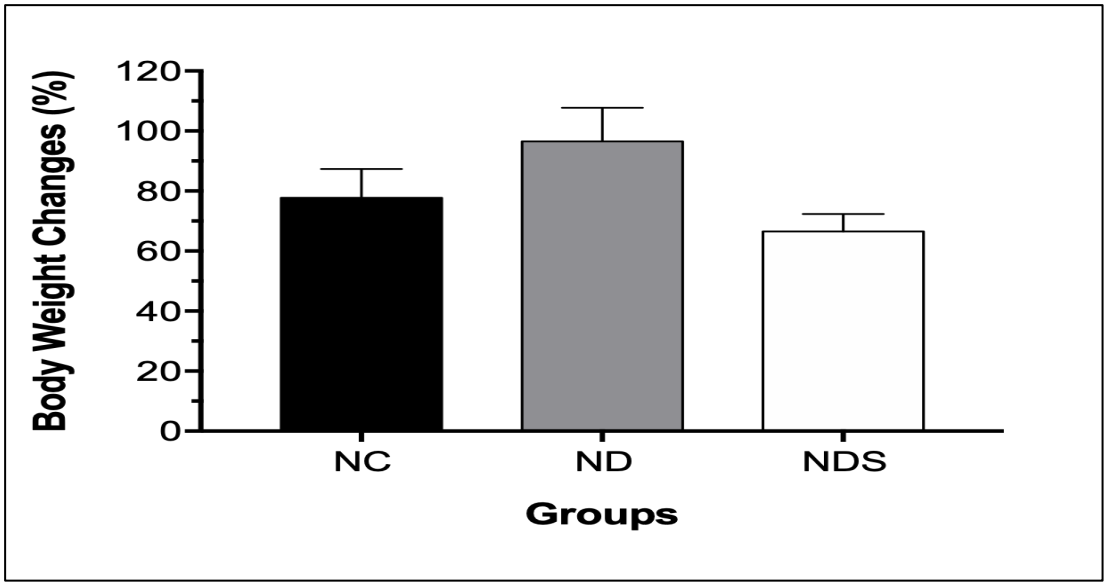


Figure 4.1 Body weight changes of normal rats after ten-weeks study duration.

(n = 10 rats per group)

There was no significant difference in body weight changes between study groups in normal rats ($p = 0.084$).

[NC: normal rats with control diet; ND: normal rats with vitamin D-deficient diet; NDS: normal rats with vitamin D-deficient diet + calcitriol supplementation]

Diabetic Rats

Baseline and final body weight of diabetic rats with corresponding body weight changes were listed in Table 4.2. Baseline body weight of all study groups in diabetic rats was similar at the beginning of the study (W_1). Throughout the 10-weeks study duration, diabetic rats receiving control diet (group DC) showed no significant difference in their body weight ($p = 0.173$). Diabetic rats receiving vitamin D-deficient diet (groups DD) and calcitriol-supplemented vitamin D-deficient diabetic rats (group DDS) gained significant weight over time ($p = 0.021$ and $p = 0.007$ respectively). However, all groups in diabetic rats had comparable final body weight at the end of 10-weeks study duration. There was no significant difference in body weight changes between study groups in diabetic rats (Figure 4.2).

Table 4.2 Body weight (BW) of diabetic rats at baseline and after ten-weeks study duration with corresponding body weight changes.

Groups	Baseline BW at W_1 (g)	Final BW at W_{10} (g)	BW Changes (%)
DC	257.80±26.00	278.60±45.28	8.54±17.14
DD	261.00±27.82	316.90±53.63 *	22.86±24.75
DDS	233.70±32.62	276.50±31.58 #	19.97±20.11
One-way ANOVA	$p = 0.088$	$p = 0.091$	$p = 0.286$

(n = 10 rats per group)

* $p < 0.05$ and # $p < 0.01$ when compared to baseline body weight.

There was no significant difference in baseline body weight, final body weight and body weight changes between study groups in diabetic rats.

[DC: diabetic rats with control diet; DD: diabetic rats with vitamin D-deficient diet; DDS: diabetic rats with vitamin D-deficient diet + calcitriol supplementation]

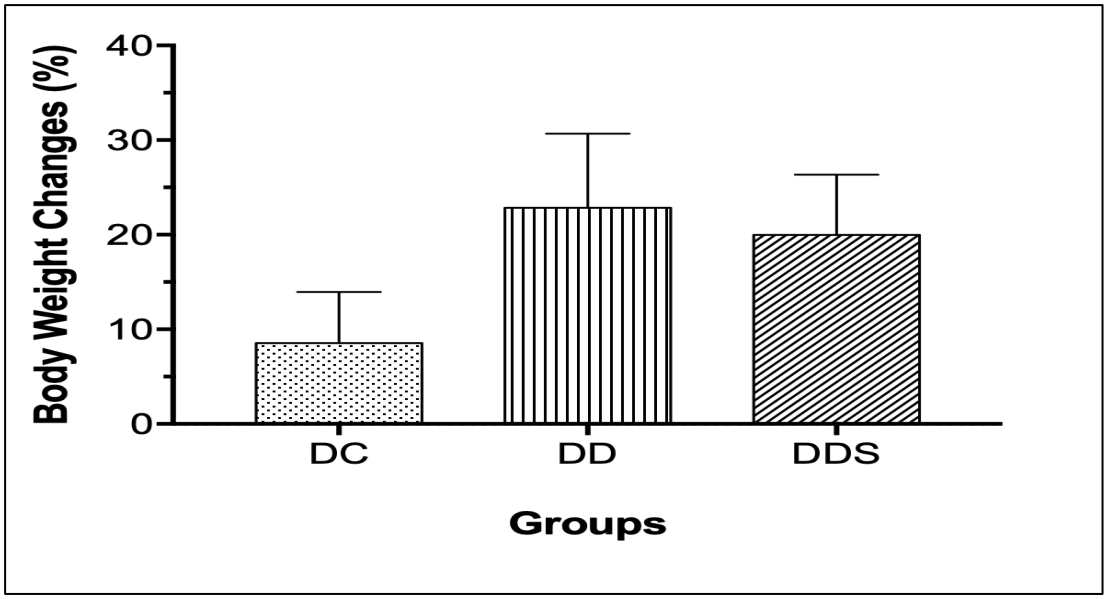


Figure 4.2 Body weight changes of diabetic rats after ten-weeks study duration.

(n = 10 rats per group)

There was no significant difference in body weight changes between study groups in diabetic rats ($p = 0.286$).

[DC: diabetic rats with control diet; DD: diabetic rats with vitamin D-deficient diet; DDS: diabetic rats with vitamin D-deficient diet + calcitriol supplementation]

4.1.2 Fasting Blood Glucose Levels

Normal Rats

Baseline and final fasting blood glucose (FBG) levels of normal rats were listed in Table 4.3. In normal rats, there was no significant difference in baseline and final FBG levels at the beginning of the study (W_1) and also at the end of 10-weeks study duration (W_{10}) between rats receiving control diet (group NC), rats receiving vitamin D-deficient diet (group ND) and vitamin D-deficient rats supplemented with calcitriol (group NDS). Throughout the 10-weeks study duration, the FBG levels of all groups in normal rats did not show significant changes over time.

Table 4.3 Baseline and final fasting blood glucose (FBG) levels of normal rats.

Groups	Baseline FBG levels at W_1 (mmol/L)	Final FBG levels at W_{10} (mmol/L)
NC	5.98±0.42	5.79±0.58
ND	6.12±0.28	6.18±1.19
NDS	6.05±0.30	5.75±0.61
One-way ANOVA	$p = 0.658$	$p = 0.463$

(n = 10 rats per group)

There were no significant changes in fasting blood glucose (FBG) levels of normal rats over ten-weeks study duration.

There was no significant difference in baseline and final fasting blood glucose (FBG) levels between study groups in normal rats.

[NC: normal rats with control diet; ND: normal rats with vitamin D-deficient diet; NDS: normal rats with vitamin D-deficient diet + calcitriol supplementation]

Diabetic Rats

Baseline and final FBG levels of diabetic rats were listed in Table 4.4. Similarly, baseline FBG levels were comparable between study groups in diabetic rats at the beginning of the study. Throughout the 10-weeks study duration, there was a significant increase in FBG levels over time observed in groups DC (p = 0.0019) and DDS (p = 0.0066), but not in group DD (p = 0.284). However, all groups showed no significant difference in final FBG levels at the end of 10-weeks study duration.

Table 4.4 Baseline and final fasting blood glucose (FBG) levels of diabetic rats.

Groups	Baseline FBG levels at W_1 (mmol/L)	Final FBG levels at W_{10} (mmol/L)
DC	25.85±4.83	31.39±2.54 *
DD	26.39±4.45	28.73±5.32
DDS	28.63±4.36	32.16±1.84 *
One-way ANOVA	p = 0.364	p = 0.097

(n = 10 rats per group).

* $p < 0.01$ when compared to baseline FBG levels.

There was no significant difference in baseline and final fasting blood glucose (FBG) levels between study groups in diabetic rats.

[DC: diabetic rats with control diet; DD: diabetic rats with vitamin D-deficient diet; DDS: diabetic rats with vitamin D-deficient diet + calcitriol supplementation]

4.1.3 Vitamin D Levels

Vitamin D levels were determined by the measurement of 25(OH)D concentrations in serum samples.

Normal Rats

In normal rats, serum vitamin D levels in rats receiving vitamin D-deficient diet (groups ND and NDS) were significantly lower compared to rats receiving control diet (group NC) ($p < 0.001$). No significant difference in vitamin D levels was observed in group NDS that received calcitriol supplementation for four weeks compared to group ND ($p = 0.2797$). (Figure 4.3). [25(OH)D - NC: 35.24±7.52 nmol/L; ND: 3.73±2.57 nmol/L; NDS: 7.61±3.41 nmol/L]

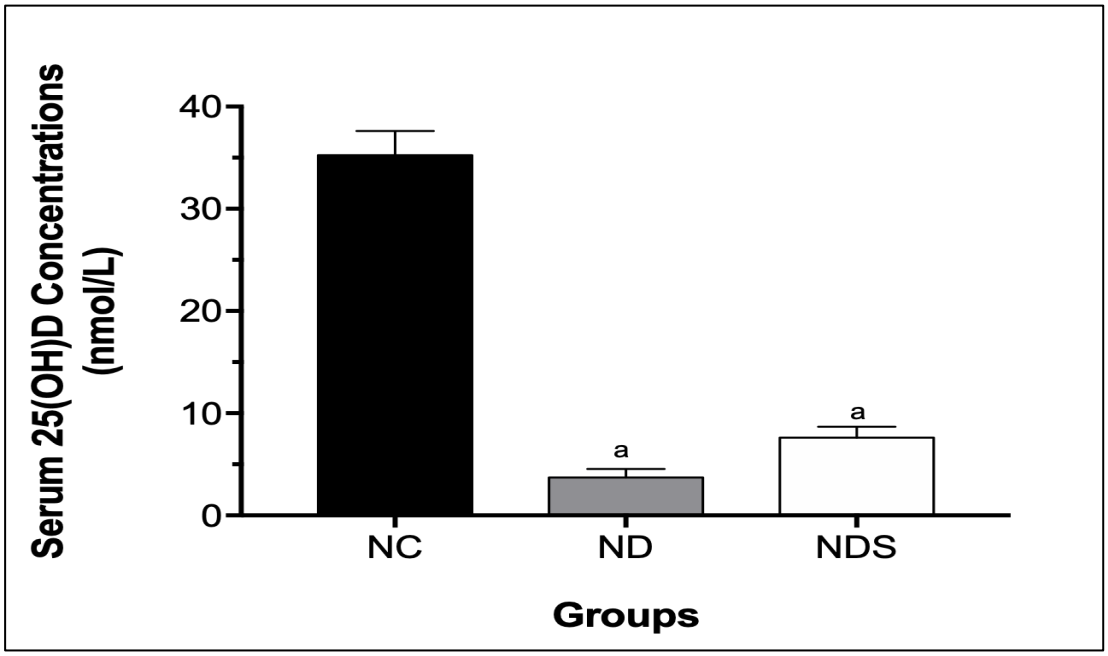


Figure 4.3 Vitamin D levels (serum 25(OH)D concentrations) of normal rats. (n = 10 rats per group).

$p < 0.001$ (One-way ANOVA); [a] $p < 0.001$ when compared to group NC.

[NC: normal rats with control diet; ND: normal rats with vitamin D-deficient diet; NDS: normal rats with vitamin D-deficient diet + calcitriol supplementation]

Diabetic Rats

A similar pattern on serum vitamin D levels was observed in diabetic rats. Diabetic rats receiving vitamin D-deficient diet (groups DD & DDS) showed significantly lower serum vitamin D levels compared to diabetic rats receiving control diet (group DC) ($p < 0.001$). However, supplementation with calcitriol for four weeks (group DDS) did not improve vitamin D levels when compared to group DD ($p = 0.995$). (Figure 4.4). [25(OH)D - DC: 40.57±8.14 nmol/L; DD: 3.55±2.25 nmol/L; DDS: 5.77±2.07 nmol/L]

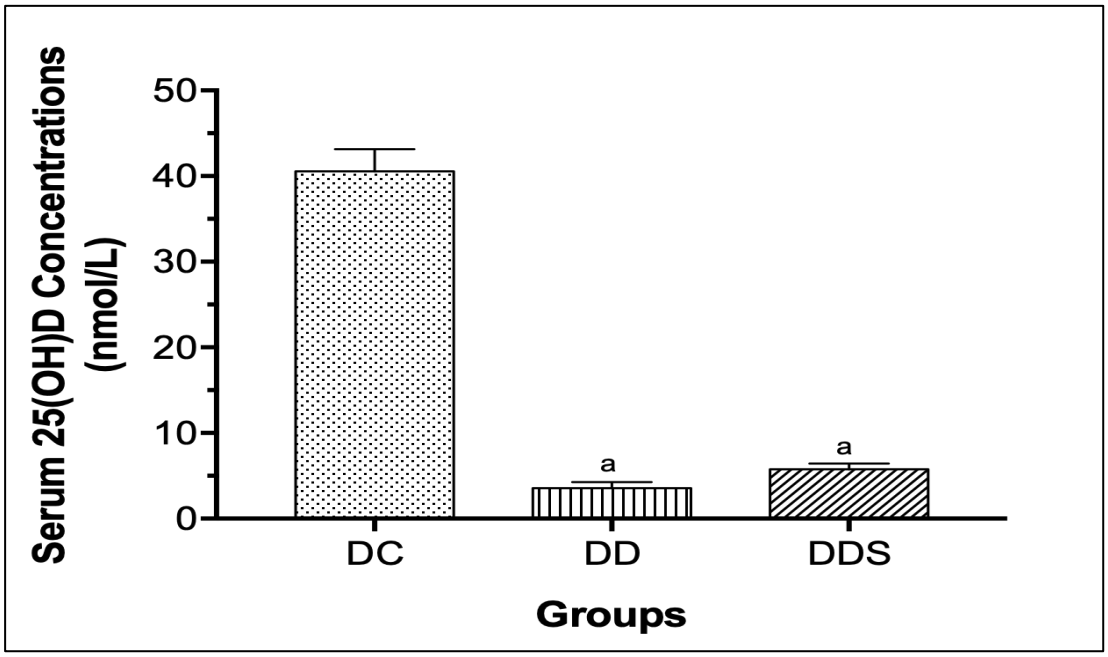


Figure 4.4 Vitamin D levels (serum 25(OH)D concentrations) of diabetic rats. (n = 10 rats per group).

$p < 0.001$ (One-way ANOVA); [a] $p < 0.001$ when compared to group DC.

[DC: diabetic rats with control diet; DD: diabetic rats with vitamin D-deficient diet; DDS: diabetic rats with vitamin D-deficient diet + calcitriol supplementation]

4.1.4 Calcium Levels

Normal Rats

In normal rats, no significant difference in serum calcium levels was observed in rats receiving control diet (group NC) and rats receiving vitamin D-deficient diet (group ND). However, serum calcium levels were significantly increased in vitamin D-deficient rats supplemented with calcitriol (group NDS) compared to groups NC (p = 0.009) and ND (p = 0.005) (Figure 4.5). [Ca - NC: 2.76±0.29 mmol/L; ND: 2.73±0.28 mmol/L; NDS: 3.19±0.32 mmol/L]

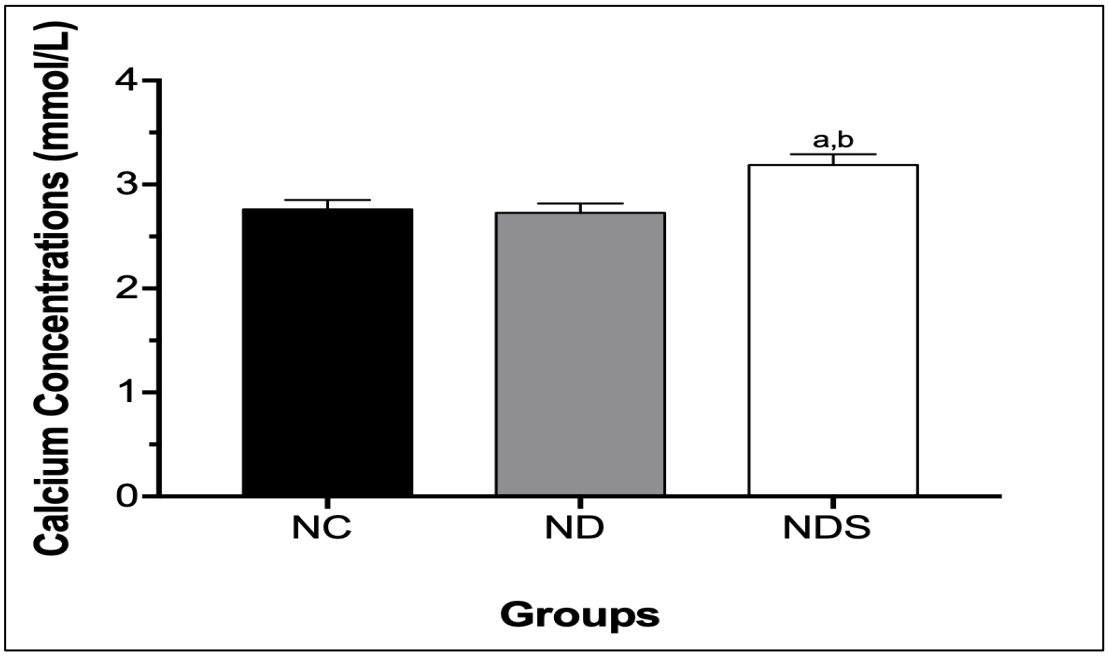


Figure 4.5 Serum calcium levels of normal rats.

(n = 10 rats per group)

p = 0.002 (One-way ANOVA)

[a] $p < 0.01$ when compared to group NC; [b] $p < 0.01$ when compared to group ND.

[NC: normal rats with control diet; ND: normal rats with vitamin D-deficient diet; NDS: normal rats with vitamin D-deficient diet + calcitriol supplementation]

Diabetic Rats

In diabetic rats, there was no significant difference in serum calcium levels observed in rats receiving vitamin D-deficient diet (groups DD and DDS) compared to rats receiving control diet (group DC). Four-weeks calcitriol supplementation to group DDS did not show significant changes in serum calcium levels when compared to group DD (Figure 4.6). [Ca - DC: 2.86±0.37 mmol/L; DD: 2.83±0.36 mmol/L; DDS: 3.15±0.18 mmol/L].

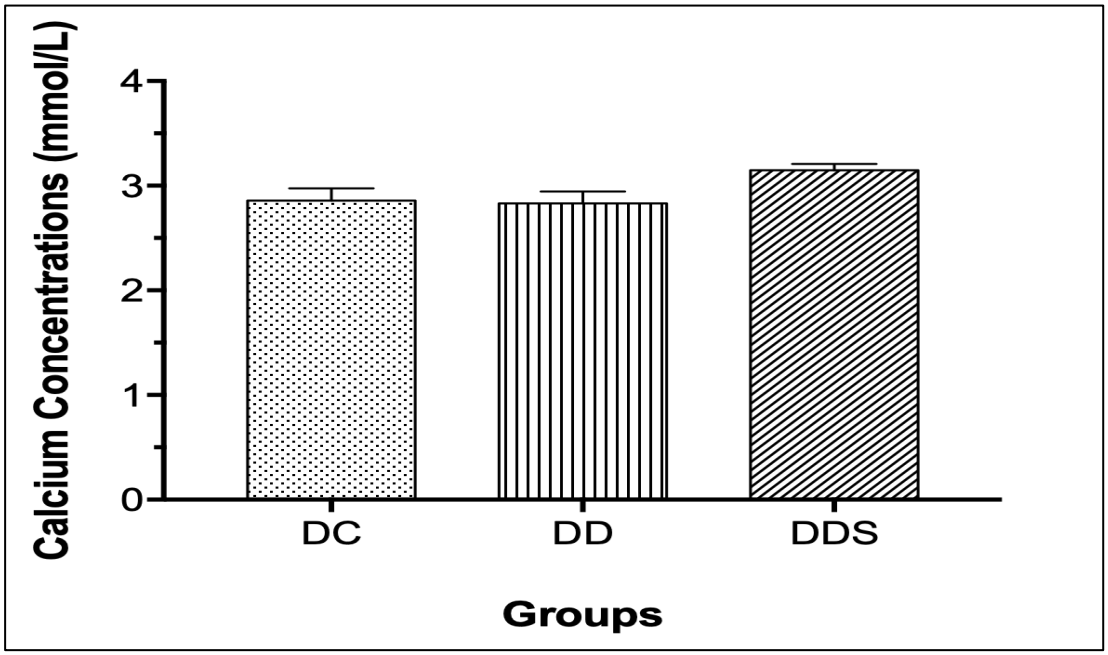


Figure 4.6 Serum calcium levels of diabetic rats.

(n = 10 rats per group)

There was no significant difference in serum calcium levels between study groups in diabetic rats ($p = 0.060$).

[DC: diabetic rats with control diet; DD: diabetic rats with vitamin D-deficient diet; DDS: diabetic rats with vitamin D-deficient diet + calcitriol supplementation]

4.1.5 Endothelium-dependent Microvascular Responses

4.1.5(a) Acetylcholine-induced Endothelium-dependent Relaxation

Normal Rats

Figure 4.7 showed the concentration-response curves and maximal relaxation (R_{max}) of acetylcholine (ACh)-induced endothelium-dependent relaxation in mesenteric arteries of normal rats. In normal rats, maximal relaxation to ACh-mediated response was significantly lower in rats receiving vitamin D-deficient diet (groups ND and NDS) compared to rats receiving control diet (group NC) ($p = 0.003$ and $p < 0.001$ respectively). Calcitriol supplementation to vitamin D-deficient rats (group NDS) showed no significant difference in maximal relaxation to ACh compared to group ND. [R_{max} - NC: 74.56±18.53%; ND: 46.52±16.11%; NDS: 41.83±15.38%]

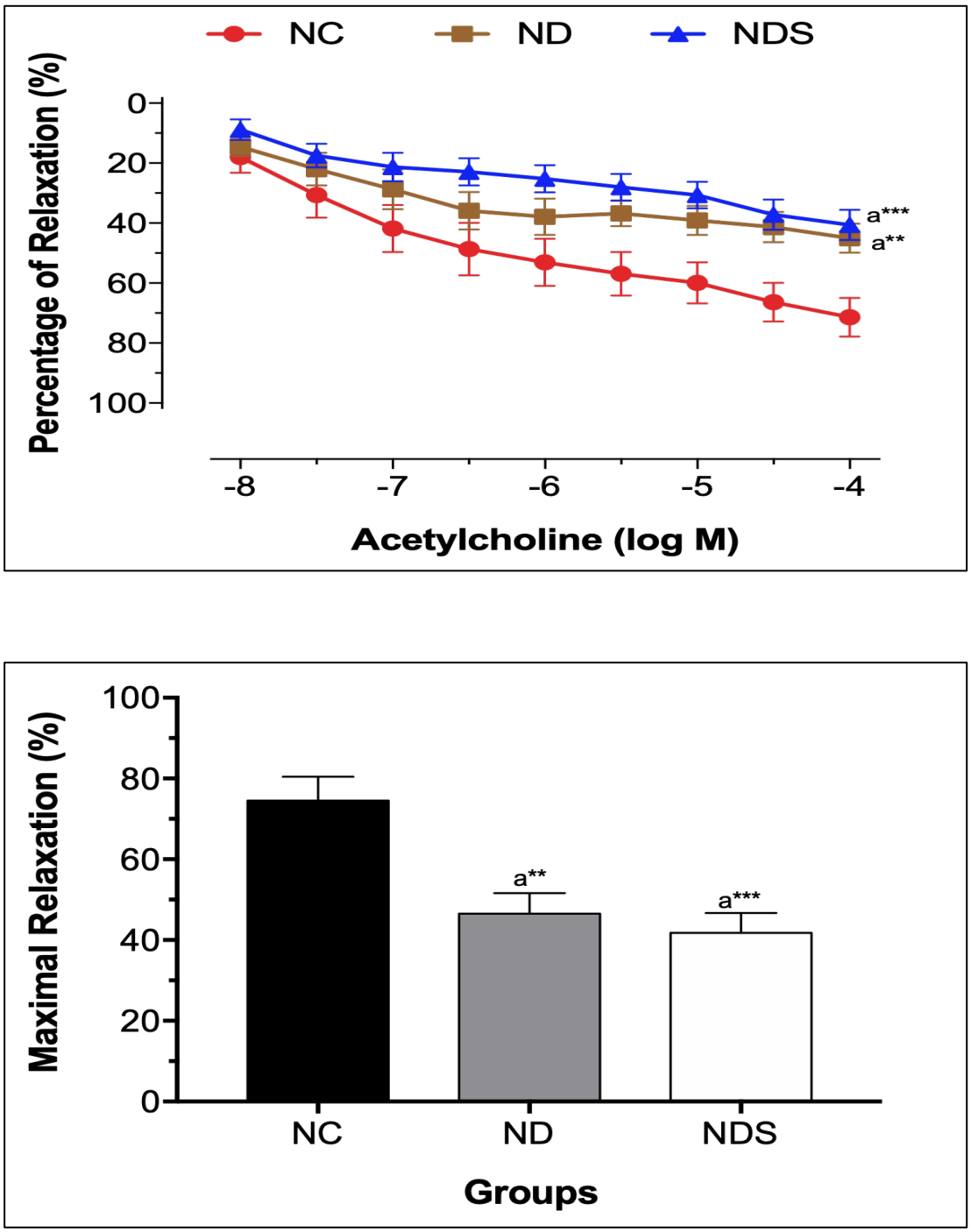


Figure 4.7 Concentration-response curves and maximal relaxation of acetylcholine-induced endothelium-dependent relaxation in mesenteric arteries of normal rats.

(n = 10 rats per group)

$p < 0.001$ (One-way ANOVA)

$^{a**}p < 0.01$ and $^{a***}p < 0.001$ when compared to group NC.

[NC: normal rats with control diet; ND: normal rats with vitamin D-deficient diet; NDS: normal rats with vitamin D-deficient diet + calcitriol supplementation]

Diabetic Rats

Figure 4.8 showed the concentration-response curves and maximal relaxation (R_{max}) of ACh-induced endothelium-dependent relaxation in mesenteric arteries of diabetic rats. In diabetic rats, rats receiving vitamin D-deficient diet (group DD) had significantly lower maximal relaxation to ACh compared to diabetic controls (group DC) (p = 0.001). Calcitriol supplementation for four weeks in group DDS showed a significant improvement in ACh-mediated maximal relaxation compared to group DD (p = 0.011) and the maximal relaxation was comparable to group DC. [R_{max} - DC: 53.48±19.97%; DD: 28.45±10.70 %; DDS: 48.40±8.41%]

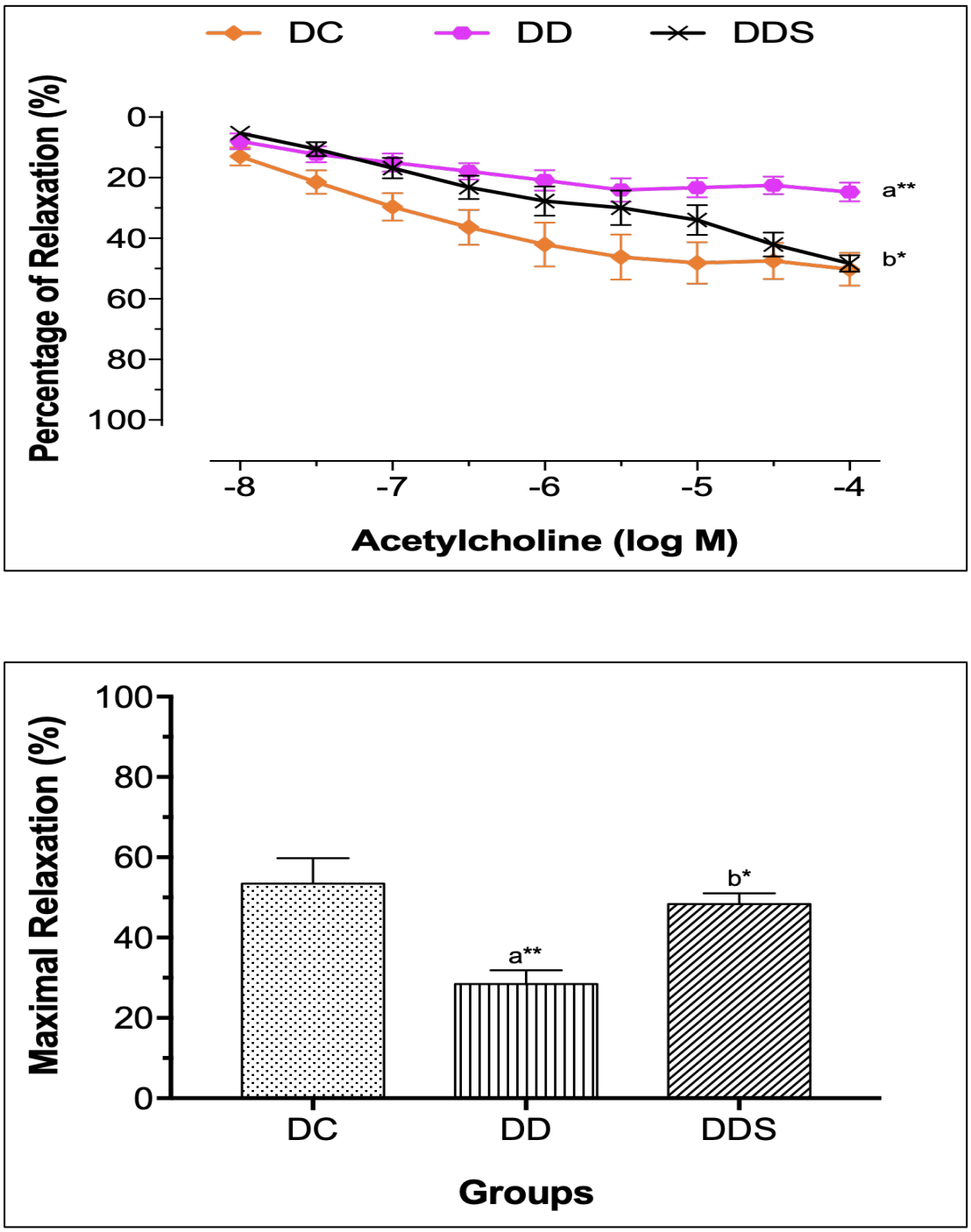


Figure 4.8 Concentration-response curves and maximal relaxation of acetylcholine-induced endothelium-dependent relaxation in mesenteric arteries of diabetic rats.

(n = 10 rats per group)

$p = 0.001$ (One-way ANOVA)

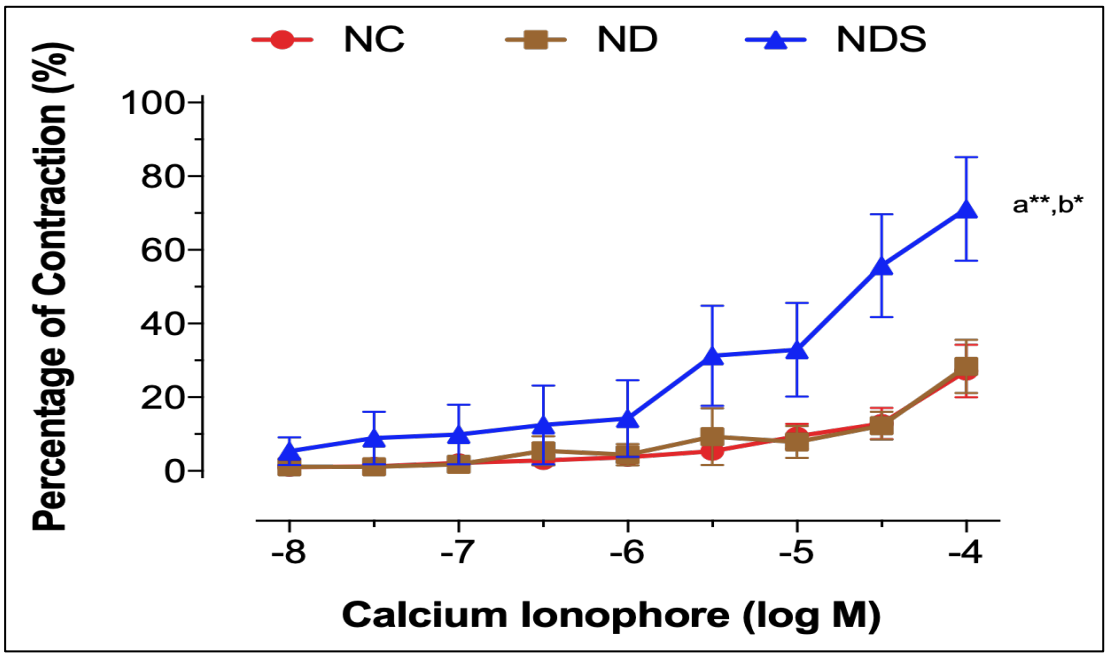
[a**] $p < 0.01$ when compared to group DC; [b*] $p < 0.05$ when compared to group DD.

[DC: diabetic rats with control diet; DD: diabetic rats with vitamin D-deficient diet; DDS: diabetic rats with vitamin D-deficient diet + calcitriol supplementation]

4.1.5(b) Calcium Ionophore-induced Endothelium-dependent Contraction

Normal Rats

Figure 4.9 showed the concentration-response curves and maximal contraction (E_{max}) of calcium ionophore (CaI)-induced endothelium-dependent contraction in mesenteric arteries of normal rats. In normal rats, there was no significant difference in maximal contraction to CaI-mediated response between rats receiving control diet (group NC) and rats receiving vitamin D-deficient diet (group ND). However, vitamin D-deficient rats with calcitriol supplementation showed significantly higher in maximal contraction to CaI compared to groups ND ($p = 0.016$) and NC ($p = 0.006$). [E_{max} - NC: 27.19±22.64%; ND: 32.31±26.20%; NDS: 74.02±40.66%]



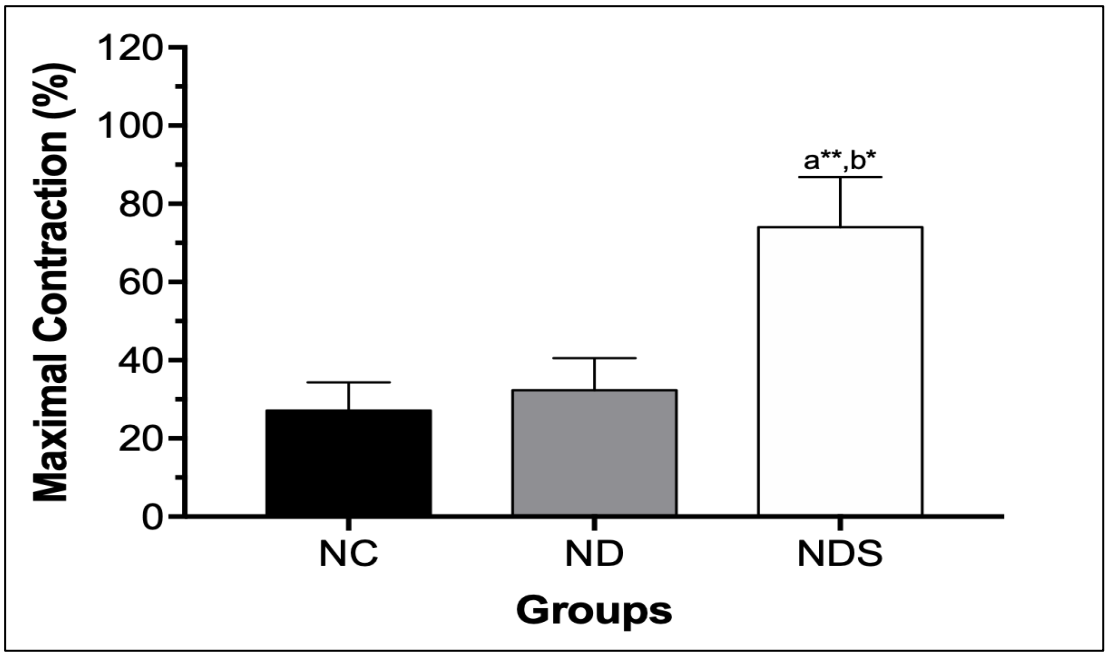


Figure 4.9 Concentration-response curves and maximal contraction of calcium ionophore-induced endothelium-dependent contraction in mesenteric arteries of normal rats.

(n = 10 rats per group)

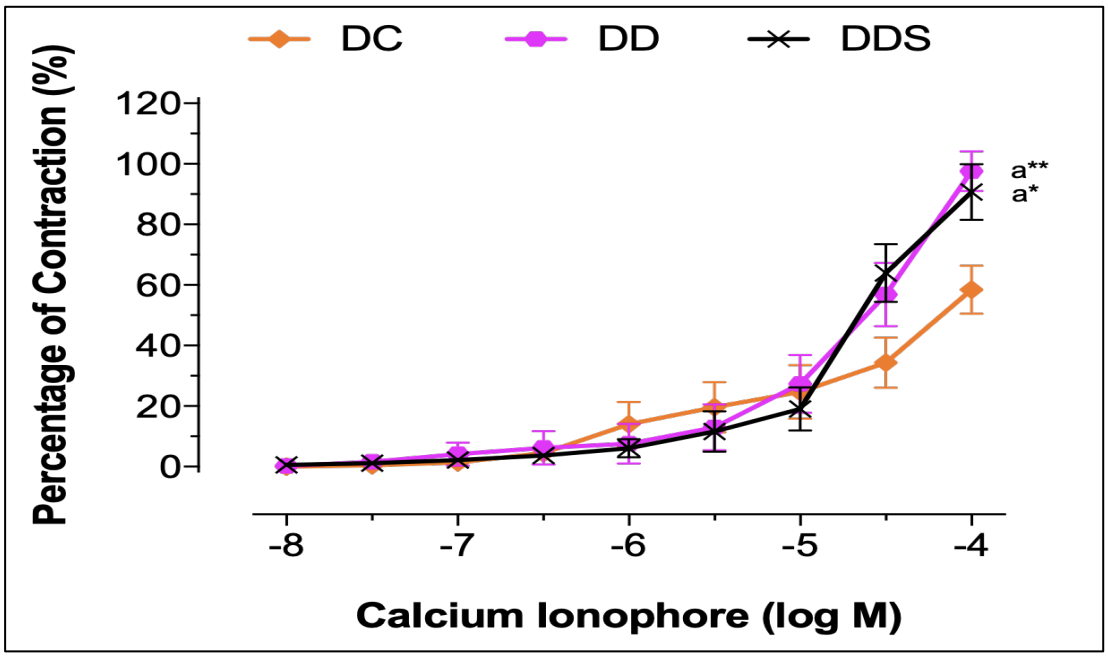
$p = 0.004$ (One-way ANOVA)

[a**] $p < 0.01$ when compared to group NC; [b*] $p < 0.05$ when compared to group ND.

[NC: normal rats with control diet; ND: normal rats with vitamin D-deficient diet; NDS: normal rats with vitamin D-deficient diet + calcitriol supplementation]

Diabetic Rats

Figure 4.10 showed the concentration-response curves and maximal contraction (E_{max}) of CaI-induced endothelium-dependent contraction in mesenteric arteries of diabetic rats. In diabetic rats, rats receiving vitamin D-deficient diet (group DD) showed significantly higher maximal contraction to CaI compared to rats receiving control diet (group DC) ($p = 0.006$). However, calcitriol supplementation in group DDS showed no significant difference in CaI-mediated maximal contraction compared to group DD and the maximal contraction remained significantly higher compared to group DC ($p = 0.028$). [E_{max} - DC: 58.78±25.59%; DD: 97.55±20.80%; DDS: 90.67±29.14%]



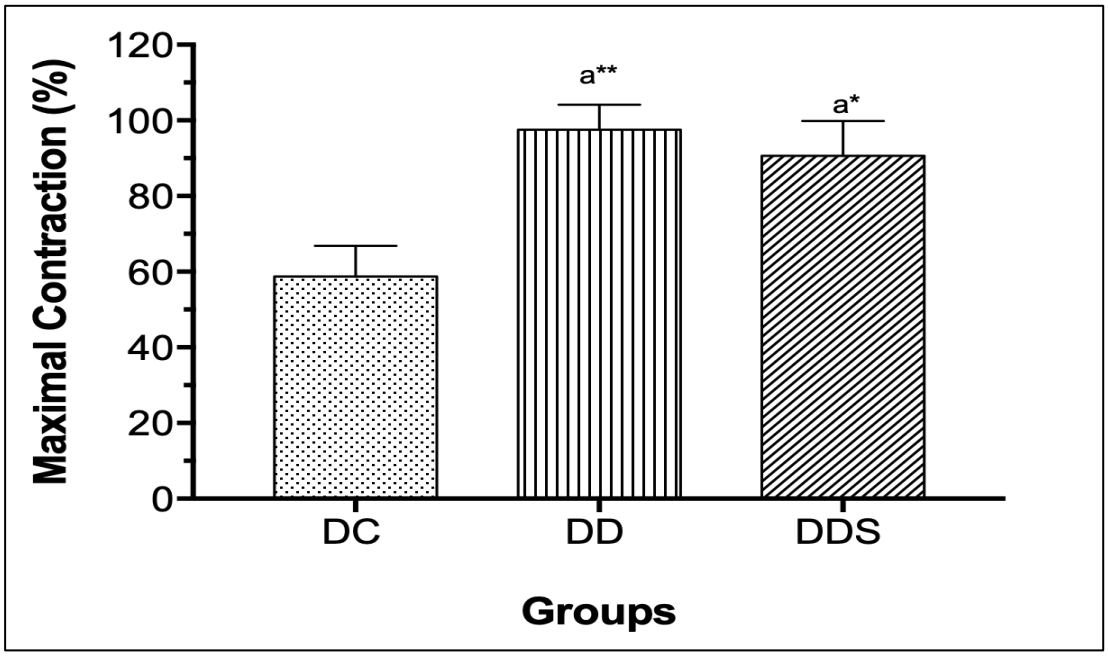


Figure 4.10 Concentration-response curves and maximal contraction of calcium ionophore-induced endothelium-dependent contraction in mesenteric arteries of diabetic rats.

(n = 10 rats per group)

$p = 0.005$ (One-way ANOVA)

[a**] $p < 0.01$ when compared to group DC; [a*] $p < 0.05$ when compared to group DC.

[DC: diabetic rats with control diet; DD: diabetic rats with vitamin D-deficient diet; DDS: diabetic rats with vitamin D-deficient diet + calcitriol supplementation]

4.1.6 Endothelium-independent Microvascular Responses

4.1.6(a) Sodium Nitroprusside-induced Endothelium-independent Relaxation

Normal Rats

Figure 4.11 showed the concentration-response curves and maximal relaxation (R_{max}) of sodium nitroprusside (SNP)-induced endothelium-independent relaxation in mesenteric arteries of normal rats. In normal rats, there was no significant difference in maximal relaxation to SNP-mediated response between rats receiving control diet (group NC) and rats receiving vitamin D-deficient diet (group ND). However, calcitriol supplementation to vitamin D-deficient rats (group NDS) showed a reduced trend in maximal relaxation to SNP compared to group ND ($p = 0.101$). Also, group NDS had significantly lower maximal relaxation to SNP compared to group NC ($p = 0.034$). [R_{max} - NC: 69.30±16.39%; ND: 65.34±25.38%; NDS: 47.01±9.58%]

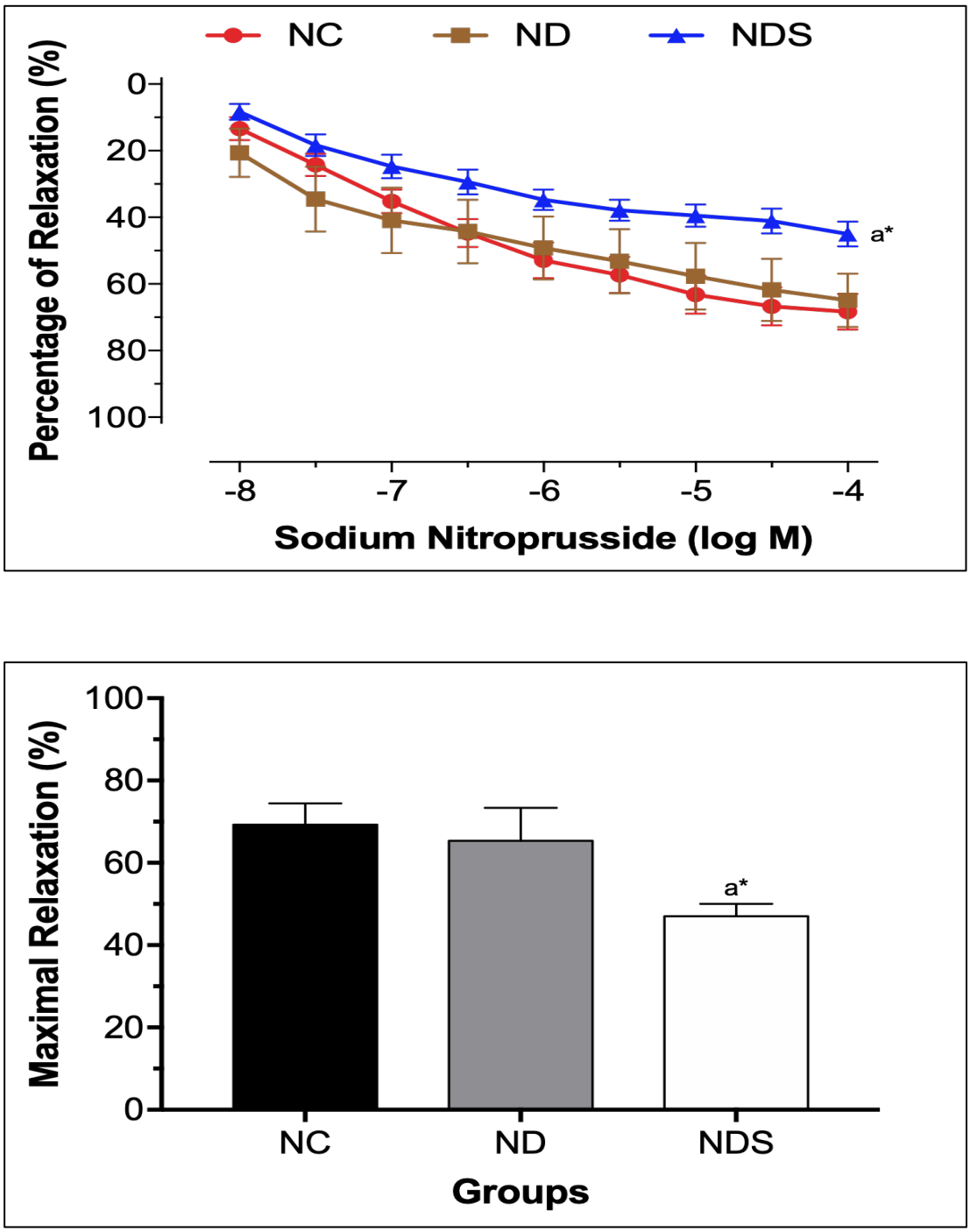


Figure 4.11 Concentration-response curves and maximal relaxation of sodium nitroprusside-induced endothelium-independent relaxation in mesenteric arteries of normal rats.

(n = 10 rats per group)

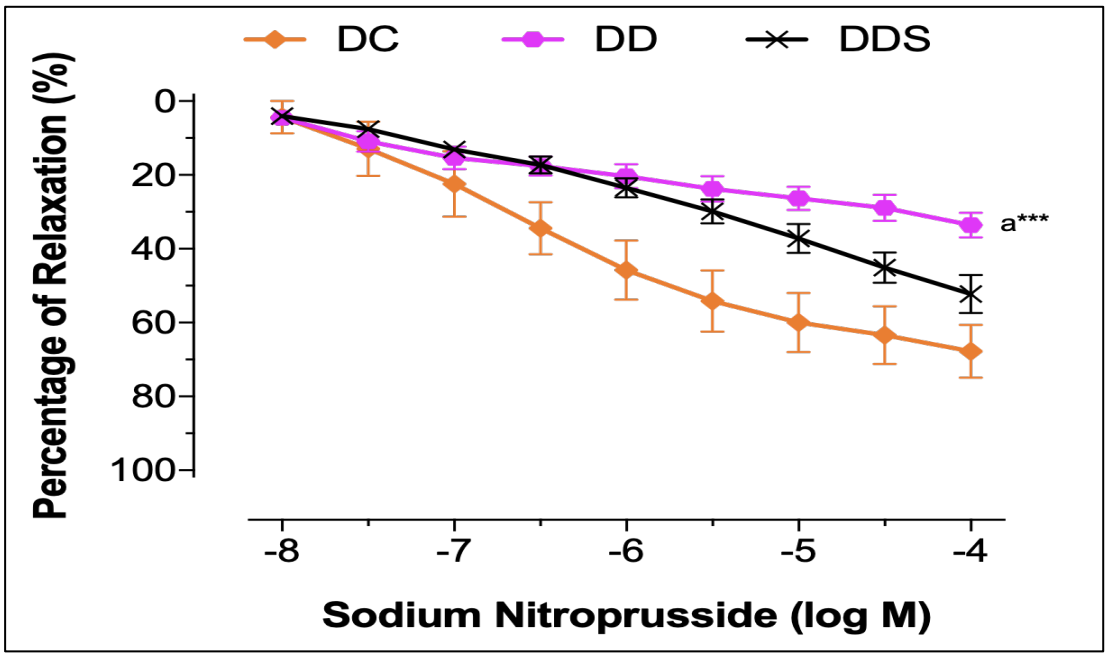
$p = 0.025$ (One-way ANOVA)

[a*] $p < 0.05$ when compared to group NC.

[NC: normal rats with control diet; ND: normal rats with vitamin D-deficient diet; NDS: normal rats with vitamin D-deficient diet + calcitriol supplementation]

Diabetic Rats

Figure 4.12 showed the concentration-response curves and maximal relaxation (R_{max}) of SNP-induced endothelium-independent relaxation in mesenteric arteries of diabetic rats. In diabetic rats, rats receiving vitamin D-deficient diet (group DD) had significantly lower maximal relaxation to SNP compared to group DC ($p < 0.001$). There was no significant difference in SNP-mediated maximal relaxation between group DDS that received calcitriol supplementation and group DD. [R_{max} - DC: 67.83±22.11%; DD: 34.42±9.44%; DDS: 52.24±16.04%]



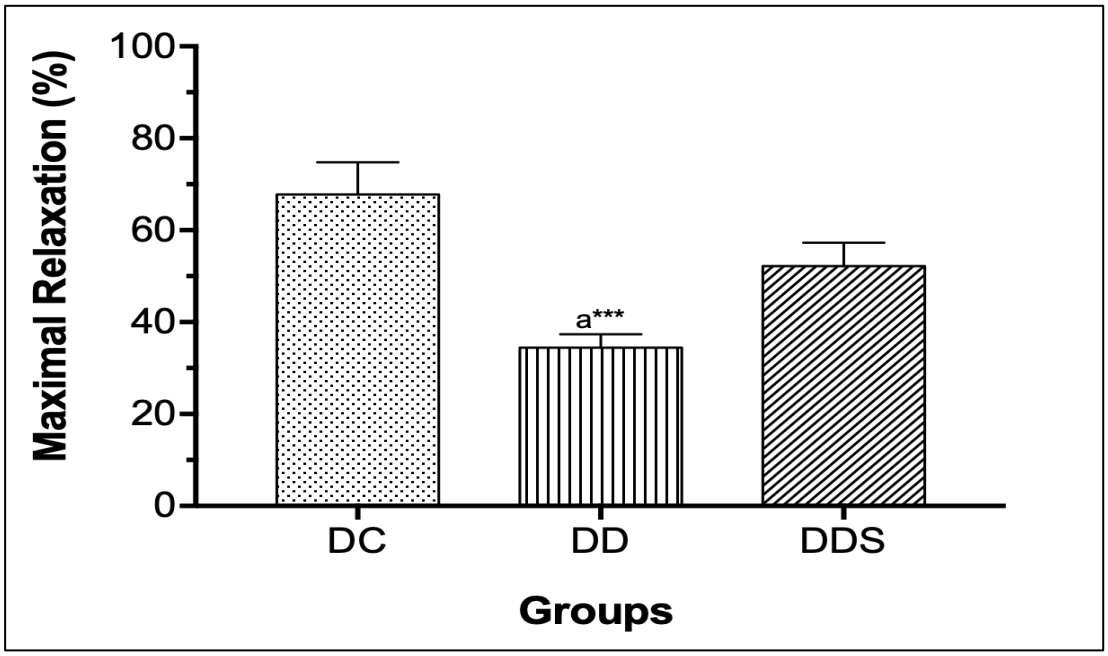


Figure 4.12 Concentration-response curves and maximal relaxation of sodium nitroprusside-induced endothelium-independent relaxation in mesenteric arteries of diabetic rats.

(n = 10 rats per group)

$p < 0.001$ (One-way ANOVA)

^{a***} $p < 0.001$ when compared to group DC.

[DC: diabetic rats with control diet; DD: diabetic rats with vitamin D-deficient diet; DDS: diabetic rats with vitamin D-deficient diet + calcitriol supplementation]

4.1.6(b) Salbutamol-induced Endothelium-independent Relaxation

Normal Rats

Figure 4.13 showed the concentration-response curves and maximal relaxation (R_{max}) of salbutamol (SB)-induced endothelium-independent relaxation in mesenteric arteries of normal rats. All groups in normal rats regardless of diet and supplementation (groups NC, ND and NDS) showed comparable maximal relaxation to SB-mediated response. [R_{max} - NC: 91.12±9.10%; ND: 96.06±4.52%; NDS: 95.74±3.47%]

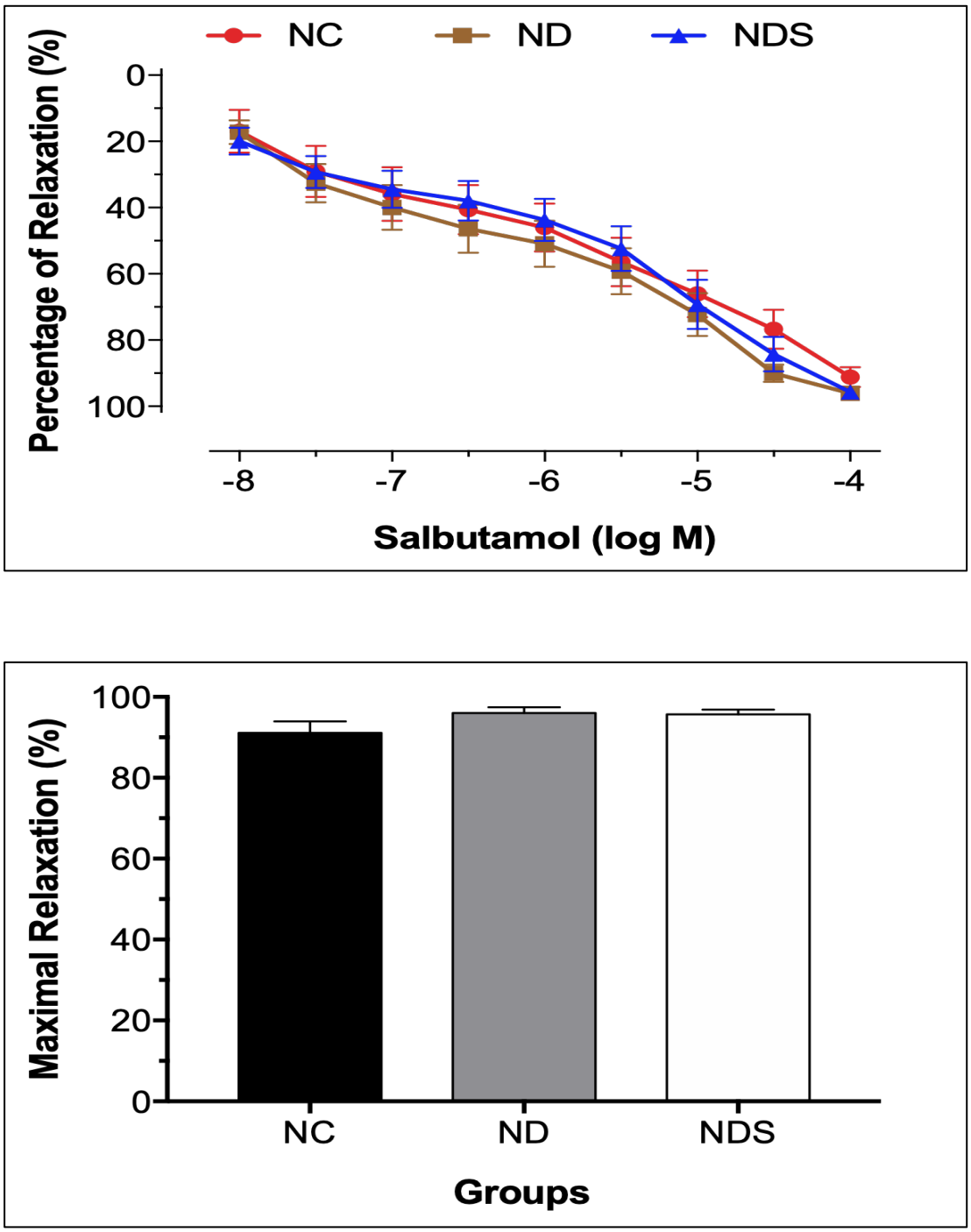


Figure 4.13 Concentration-response curves and maximal relaxation of salbutamol-induced endothelium-independent relaxation in mesenteric arteries of normal rats.

(n = 10 rats per group)

There was no significant difference between study groups in normal rats ($p = 0.156$).

[NC: normal rats with control diet; ND: normal rats with vitamin D-deficient diet; NDS: normal rats with vitamin D-deficient diet + calcitriol supplementation]

Diabetic Rats

Figure 4.14 showed the concentration-response curves and maximal relaxation (R_{max}) of SB-induced endothelium-independent relaxation in mesenteric arteries of diabetic rats. Similarly, all groups in diabetic rats (groups DC, DD and DDS) showed no significant difference in SB-mediated maximal relaxation ($p = 0.628$). [R_{max} - DC: 91.98±6.58%; DD: 86.28±15.44%; DDS: 90.92±17.34%]

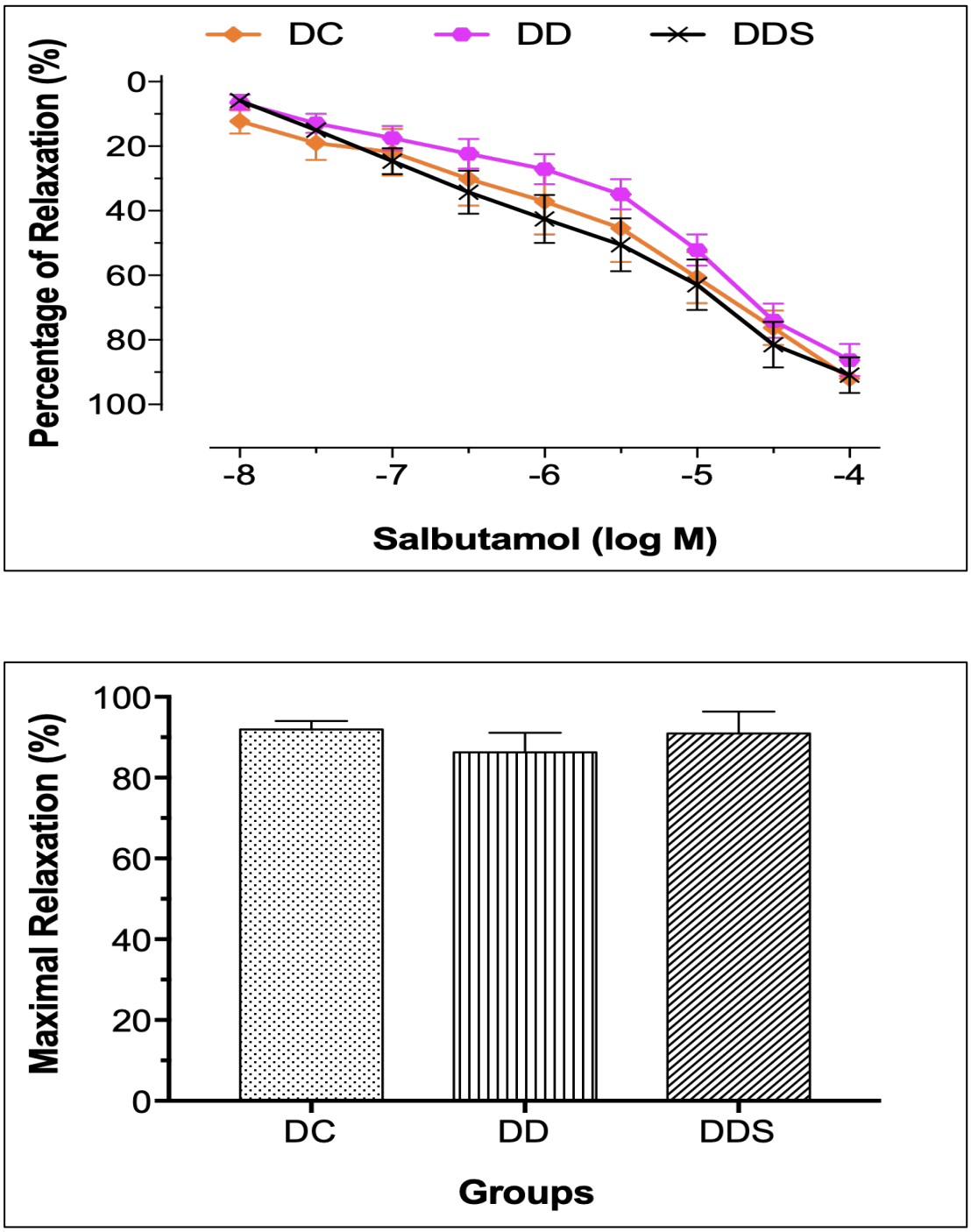


Figure 4.14 Concentration-response curves and maximal relaxation of salbutamol-induced endothelium-independent relaxation in mesenteric arteries of diabetic rats.

(n = 10 rats per group)

There was no significant difference between study groups in diabetic rats ($p = 0.628$).

[DC: diabetic rats with control diet; DD: diabetic rats with vitamin D-deficient diet; DDS: diabetic rats with vitamin D-deficient diet + calcitriol supplementation]

4.1.6(c) Phenylephrine-induced Endothelium-independent Contraction

Normal Rats

Figure 4.15 showed the concentration-response curves and maximal contraction (E_{max}) of phenylephrine (PE)-induced endothelium-independent contraction in mesenteric arteries of normal rats. All groups in normal rats (groups NC, ND and NDS) had comparable PE-mediated maximal contraction ($p = 0.358$). [E_{max} - NC: 118.50±40.31%; ND: 117.10±42.94%; NDS: 142.60±47.99%]

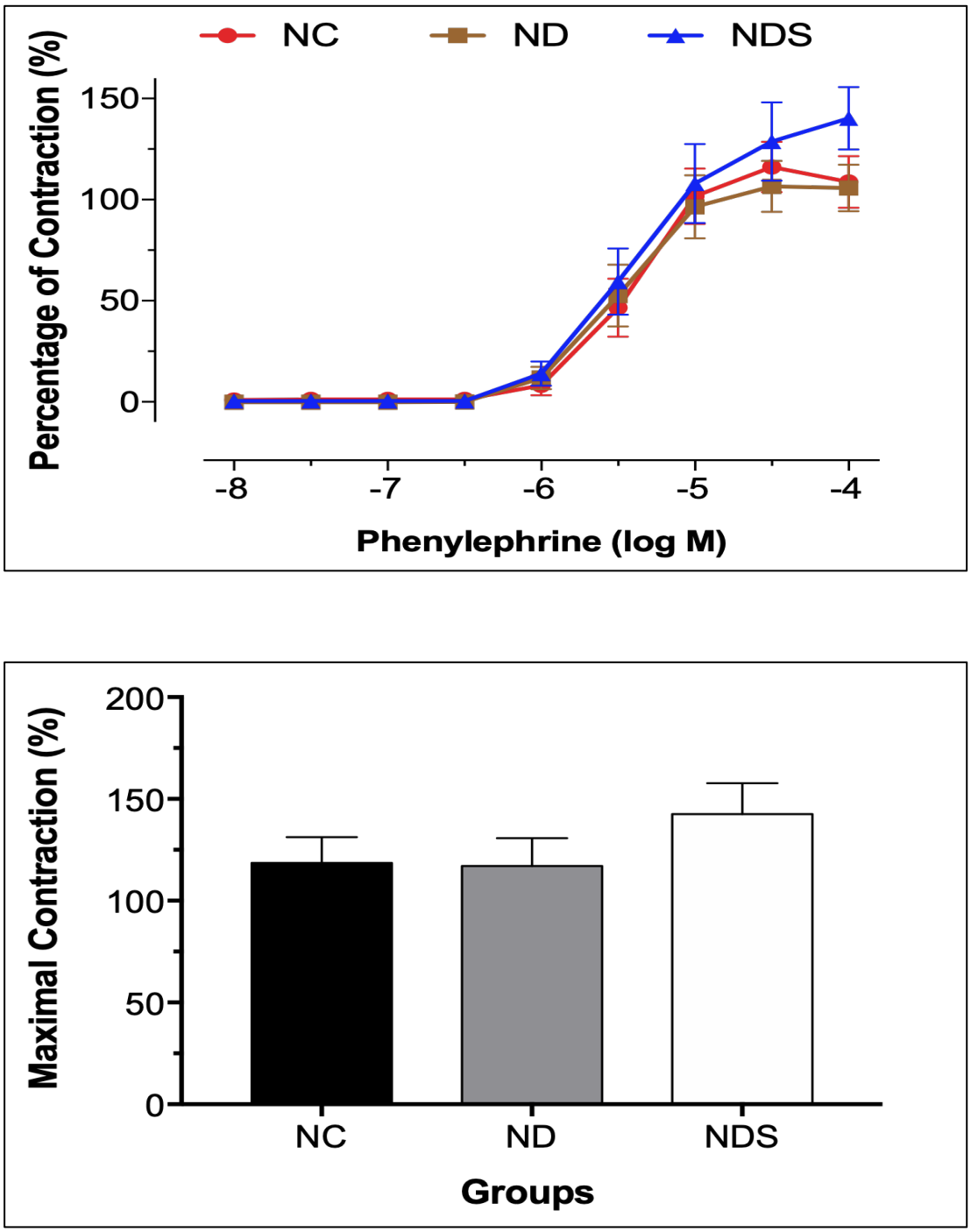


Figure 4.15 Concentration-response curves and maximal contraction of phenylephrine-induced endothelium-independent contraction in mesenteric arteries of normal rats.

(n = 10 rats per group)

There was no significant difference between study groups in normal rats ($p = 0.358$).

[NC: normal rats with control diet; ND: normal rats with vitamin D-deficient diet; NDS: normal rats with vitamin D-deficient diet + calcitriol supplementation]

Diabetic Rats

Figure 4.16 showed the concentration-response curves and maximal contraction of PE-induced endothelium-independent contraction in mesenteric arteries of diabetic rats respectively. Similarly, all groups in diabetic rats (groups DC, DD and DDS) showed comparable maximal contraction to PE-mediated response ($p = 0.577$). [E_{max} - DC: 148.30±63.77%; DD: 141.50±36.15%; DDS: 168.70±72.90%]

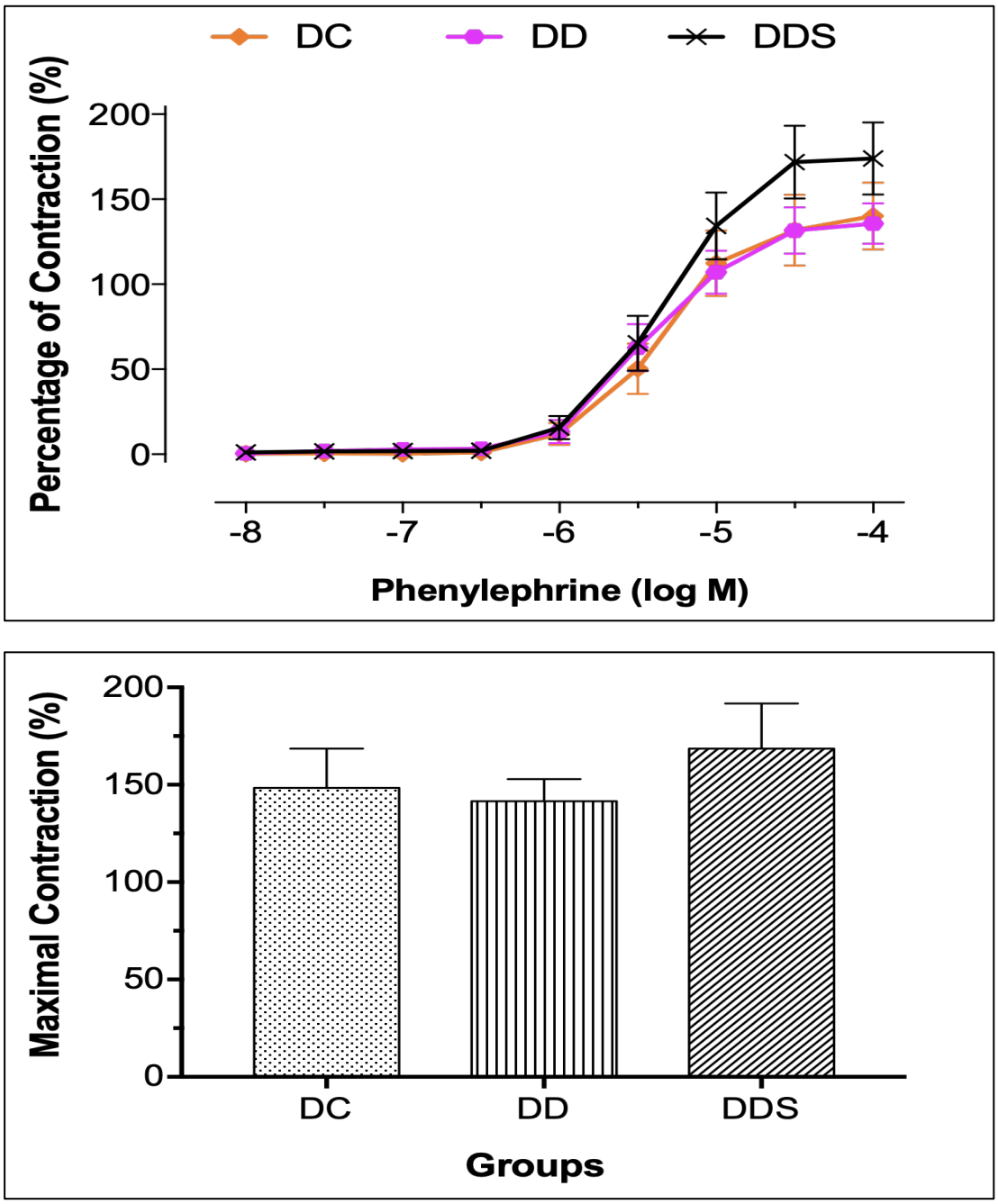


Figure 4.16 Concentration-response curves and maximal contraction of phenylephrine-induced endothelium-independent contraction in mesenteric arteries of diabetic rats.

(n = 10 rats per group)

There was no significant difference between study groups in diabetic rats ($p = 0.577$).

[DC: diabetic rats with control diet; DD: diabetic rats with vitamin D-deficient diet; DDS: diabetic rats with vitamin D-deficient diet + calcitriol supplementation]

4.1.7 Oxidative Stress Parameters

4.1.7(a) Superoxide Dismutase Levels

Normal Rats

In normal rats, SOD levels of rats receiving vitamin D-deficient diet (group ND) were significantly lower than rats receiving control diet (group NC) ($p = 0.002$). Calcitriol supplementation to vitamin D-deficient rats (group NDS) did not improve SOD levels when compared to group ND and the SOD levels remained significantly lower than group NC ($p = 0.003$) (Figure 4.17). [SOD - NC: 27.75±10.63 U/mg protein; ND: 13.72±6.02 U/mg protein; NDS: 14.07±7.57 U/mg protein]

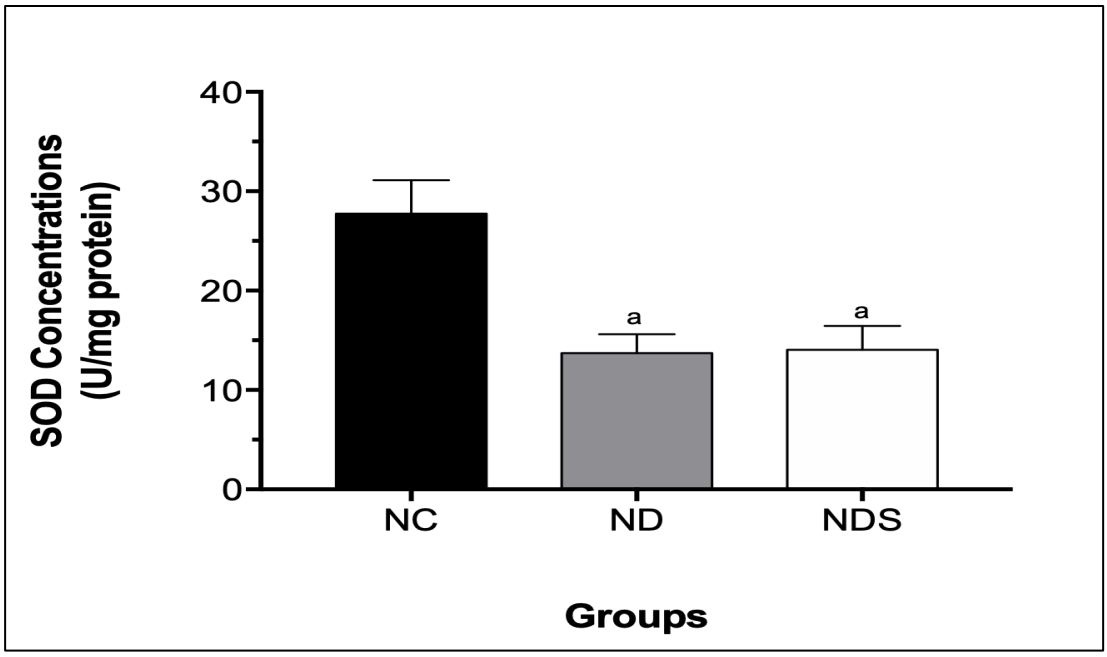


Figure 4.17 Superoxide dismutase levels of normal rats.

(n = 10 rats per group)

[a] $p < 0.001$ (One-way ANOVA)

[a] $p < 0.01$ when compared to group NC.

[NC: normal rats with control diet; ND: normal rats with vitamin D-deficient diet; NDS: normal rats with vitamin D-deficient diet + calcitriol supplementation]

Diabetic Rats

In diabetic rats, rats receiving vitamin D-deficient diet (group DD) had significantly lower SOD levels than diabetic controls (group DC) (p = 0.030). However, calcitriol supplementation significantly increased SOD levels in group DDS compared to group DD (p = 0.007) and the SOD levels were comparable to group DC (Figure 4.18). [SOD - DC: 25.58±5.83 U/mg protein; DD: 12.38±4.28 U/mg protein; DDS: 28.40±16.95 U/mg protein]

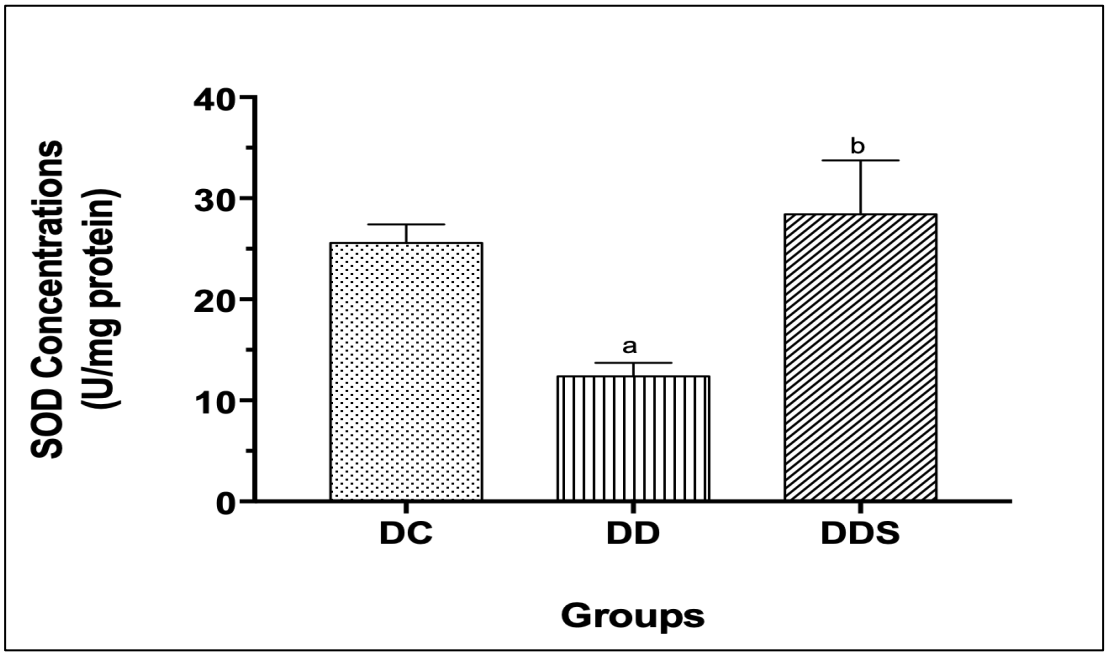


Figure 4.18 Superoxide dismutase levels of diabetic rats.

(n = 10 rats per group)

$p < 0.001$ (One-way ANOVA)

[a] $p < 0.05$ when compared to group DC; [b] $p < 0.01$ when compared to group DD.

[DC: diabetic rats with control diet; DD: diabetic rats with vitamin D-deficient diet; DDS: diabetic rats with vitamin D-deficient diet + calcitriol supplementation]

4.1.7(b) Malondialdehyde Levels

Normal Rats

In normal rats, there was no statistical difference in MDA levels between all study groups (groups NC, ND and NDS) (p = 0.165) (Figure 4.19). [MDA - NC: 2.14±0.91 nmol/mg protein; ND: 3.12±2.02 nmol/mg protein; NDS: 1.85±1.39 nmol/mg protein]

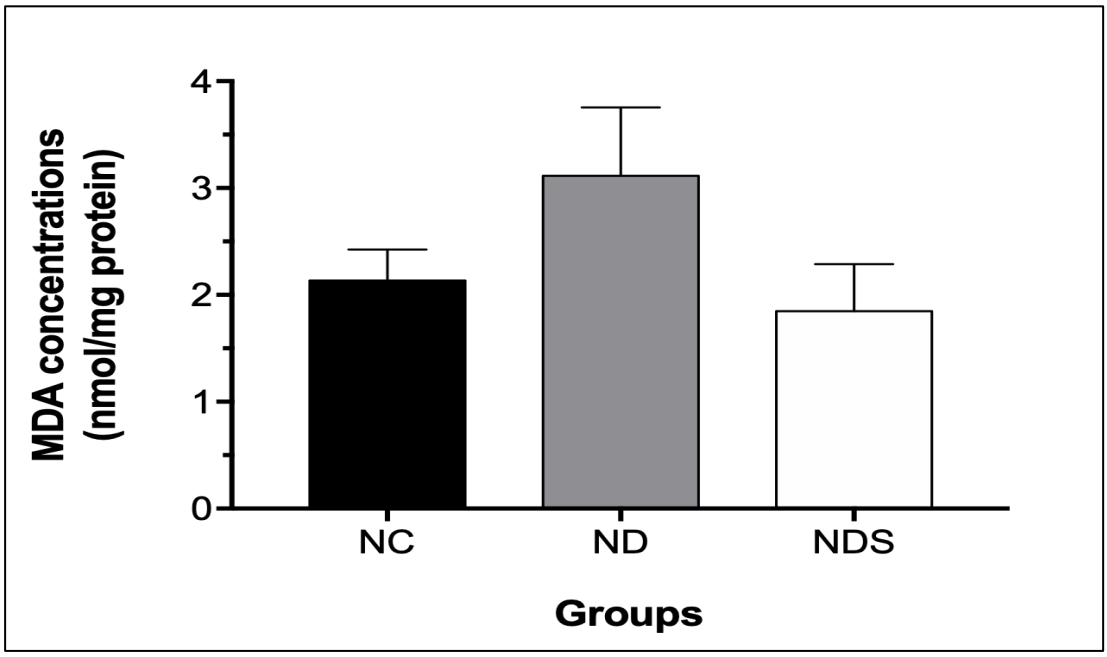


Figure 4.19 Malondialdehyde levels of normal rats.

(n = 10 rats per group)

There was no significant difference between study groups in normal rats (p = 0.165).

[NC: normal rats with control diet; ND: normal rats with vitamin D-deficient diet; NDS: normal rats with vitamin D-deficient diet + calcitriol supplementation]

Diabetic Rats

Diabetic rats receiving vitamin D-deficient diet (group DD) had significantly higher MDA levels than diabetic controls (group DC) (p = 0.006). Calcitriol supplementation to vitamin D-deficient rats (group DDS) significantly reduced MDA levels when compared to group DD (p = 0.010) and the MDA levels were comparable to group DC (Figure 4.20). [MDA - DC: 1.85±1.57 nmol/mg protein; DD: 4.87±2.53 nmol/mg protein; DDS: 2.06±1.60 nmol/mg protein]

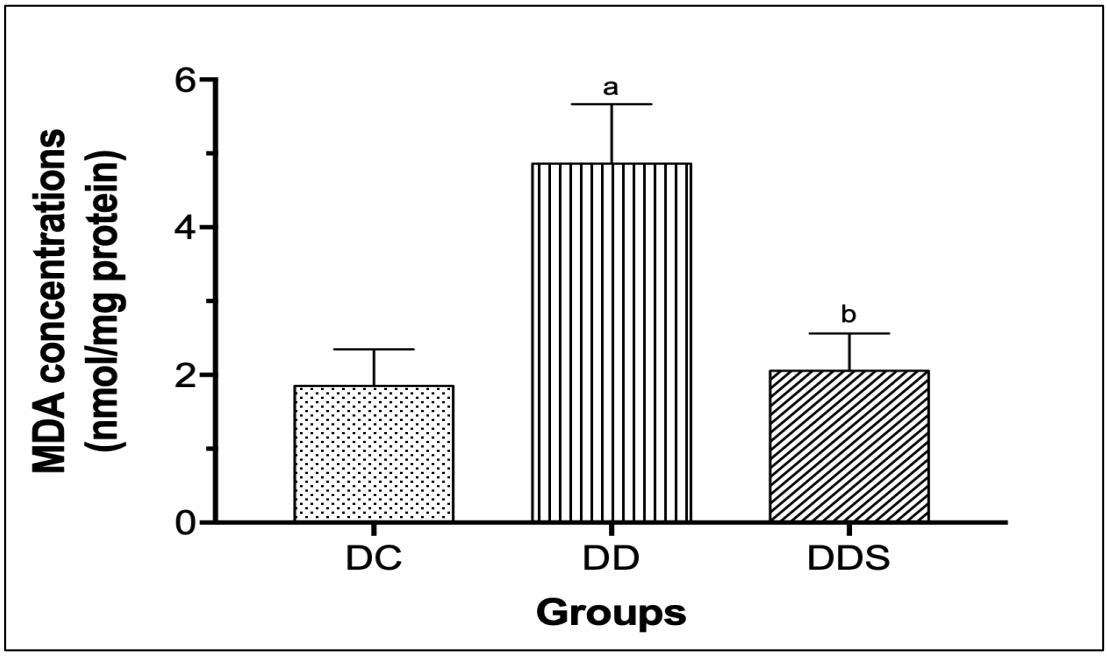


Figure 4.20 Malondialdehyde levels of diabetic rats.

(n = 10 rats per group)

p = 0.003 (One-way ANOVA)

[a] $p < 0.01$ when compared to group DC; [b] $p < 0.05$ when compared to group DD.

[DC: diabetic rats with control diet; DD: diabetic rats with vitamin D-deficient diet; DDS: diabetic rats with vitamin D-deficient diet + calcitriol supplementation]

4.1.8 Protein Expression and Localization of eNOS

Normal Rats

By performing Western blotting, a single protein band with protein size of approximately 133 kDa was detected as eNOS while another single protein band with protein size of approximately 42 kDa was identified as ß-actin.

In normal rats, the expression levels of eNOS were significantly lower in the mesenteric arteries of rats receiving vitamin D-deficient diet (group ND) compared to rats receiving control diet (group NC) ($p < 0.001$). However, the expression levels of eNOS in the mesenteric arteries of calcitriol-supplemented vitamin D-deficient rats (group NDS) were comparable to group ND and the protein expression levels remained significantly lower than group NC ($p < 0.001$) (Figure 4.21). [eNOS- NC: 0.61±0.16 arbitrary unit; ND: 0.29±0.03 arbitrary unit; NDS: 0.39±0.04 arbitrary unit]

In normal rats, immunohistochemical staining demonstrated that the intensity of immunoreactive eNOS protein was lower in the mesenteric arteries of rats receiving vitamin D-deficient diet (group ND) compared to rats receiving control diet (group NC). The intensity of eNOS in the mesenteric arteries of calcitriol-supplemented vitamin D-deficient rats (group NDS) was comparable to group ND (Figure 4.22).

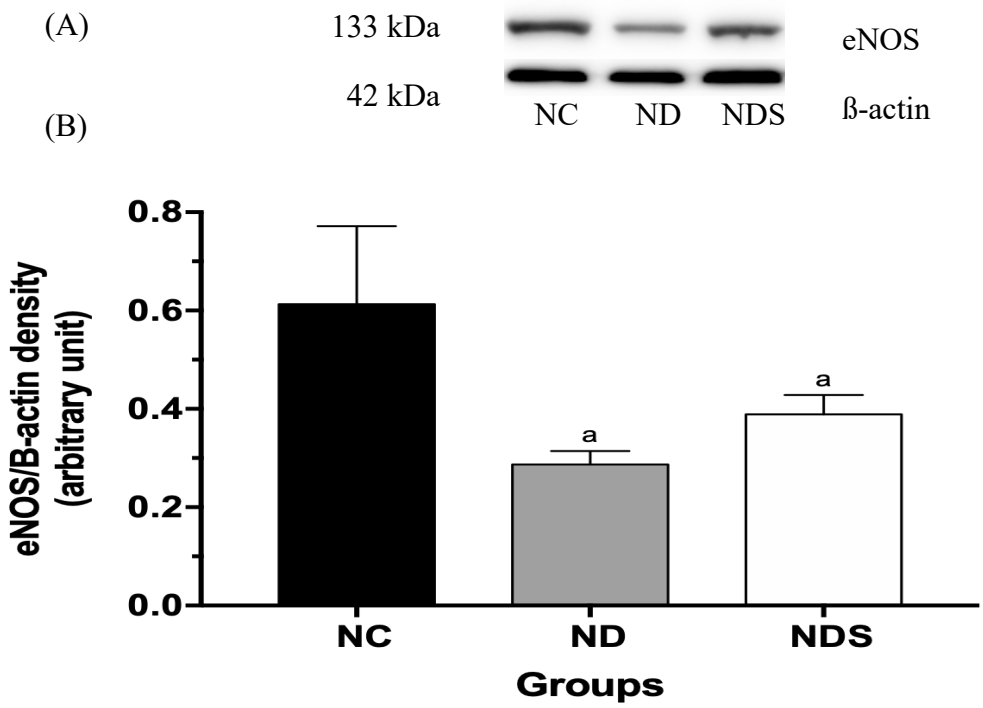


Figure 4.21 (A) Representative Western blot results demonstrated eNOS protein expression in mesenteric arteries of normal rats. (B) Graphical representation of the data normalised to ß-actin.

(n = 10 rats per group).

$p = 0.001$ (One-way ANOVA); [a] $p < 0.001$ when compared to group NC.

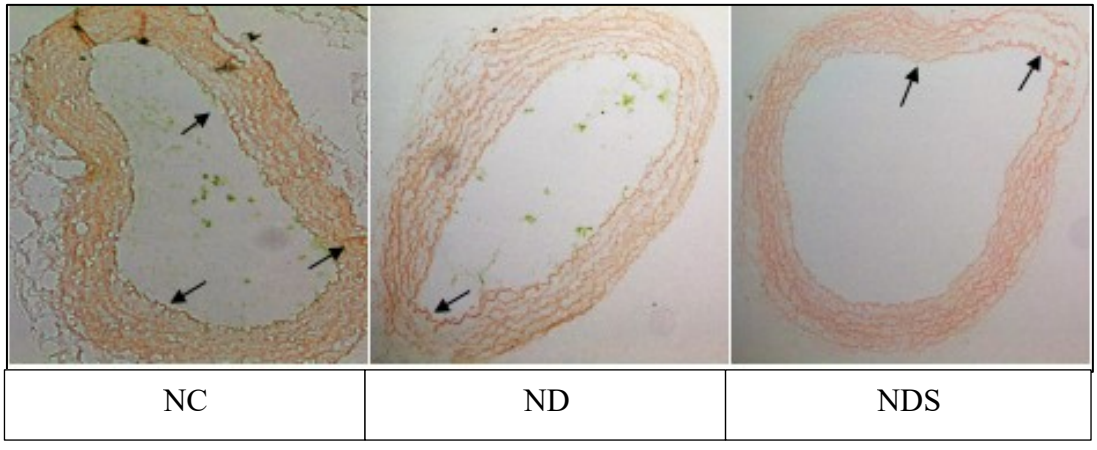


Figure 4.22 Representative immunohistochemical staining results demonstrated the localization of eNOS in mesenteric arteries of normal rats.

Reddish-brown staining with arrow pointing indicates the presence of eNOS. Magnification 100X.

[NC: normal rats with control diet; ND: normal rats with vitamin D-deficient diet; NDS: normal rats with vitamin D-deficient diet + calcitriol supplementation]

Diabetic Rats

Similarly, a single protein band with protein size of approximately 133 kDa detected as eNOS while another single protein band with protein size of approximately 42 kDa identified as ß-actin were visible by performing Western blotting.

In diabetic rats, the expression levels of eNOS were significantly lower in the mesenteric arteries of rats receiving vitamin D-deficient diet (group DD) compared to rats receiving control diet (group DC) ($p < 0.001$). Calcitriol supplementation improved the expression levels of eNOS in the mesenteric arteries of calcitriol-supplemented vitamin D-deficient rats (group DDS) compared to group DD ($p < 0.001$) (Figure 4.23). [eNOS - DC: 0.21±0.01 arbitrary unit; DD: 0.09±0.02 arbitrary unit; DDS: 0.15±0.02 arbitrary unit]

In diabetic rats, immunohistochemical staining demonstrated that the intensity of immunoreactive eNOS protein was lower in the mesenteric arteries of rats receiving vitamin D-deficient diet (group DD) compared to rats receiving control diet (group DC). Calcitriol-supplemented vitamin D-deficient rats (group DDS) showed higher eNOS intensity in their mesenteric arteries compared to group DD and the eNOS intensity was comparable to group DC (Figure 4.24).

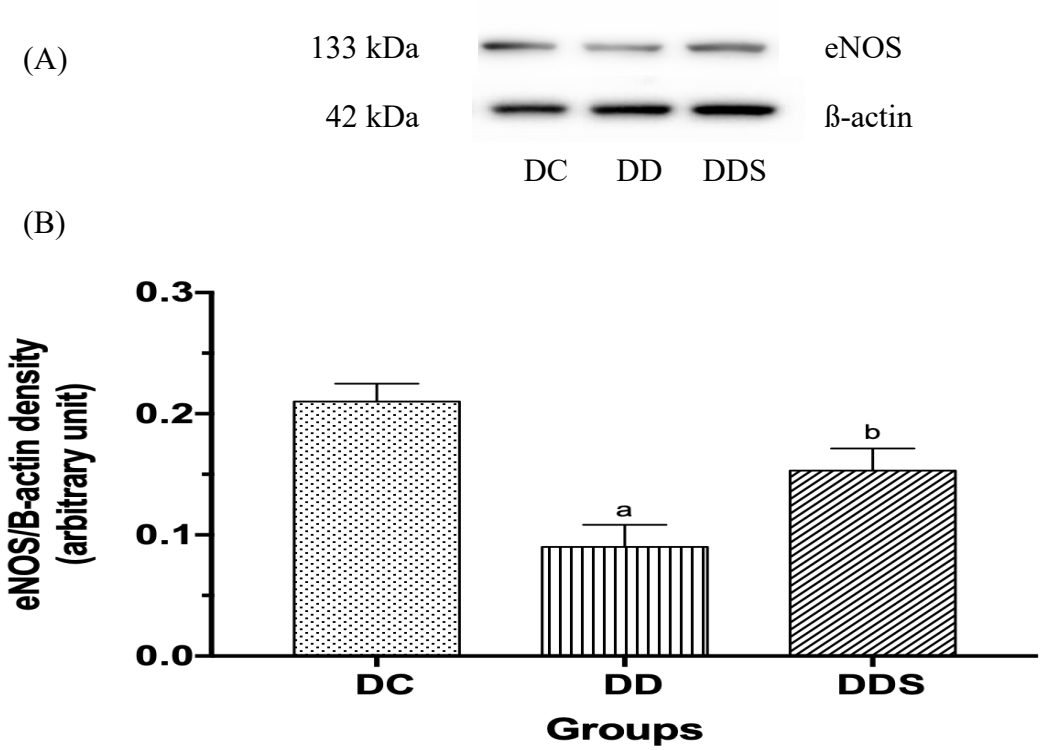


Figure 4.23 (A) Representative Western blot results demonstrated eNOS protein expression in mesenteric arteries of diabetic rats. (B) Graphical representation of the data normalised to ß-actin.

(n = 10 rats per group)

$p < 0.001$ (One-way ANOVA)

[a] $p < 0.001$ when compared to group DC; [b] $p < 0.001$ when compared to group DD.

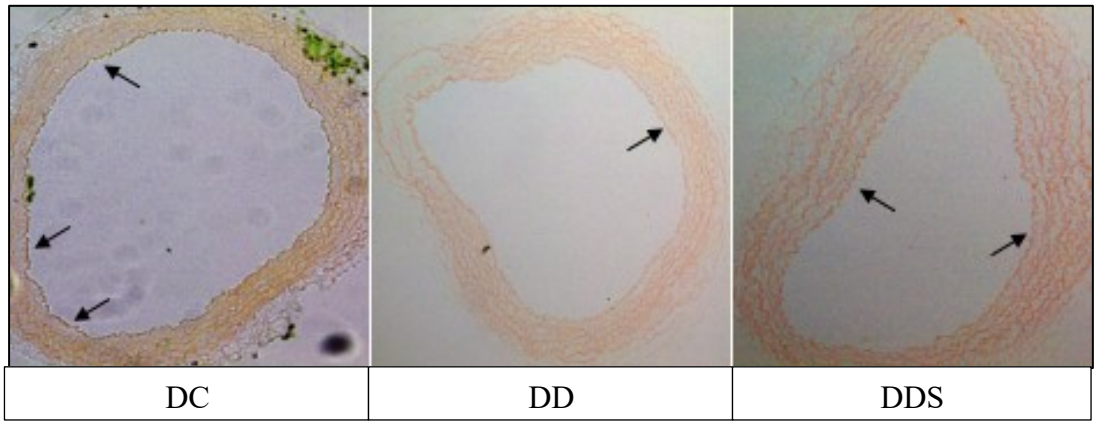


Figure 4.24 Representative immunohistochemical staining results demonstrated the localization of eNOS in mesenteric arteries of diabetic rats.

Reddish-brown staining with arrow pointing indicates the presence of eNOS. Magnification 100X.

[DC: diabetic rats with control diet; DD: diabetic rats with vitamin D-deficient diet; DDS: diabetic rats with vitamin D-deficient diet + calcitriol supplementation]

4.2 Human Study

Normally distributed numerical data determined by independent t-test were presented as mean±standard deviation (SD) while non-normally distributed data by Mann-Whitney test were expressed as median±interquartile range (IQR) in the tables. Pearson Chi-Square test was used to determine the differences for categorical variables between two study groups, expressed as number (%). Data were presented as mean±standard error of mean (SEM) in the figures. In all cases, the *p* value of less than 0.05 was considered as statistically significant.

4.2.1 Background Characteristics

Table 4.5 showed the background characteristics of vitamin D non-deficient diabetic patients (group DNP) and vitamin D-deficient diabetic patients (group DDP) in this study. The present study showed that vitamin D levels of group DDP were significantly lower than group DNP ($p < 0.001$). The mean age of group DDP was significantly lower than group DNP ($p = 0.001$); diabetic patients with vitamin D deficiency were significantly younger. There was no significant difference in their gender, body mass index, blood pressures (both systolic and diastolic), total cholesterol levels and serum calcium levels between these two study groups. Vitamin D deficiency in diabetic patients (group DDP) did not show significant difference in glycaemic parameters such as FBG and HbA_{1c} levels compared to group DNP. Based on the medical history of both study groups, eight patients in group DNP had hypertension while seven were treated for hypercholesterolemia. In group DDP, there were 11 patients treated for hypertension, and seven patients had hypercholesterolemia. However, there was no significant difference in the underlying diseases between these study groups.

Table 4.5 Background characteristics of diabetic patients.

Parameters	**Group DNP (n = 10)**	**Group DDP (n = 13)**	***p* value**
Male; n (%) / Female; n (%)	5 (50.00) / 5 (50.00)	6 (46.15) / 7 (53.85)	0.855
Age (years old)	63.20 (6.16)	49.46 (9.87)	0.001*
Body Mass Index, BMI (kg/m^2)	23.34 (2.70)	26.15 (6.64)	0.184
Systolic Blood Pressure, SBP (mm/Hg)	140.40 (19.77)	139.31 (13.71)	0.877
Diastolic Blood Pressure, DBP (mm/Hg)	76.20 (9.59)	76.92 (12.22)	0.879
Total Cholesterol Levels (mmol/L)	4.27 (1.45)	4.23 (0.90)	0.938
Serum 25(OH)D Levels (nmol/L)	66.98 (12.73)	37.73 (6.82)	<0.001*
Fasting Blood Glucose, FBG Levels (mmol/L)	6.95 (2.10)	8.56 (2.63)	0.128
Glycated Haemoglobin, HbA_{1c} Levels (%)	9.25 (2.98)	9.70 (1.79)	0.679
Calcium Levels (mmol/L)	2.09 (0.27)	1.94 (0.17)	0.156
Underlying Diseases; n (%)			
Hypertension	8 (80.00)	11 (84.62)	0.772
Hypercholesterolemia	7 (70.00)	7 (53.85)	0.431

Numerical data were expressed in mean (SD); Categorical data were expressed in number (%).

* indicates a statistically significant difference between two study groups.

[DNP: vitamin D non-deficient diabetic patients; DDP: vitamin D-deficient diabetic patients]

4.2.2 Oxidative Stress Parameters

Previous studies reported that age might be one of the potential covariates that affects oxidative stress levels. In this study, there was significant difference in age between two study groups. Hence, the analysis of covariance (ANCOVA) was performed to adjust the influence of this variable in oxidative stress analysis.

4.2.2(a) Superoxide Dismutase Levels

As illustrated in Table 4.6, the subcutaneous artery of diabetic patients with vitamin D deficiency (group DDP) showed significantly lower SOD levels compared to vitamin D non-deficient diabetic patients (group DNP) ($p = 0.001$). However, after adjusting for age as the covariate, the difference is no longer statistically significant at $p < 0.05$. A lower trend for SOD levels was still be seen in the vitamin D-deficient group compared to non-deficient group ($p = 0.072$).

Table 4.6 Superoxide dismutase levels of diabetic patients.

	DNP (n = 10)	**DDP (n = 13)**	**Mean Difference (95% CI)**	***p* value**
SOD levels (U/mg protein)	2.74±1.49	0.94±0.80	1.80 (0.80, 2.80)	0.001*
Adjusted SOD levels (U/mg protein)	2.37±1.30	1.22±1.26	1.15 (-0.11, 2.40)	0.072

Values were expressed in mean±standard deviation.

Adjustment for age was made using ANCOVA.

* indicates a statistically significant difference between two study groups.

[SOD: superoxide dismutase; CI; confidence interval; DNP: vitamin D non-deficient diabetic patients; DDP: vitamin D-deficient diabetic patients]

4.2.2(b) Malondialdehyde Levels

As showed in Table 4.7, the subcutaneous artery of diabetic patients with vitamin D deficiency (group DDP) showed significantly higher MDA levels compared to vitamin D non-deficient diabetic patients (group DNP) (p = 0.017). After the adjustment of age as the covariate, the significant difference in MDA levels between two study groups persisted (p = 0.005).

Table 4.7 Malondialdehyde levels of diabetic patients.

	DNP (n = 10)	**DDP (n = 13)**	**Mean Difference (95% CI)**	***p* value**
MDA levels (nmol/mg protein)	10.60±3.79	16.71±6.63	-6.11 (-11.01, -1.22)	0.017*
Adjusted MDA levels (nmol/mg protein)	8.76±6.33	18.12±6.12	-9.36 (-15.50, -3.23)	0.005*

Values were expressed in mean±standard deviation.

Adjustment for age was made using ANCOVA.

* indicates a statistically significant difference between two study groups.

[MDA: malondialdehyde; CI: confidence interval; DNP: vitamin D non-deficient diabetic patients; DDP: vitamin D-deficient diabetic patients]

CHAPTER 5

DISCUSSION

5.1 Animal Study

5.1.1 Body Weight

The measurement of body weight is a common way to monitor and determine the general health status of an animal. Baseline body weight, measured on the day of successful diabetes induction, was comparable at the beginning of the study for all study groups in normal and diabetic rats.

Normal Rats

In normal rats, significant body weight gain over 10-weeks study duration was observed for all study groups. All the groups in normal rats had comparable final body weight after 10-weeks study period. The similar rate of weight gain indicates that the rats, regardless of their vitamin D status, undergo normal growing process over time.

Normal rats with vitamin D deficiency showed no significant difference in body weight changes compared to controls. This is in contrast with an increasing body of evidence that implied 25(OH)D levels are inversely associated with body fat mass, involved in weight regulation (Konradsen *et al*., 2008; Kremer *et al.*, 2009). Asymptomatic subjects with lower 25(OH)D levels showed higher weight gain and body fat compared to controls with optimal vitamin D status (Erin *et al.*, 2012). The possible mechanism proposed as low 25(OH)D levels trigger vitamin D receptors (VDR) located on adipocytes via 25(OH)D signalling, to promote greater adiposity through the regulation of parathyroid hormone (PTH) and the modulation of

adipogenesis. Low vitamin D levels increase the secretion of PTH that promotes calcium influx into adipocytes, thus induces lipogenesis and inhibits lipolysis, leading to fat accumulation and weight gain. Low 1,25(OH)D_2 levels might also modulate adipogenesis, leads to excess differentiation of preadipocytes to adipocytes (McCarty and Thomas, 2003; Zemel, 2003). This as well explained the findings of the prevalence of vitamin D deficiency is more pronounced in overweight and obese populations (Konradsen *et al.*, 2008).

The present study found that calcitriol-supplemented vitamin D-deficient rats showed the reduced trend of body weight changes ($p = 0.0864$) compared to vitamin D-deficient rats. This is in line with a recent study that reported vitamin D supplementation induces weight loss in obese children with vitamin D deficiency, in a 12-months intervention program (Szlagatys-Sidorkiewicz *et al.*, 2017). Hydroxyvitamin D supplementation has also been shown to reduce fat mass and increase lean mass in rats (Siddiqui *et al.*, 2008). Adequate vitamin D levels will lower PTH levels to diminish the calcium influx into adipocytes, thus increases lipolysis and induces apoptosis in adipocytes, leading to decreased fat mass and weight loss (Sergeev, 2009). Another possible reason for the weight loss might be due to vitamin D-induced hypercalcaemia as reported in mice (Yoshioka *et al.*, 2017). Elevated calcium levels induced by cholecalciferol supplementation in mice lead to transient weight loss, presumably due to hypercalcaemia that disrupts calcium homeostasis and enhances hepatotoxicity. This explanation is in line with the present finding that showed the reduced trend of body weight changes in calcitriol-supplemented vitamin D-deficient normal rats, presumably due to vitamin D-induced hypercalcaemia.

Diabetic Rats

The diabetic controls showed no significant increase in body weight throughout 10-weeks study period. This might due to the course of diabetes that interrupts weight gain in rats. At the onset of the disease, insufficient insulin alters the glucose metabolism for energy demand; preventing the body from getting glucose from the blood into the body cells as energy supply. Thus, the body starts breaking down fat and muscle tissue, predisposing to the reduction in body weight. Despite the impact of this disease, diabetic rats with vitamin D deficiency, both with and without calcitriol supplementation, incurred a significant increase in body weight changes after 10-weeks study duration. Vitamin D deficiency coupled with diabetes interrupts glucose and lipid homeostasis by reducing the production and secretion of adipokines such as adiponectin, thus promoting greater adiposity (as discussed in normal rats) and contributing to the onset of obesity or weight gain over time (Berridge, 2017). Nevertheless, calcitriol-supplemented vitamin D-deficient diabetic rats showed similar final body weight after 10-weeks study period and comparable body weight changes compared to vitamin D-deficient diabetic rats.

Comparing between normal and diabetic rats, diabetic rats in their respective groups gained much lower weight over 10-weeks study duration compared to normal rats, regardless of vitamin D status. Their final body weight and body weight changes were also significantly lower than respective study groups in normal rats. Reduced rate of body weight gain in the rats usually serves as an indicator of deteriorating health in the animal models (OECD, 2002a).

5.1.2 Fasting Blood Glucose Levels

Normal Rats

FBG levels of all study groups in normal rats did not show significant changes over time. FBG levels of normal rats, regardless of vitamin D status, remained similar until the end of the study. All normal rats had FBG levels within the normal range throughout the study duration.

Diabetic Rats

Diabetic rats had higher baseline FBG levels than normal rats for all categories of vitamin D status. This showed the effectiveness of single dose streptozotocin injected intraperitoneally to the rats in developing diabetic models for this study. Baseline FBG levels for all groups in diabetic rats were comparable. Even though diabetic controls and calcitriol-supplemented vitamin D-deficient diabetic rats showed a significant increase in their final FBG levels compared to their baseline levels, the final FBG levels between all study groups showed no significant difference. FBG levels of diabetic rats persist within the diabetes range (more than 16.6 mmol/L) until the end of the study indicates that the condition of hyperglycaemia developed in diabetic rats was preserved throughout the study duration.

5.1.3 Vitamin D Levels

Normal Rats

Vitamin D-deficient normal rats demonstrated a markedly reduced serum 25(OH)D levels compared to controls. This indicates that the 10-weeks duration of giving a vitamin D-deficient diet has successfully developed and maintained the rats

with vitamin D deficiency. According to Stavenuiter *et al.* (2015), the condition of vitamin D deficiency can be safely induced in laboratory rats with negligible effect of PTH within three weeks of giving vitamin D-deficient diet. Inadequate nutritional intake of vitamin D from daily diet with controlled exposure to ultraviolet B radiation attenuates the subsequent hydroxylation processes of vitamin D in the body, leading to the reduced serum 25(OH)D levels in vitamin D-deficient rats.

The present finding discovered that calcitriol supplementation failed to increase the reduced serum 25(OH)D levels in vitamin D-deficient rats. This is reasonably explained by the type of vitamin D analogue given to the supplemented group. Vitamin D-deficient rats were supplemented with calcitriol ($1,25(OH)_2D$) instead of calcidiol (25(OH)D) in the present study. Calcitriol is the physiologically active metabolite converted from 25(OH)D in the presence of 25(OH)D-1α-hydroxylase during second hydroxylation process in the kidney (Christakos *et al.*, 2012). This explained why serum 25(OH)D levels remained low although calcitriol was given to the supplemented group.

Diabetic Rats

Similarly, significantly lower serum 25(OH)D levels were observed in vitamin D-deficient diabetic rats compared to diabetic controls. Calcitriol supplementation to vitamin D-deficient diabetic rats failed to improve serum 25(OH)D levels in the supplemented rats. Although serum 25(OH)D levels were not improved by calcitriol supplementation, the serum concentrations of circulating $1,25(OH)_2D$, the active form of vitamin D which accounts for its physiological effects, will likely be higher.

However, the measurement of serum $1,25(OH)_2D$ levels is not conducted in this study. This is because measuring the exact serum calcitriol levels is not practically

feasible due to its lipophilic nature and low circulating concentrations at picomolar levels (Wootton, 2005). Furthermore, calcitriol has a shorter half-life (Jones, 2008) and its levels are mainly manipulated by PTH (Zerwekh, 2008), thus the outcome might not conclusively represent the exact value of calcitriol concentrations in the blood. Thus, the measurement of 25(OH)D levels remained to be the gold standard used to represent the vitamin D status in the body; it circulates at 1000-fold higher concentrations compared to calcitriol (Alshahrani and Aljohani, 2013).

Despite the limitation in the measurement of serum calcitriol levels, this study still proposed to supplement the rats with calcitriol instead of inactive vitamin D_3 analogues or vitamin D_2 supplementation. This is considering the fact that other vitamin D analogues might have a different binding capacity to vitamin D binding protein; and the diabetic or disease models may have difficulties to convert vitamin D to its active metabolites (Aronov *et al.*, 2008). Impaired kidney function due to diabetic kidney disease develops in approximately 30% of T1DM patients; this may affect the conversion of 25(OH)D to calcitriol which normally occurs at the kidney (Alicic *et al.*, 2017). Hence, calcitriol is preferred as it is the active metabolite of vitamin D that can directly exert its physiological role without biological conversion.

The present study also discovered that vitamin D levels between normal and diabetic rats in their respective categories of vitamin D status were similar. This showed that diabetes does not significantly exacerbate the condition of vitamin D deficiency in rat models in the present study.

5.1.4 Calcium Levels

$1,25(OH)_2D$ and PTH are the main hormonal regulators of ionised calcium. Theoretically, vitamin D facilitates the absorption of calcium from food through the

intestinal wall and transports it into the circulatory system, through the interaction with VDR. High vitamin D levels improve intestinal calcium absorption, thus increasing calcium levels in the blood, which is essential for calcium homeostasis and bone metabolism (Heaney *et al.*, 1997). Low vitamin D levels lead to the defect in intestinal calcium absorption and subsequently lower intracellular calcium levels. Hypocalcaemia was reported in vitamin D-deficient models and also VDR knockout mice (Erben *et al.*, 2002). Low calcium levels induced by low vitamin D levels promotes secondary hyperparathyroidism. The increased production and secretion of PTH, which in turn stimulates the release of more calcium ions from large calcium stores in the bones into the bloodstream, in an attempt to increase calcium levels (Fraser, 2009).

Normal Rats

Vitamin D-deficient normal rats showed comparable calcium levels compared to controls. This might due to calcium homeostasis that maintains the calcium levels in the blood even when the body does not get enough vitamin D from diet or through sunlight exposure. Bone reserves might serve as the protective measure to sustain the calcium levels in the blood regulated by PTH (Lieben and Carmeliet, 2013). This explains why 10-weeks duration of having a vitamin D-deficient diet successfully reduced vitamin D levels in rats, but calcium levels remained constant in this study.

Serum calcium levels in calcitriol-supplemented vitamin D-deficient rats were significantly higher compared to vitamin D-deficient rats, as well as compared to controls. Calcitriol enhances intestinal calcium absorption in normal rats, which in excess might interfere with calcium homeostasis, leading to the unfavourable effects of hypercalcaemia. Nevertheless, calcitriol-supplemented vitamin D-deficient non-

diabetic rats in this study did not show any observable sign of vitamin D toxicity and/or hypercalcaemia such as retarded growth, dehydration, polyuria, polydipsia and foamy urine throughout the study period. The dosage of calcitriol supplementation in the present study was chosen based on previous studies which gave the same dose of calcitriol (0.15 µg/kg/day) to control rats, for four and 13 weeks by subcutaneous injection (Mancuso *et al.*, 2008; Lee *et al.*, 2014) and for 18 weeks by oral gavage (Dong *et al.*, 2012). Similar studies and even longer duration of calcitriol supplementation did not report signs of hypercalcaemia in their control groups.

Diabetic Rats

Similarly, vitamin D deficiency in diabetic rats showed no significant difference in calcium levels compared to diabetic controls, mainly due to calcium homeostasis being regulated by PTH. However, the same dosage of calcitriol supplemented to vitamin D-deficient diabetic rats showed different finding as in normal rats. Calcitriol supplemented to vitamin D-deficient diabetic rats did not significantly potentiate serum calcium levels. The same amount of calcitriol given to diabetic rats might not be fully utilised for its physiological role in facilitating intestinal calcium absorption as those given to normal rats. The course of diabetes may deteriorate intestinal calcium absorption, leading to a reduction in calcium uptake into the body for calcium metabolism (Wong *et al.*, 2017).

Comparing between calcitriol-supplemented vitamin D-deficient normal and diabetic rats, their serum calcium levels were similar. The different findings in their vascular and oxidative stress parameters in the present study might possibly be due to the actual amounts of free calcium ions in the circulatory system. The calcium levels measured in the blood are the total amounts of calcium ions, which consist of

free/ionised calcium ions, calcium ions bound to proteins like albumin and calcium ions bound to various organic anions.

Free calcium ions are the most active functional group responsible for its physiological role. High calcium levels in normal rats possibly indicate higher free calcium ions, which in excess might be responsible for hypercalcaemia. Unlike diabetic rats that received vitamin D supplementation, which might experience the alteration in vitamin D metabolism; the same dosage of calcitriol might not be fully utilised for improving intestinal calcium absorption, or the calcium ions might in a higher ratio of bounded form than in free form (McNair *et al.*, 1983). This might well explain why calcitriol supplementation to vitamin D-deficient diabetic rats showed favourable outcome in vascular parameters, although their serum calcium levels were similar to those calcitriol-supplemented vitamin D-deficient rats with hypercalcaemia.

5.1.5 Microvascular Tissue Oxidative Stress Status

Oxidative stress reflects an imbalance between the production of free radicals and the effectiveness of antioxidant defence system to counteract them (Betteridge, 2000). Oxidative stress plays a dominant role in the development of vascular complications, particularly in diabetic subjects (Pham-Huy *et al.*, 2008). The improvement in oxidative stress parameters, indicating reduced oxidative stress levels, is likely to confer vascular protection.

5.1.5(a) Superoxide Dismutase Levels

Superoxide dismutase (SOD) is the antioxidant enzyme that acts as a major defender in antioxidant defence system to encounter oxidative stress and subsequently reduce the risk of cellular injury (Tiwari *et al.*, 2013). SOD catalyses the dismutation

of superoxide anion to hydrogen peroxide and molecular oxygen (Evans *et al.*, 2003). Accumulating evidence reported that the levels and activity of SOD may be low and diminished due to its depletion, in order to counteract with excess free radicals over time (Giacco and Brownlee, 2010; Shukla *et al*, 2012; Kayama *et al.*, 2015). The depletion of antioxidant enzymes may render the animal models more susceptible to ROS attack and more vulnerable to oxidative damage.

Normal Rats

Vitamin D-deficient normal rats exhibited significantly lower SOD levels compared to controls. Lower SOD levels found in vitamin D-deficient rats may be a compensatory response to combat with excess ROS induced by vitamin D deficiency. Vitamin D deficiency impairs mitochondrial respiratory functions (excessive respiration leads to elevated ROS production), upregulates the gene expression of NADPH oxidase (the key producer of ROS) and downregulates cellular antioxidants expression, leading to the elevated formation of free radicals and ROS (Dong *et al.*, 2012; Wimalawansa, 2019). There was a significant positive correlation between serum 25(OH)D levels and erythrocyte SOD activity in vitamin D-deficient subjects receiving vitamin D_3 supplementation (Javanbakht *et al.*, 2010); reduced SOD levels have been reported in vitamin D-deficient subjects (Tarcin *et al.*, 2009).

Calcitriol supplementation to vitamin D-deficient rats showed comparable SOD levels as in non-supplemented vitamin D-deficient rats. SOD levels are most likely to be increased by calcitriol supplementation in the present study. However, hypercalcaemia induced by calcitriol supplementation might induce the generation of ROS in calcitriol-supplemented vitamin D-deficient rats. Augmented oxidative stress depletes SOD levels, this possibly explained why SOD levels remained low even after

four-weeks calcitriol treatment in vitamin D-deficient rats with hypercalcaemia. High calcium levels induced by vitamin D supplementation presumably potentiate oxidative stress by decreasing total antioxidant activities (Yoshioka *et al.*, 2017).

Diabetic Rats

Likewise, vitamin D-deficient diabetic rats showed markedly reduced SOD levels compared to diabetic controls. SOD levels were depleted presumably as a result of its antioxidant defence activities. Reduced SOD levels and activity have been reported in diabetic animal models (Hamden *et al.*, 2009; George *et al.*, 2012; Alatawi *et al.*, 2018). Hyperglycaemia in diabetes could serve as an additional source of mitochondrial ROS overproduction by both enzymatic and non-enzymatic pathways, rendering diabetics to augmented oxidative stress (Tiganis, 2011). Reduced SOD levels may exacerbate oxidative stress status, which is one of the risk factors preceding the development of atherosclerosis and secondary complications in diabetics. However, there were other studies that reported preserved or increased SOD levels in diabetic models (Bandeira *et al., 2012*; Jang *et al.*, 2000). The difference in findings is due mainly to the disease duration of diabetes. There was a triphasic change in SOD levels in diabetes pathogenesis. SOD levels might first increase to encounter with increased ROS levels, preserved when the antioxidant defence mechanism is ongoing and might lastly decrease or deplete due to long-term defence process.

Vitamin D-deficient diabetic rats given calcitriol supplementation showed a significant increase in SOD levels to values similar as diabetic controls. The outcome is similar with the animal studies conducted in diabetic Wistar rats supplemented with four-weeks calcitriol (Hamden *et al.*, 2009), two-weeks cholecalciferol (George *et al.*, 2012) and four-weeks vitamin D_3 with calcium (Alatawi *et al.*, 2018) respectively.

However, there were contradictory findings that reported T2DM patients with suboptimal vitamin D levels receiving vitamin D_3 tablets once daily for 12 weeks, did not significantly improve SOD levels (Yiu *et al.*, 2013). The types of vitamin D analogues provided to the study models are the main determinant for a favourable outcome. Calcitriol is more efficient in its physiological role as it is the active metabolite compared to other vitamin D_3 analogues such as cholecalciferol and calcidiol. Likewise, vitamin D_3 is more efficacious compared to vitamin D_2 as vitamin D_3 is the type of vitamin D naturally exists in the body.

5.1.5(b) Malondialdehyde Levels

The measurement of the levels of ROS (such as superoxide anion) is not practical, in view that the free radical chain reactions occurred almost instantaneously. An exceptional increase in free radicals that is unable to be sufficiently scavenged by antioxidant enzymes initiates the free radical chain reactions, predisposing to peroxidative injury. MDA is one of the by-products of polyunsaturated fatty acid peroxidation in the cells. It is the most frequently used biomarker of oxidative stress in CVD (Khoubnasabjafari *et al.*, 2015). The measurement of MDA levels is reliably documented as the gold standard to indicate the levels of cellular peroxidative injury attributable to augmented ROS levels (Lefevre *et al*., 1998); higher MDA levels represent higher ROS levels that ultimately lead to higher degree of lipid peroxidation.

Normal Rats

Vitamin D deficiency in normal rats did not show significant changes in MDA levels compared to controls. Vitamin D deficiency in normal rats induces the generation of excess ROS, which is likely to be efficiently removed by the antioxidant SOD. Hence, vitamin D deficiency did not induce lipid peroxidation in normal rats,

due to the protective role played by the antioxidant enzymes. This is well explained by the reduced SOD levels in conjunction with preserved MDA levels as detected in mesenteric arteries of vitamin D-deficient rats in the present study.

Calcitriol supplementation to vitamin D-deficient rats showed comparable MDA levels as in vitamin D-deficient rats. This may be explained that the antioxidant activities by SOD are most likely sufficient to convey vascular protection from peroxidation injury in calcitriol-supplemented vitamin D-deficient rats.

Diabetic Rats

Vitamin D-deficient diabetic rats showed significantly increased MDA levels compared to diabetic controls. Chronic hyperglycaemia coupled with vitamin D deficiency in diabetes induces excess ROS production and diminishes the antioxidant activity by SOD, leading to augmented oxidative stress and subsequently peroxidative injury. Increased MDA levels have been well reported in diabetic animal models (Hamden *et al.*, 2009; George *et al.*, 2012; Alatawi *et al.*, 2018).

Calcitriol supplementation to vitamin D-deficient diabetic rats successfully reversed the enhanced MDA levels back to values similar as diabetic controls. This is in line with animal studies that exhibited administration of vitamin D_3 to diabetic rats reduced lipid peroxidation as indicated by decreased TBARS levels in hepatic and renal tissues (Hamden *et al.*, 2009) and in the liver (George *et al.*, 2012), as well as reduced serum MDA levels (Alatawi *et al.*, 2018) of diabetic Wistar rats. In human studies, serum MDA levels of vitamin D-deficient T2DM subjects receiving vitamin D_3 supplementation for 12 weeks was significantly reduced compared to T2DM subjects receiving placebo (Shab-Bidar *et al.*, 2015). However, T2DM patients with hyperlipidaemia with unknown baseline vitamin D levels receiving calcitriol for 12

weeks did not show the improvement in MDA levels (Eftekhari *et al*., 2014). Baseline vitamin D levels of the diabetic subjects might possibly be one of the crucial factors to obtain a favourable outcome of vitamin D supplementation. Vitamin D supplementation may not be adequate to sufficiently increase the severe deficient baseline vitamin D levels of the disease models to the optimal levels for favourable therapeutic effects.

Oxidative stress analysis in the present study showed different findings in MDA and SOD levels of normal and diabetic rats; MDA levels were preserved in non-diabetic rats with vitamin D deficiency but were increased in vitamin D-deficient diabetic rats. SOD levels were reduced in both diabetic and non-diabetic rats with vitamin D deficiency. Reduced SOD levels representing a compensatory response in combating excess ROS, in order to prevent the propagation of free radical chain reactions that ultimately lead to vascular peroxidative injury. Reduced SOD levels with preserved MDA levels as observed in normal rats, indicates that the antioxidant system was effective in protecting the blood vessels from lipid peroxidative injury. However, the antioxidant defence system may not be as effective in diabetic models. SOD might be depleted as the consequence of counteracting more ROS, subsequently exacerbates vascular oxidative stress status, leading to vascular peroxidative injury (higher MDA levels) as seen in diabetic rats.

5.1.6 eNOS Protein Expression

eNOS plays an important role in endothelium-dependent relaxation as reported in animal models (Chataigneau *et al.*, 1999). The activity of eNOS is regulated by the changes in intracellular Ca^{2+} concentrations which will affect its binding with calmodulin. eNOS binds to calmodulin to activate the transfer of electron from

NADPH to heme iron, which then catalyses the production of NO from L-arginine and molecular oxygen in endothelial cells (Abu-Soud *et al.*, 1994). NO plays a dominant role in regulating vascular tone and acts as the major vasodilator responsible for endothelium-dependent relaxation in most vessels (Palmer *et al.*, 1987; Furchgott and Vanhoutte, 1989; Moncada and Higgs, 1993). Studies have reported that eNOS control (+/+) mice showed the presence of endothelium-dependent relaxation, whereas eNOS knockout (-/-) mice demonstrated the absence of relaxation in aorta, carotid and coronary arteries.

Normal Rats

Vitamin D-deficient normal rats showed reduced protein expression of eNOS in their mesenteric arterial tissue compared to controls. Vitamin D is involved in enhancing the transcription of eNOS and non-genomic activation of eNOS, to promote NO production in endothelial cells. Reduced expression of eNOS was discovered in VDR mutant mice (Andrukhova *et al.*, 2014) and vitamin D-deficient rats (Daniele *et al.*, 2014).

There was no significant difference in protein expression of eNOS between calcitriol-supplemented vitamin D-deficient rats and vitamin D-deficient rats. This explained best by high calcium levels found in the supplemented rats that interfere with the activity of eNOS. The unfavourable effects of hypercalcaemia downregulate the eNOS protein expression. This is supported by previous study that implied negative correlation between NO production and calcitriol levels was found in peripheral-blood mononuclear cells from hypercalcaemia patients (Chang *et al.*, 2004).

Diabetic Rats

Vitamin D-deficient diabetic rats showed reduced protein expression of eNOS compared to diabetic controls. Chronic hyperglycaemia and presumably augmented oxidative stress have been suggested to relate with reduced eNOS protein expression in diabetics (Srinivasan *et al.*, 2004). Decreased eNOS protein expression and levels were reported in cultured human coronary arterial and aortic endothelial cells that were incubated in media with elevated glucose concentrations (Ding *et al.*, 2000; Srinivasan *et al.*, 2004), and also in subcutaneous arteries of diabetic patients (Mokhtar *et al.*, 2016). However, these observations are not in line with the finding that implied diabetes might not change the expression of total eNOS protein but may decrease the proportion of eNOS expressed as a dimer (Leo *et al*., 2011). A higher ratio of eNOS monomer to its dimer is likely associated with oxidative-driven events that lead to reduced production of NO by eNOS and thus impaired endothelium-dependent relaxation. However, the levels of total eNOS may become normal as diabetes progresses, as part of the mechanisms for endothelial protection (Leo *et al*., 2011).

Calcitriol supplementation to vitamin D-deficient diabetic rats showed significant improvement in protein expression of eNOS. This may be explained by the improvement in the oxidative stress status as indicated by reduced MDA and increased SOD levels found in the mesenteric arterial tissue of calcitriol-supplemented diabetic rats.

5.1.7 Microvascular Endothelial Function

The delicate balance between endothelium-dependent relaxation and endothelium-dependent contraction maintains vascular homeostasis and ensures fine regulation of vascular tone. Endothelial dysfunction is characterised by the imbalance

between the role of endothelium-derived vasoactive substances, generally due to reduced production of EDRF or/and augmented secretion of EDCF. Endothelial dysfunction which serves as an independent prognostic cardiovascular marker, can be early manifested in the microcirculation involving smaller arteries. Endothelial dysfunction can be initiated either as a consequence of reduced production and release of NO and/or by increased inactivation of NO due to the overproduction of ROS (Bauer and Sotnikova, 2010).

5.1.7(a) Endothelium-dependent Relaxation

Normal Rats

The present study showed that endothelium-dependent relaxation in mesenteric arteries of normal rats with vitamin D deficiency was significantly impaired compared to controls. This is in accordance with the previous findings that showed chronic vitamin D insufficiency decreased endothelium-dependent vasodilation in rats' mesenteric arteries (Tare *et al.*, 2011) and reduced brachial flow-mediated dilation in healthy subjects (Jablonski *et al.*, 2011). Likewise, endothelium-specific deletion of vitamin D receptors blunts endothelium-dependent relaxation in the mouse. Epidemiological studies also implied that low vitamin D levels are associated with impaired vascular function. Reduced protein expression and presence of eNOS as detected in the mesenteric arteries of vitamin D-deficient rats, appear to account for the blunting of ACh-induced endothelium-dependent relaxation in vitamin D-deficient rats in the present study. Reduced eNOS levels attenuate the production of NO, and hence curtail its role as the major vasodilator, leading to impaired endothelium-dependent relaxation.

In addition, a wealth of evidence also demonstrated the role of augmented oxidative stress that reduces NO bioavailability, in leading to vascular dysfunction (Ungvari *et al.*, 2008; Gori and Munzel, 2011). Excess ROS induced by vitamin D deficiency (details please refer to Section 2.7) increases the monomerization of the enzyme eNOS, leading to the dissociation of eNOS dimer to its monomer (Yang *et al*., 2009). The uncoupling of eNOS alters its function as the NO producer, accounts for the impairment in NO-mediated relaxation of mesenteric arteries. Furthermore, the uncoupled and dysfunctional eNOS turns to be the producer of superoxide anion, further exacerbates oxidative stress status (Leo *et al.*, 2011).

Endogenous antioxidant enzymes, particularly SOD, play a crucial role in scavenging excess superoxide anion produced in the cells to prevent peroxidative injury. In the present study, reduced SOD levels in conjunction with preserved MDA levels in vitamin D-deficient rats might represent a compensatory mechanism of SOD antioxidant activity in combating excess ROS to protect the blood vessels from lipid peroxidation. The diminished antioxidant enzymes might subsequently exacerbate the imbalance condition of vascular oxidative stress.

Calcitriol supplementation to vitamin D-deficient normal rats did not improve the impaired endothelium-dependent relaxation. The protein expression of eNOS in the mesenteric arteries of calcitriol-supplemented vitamin D-deficient rats remained low and comparable to vitamin D-deficient rats. The MDA and SOD levels were also comparable between vitamin D-deficient and calcitriol-supplemented normal rats. These observations implied that calcitriol supplementation did not exhibit favourable effects on vascular and oxidative stress parameters in non-diabetic vitamin D-deficient rats. This might well be explained by the unfavourable condition of hypercalcaemia

induced by calcitriol supplementation in the supplemented rats. High calcium levels may inhibit basal NO release from endothelial cells, leading to impaired NO-mediated vasorelaxation in calcitriol-supplemented rats. Hypercalcaemia has been suggested as one of the possible factors in the progression of endothelial dysfunction, as reported in both animal and human models (Lopez-Jaramillo *et al*., 1990; Nilsson *et al*., 2001).

Diabetic Rats

Accumulating findings showed that endothelial dysfunction, which is defined as impaired endothelium-mediated relaxation, is evident in mesenteric arteries of diabetic animal models (Lagaud *et al.*, 2001; Pannirselvam *et al.*, 2002; Leo *et al.*, 2011). In the present study, the impairment in endothelium-dependent relaxation was more prominent in mesenteric arteries of vitamin D-deficient diabetic rats than in normal rats. This is possibly associated with the course of the disease in diabetic models. In addition to the pathology of hyperglycaemia, the condition of vitamin D deficiency might be the additional possible underlying mechanism involved in exacerbating oxidative stress status. The increased superoxide anion production induced by hyperglycaemia and vitamin D deficiency impairs the catalytic activity of eNOS by causing eNOS uncoupling to become a dysfunctional superoxide-generating enzyme, severely compromising cellular NO generation by eNOS (Alderton *et al.*, 2001; De Pascali *et al.*, 2014). Under these pathophysiological conditions, excess superoxide anion scavenges NO produced by eNOS avidly with the resulting formation of peroxynitrite, reduces considerably the bioavailability of NO as the major vasodilator, predominantly leads to the attenuated endothelium-dependent relaxation in diabetic models. Reduced endothelium-dependent relaxation has also been consistently reported to be associated with increased oxidative stress levels.

Interestingly, the effectiveness of calcitriol supplementation on ACh-induced endothelium-dependent relaxation in vitamin D-deficient normal and diabetic rats showed contradictory finding. The same dosage of calcitriol given to the supplemented group in diabetic rats significantly reversed the impaired endothelium-dependent relaxation back to similar values as in diabetic controls. The unfavourable effects of hypercalcaemia were not observed in calcitriol-supplemented diabetic rats, although their calcium levels were similar to those calcitriol-supplemented non-diabetic rats. This is possibly due to the actual levels of free calcium ions that account for its physiological role between these two groups were different. Calcitriol-supplemented non-diabetic rats might have higher free calcium ions compared to diabetic rats; leading to the unfavourable effects of hypercalcaemia.

Calcitriol supplementation in vitamin D-deficient diabetic rats significantly increased SOD and reduced MDA levels, indicating an improvement in oxidative stress status. The protein expression of eNOS in the mesenteric arterial tissue of vitamin D-deficient diabetic rats with calcitriol supplementation also showed a significant improvement. Reduced oxidative stress attenuates the propagation of oxidative-driven adverse events, coupled with the enhanced protein expression of eNOS that indicates increased NO bioavailability, thus the impairment in endothelium-dependent relaxation was successfully ameliorated.

5.1.7(b) Endothelium-dependent Contraction

Endothelium-dependent contraction is evoked by endothelium-derived contracting factors, such as endothelial COX-derived prostanoid. These vasoconstrictor prostanoids diffuse into underlying VSMC and directly activate respective receptors located on the VSMC for subsequent contractile responses. To

initiate endothelium-dependent contractions, an increase in intracellular calcium concentrations is essential. Calcium ionophore is the carrier that facilitates the transport of calcium ions across the endothelial cell membrane into EC, hence intracellular calcium levels are increased, then the calcium-dependent calmodulin mechanism is activated in the endothelium for subsequent NO production. By incubating the vessels with $_{L}$-NAME (the eNOS inhibitor), the catalytic activity of eNOS for NO production is inhibited, causing an immediate potentiation of EDCF-mediated responses.

Normal Rats

The present finding discovered that endothelium-dependent contraction in vitamin D-deficient rats was similar compared to controls. This is likely due to vitamin D-deficient normal rats in the present study exhibited well-controlled ROS levels, as shown by similar MDA levels compared to controls. Otherwise, excess ROS stimulates the activity of COX in endothelial or smooth muscle cells to release prostanoid, augmenting vasoconstriction (Tang and Vanhoutte, 2009).

Surprisingly, calcitriol supplementation to vitamin D-deficient normal rats significantly augmented endothelium-dependent contraction. This is in contrast with a study that reported calcitriol reduces the increased cytosolic free calcium concentrations in endothelial cells, leading to the decrease in endothelium-dependent contraction of aortic rings of the spontaneous hypertensive rat (Wong *et al.*, 2008). The difference in finding is possibly due to the increased calcium levels induced by calcitriol supplementation as seen in the supplemented group in this study. Hypercalcaemia amplifies the enhanced intracellular calcium levels, leading to increased vascular resistance and triggering vasoconstriction (Moussawi *et al.*, 2018).

Diabetic Rats

In the present study, endothelium-dependent contraction was augmented in mesenteric arteries of vitamin D-deficient diabetic rats compared to diabetic controls. In certain pathological conditions such as diabetes, attenuated NO production initiates the contraction of VSMC (Shi *et al.*, 2007). The insufficiency of NO bioavailability induces the production of endothelium-derived vasoconstrictor prostanoids and also endothelin-1 (Vanhoutte *et al.*, 2017), leading to vasoconstriction which amplifies the degree of endothelial dysfunction. An enhanced endothelium-dependent contraction in diabetes might also relate to hyperglycaemia-induced augmented oxidative stress and upregulation of COX-2 protein expression, as reported in human aortic endothelial cells cultured in high glucose medium (Cosentino *et al.*, 2003). Vitamin D deficiency in addition to hyperglycaemia in vitamin D-deficient diabetic rats, coupled with enhanced oxidative stress and possibly vascular inflammation induce the overproduction of superoxide anions. Superoxide anions effectively inactivate EDRF, favouring the production of cyclooxygenase product responsible for vasoconstriction (such as thromboxane A_2), leading to augmented endothelium-dependent vasoconstriction and hence endothelial dysfunction (Tang and Vanhoutte, 2009).

Calcitriol supplementation to vitamin D-deficient diabetic rats did not show significant improvement in reducing endothelium-dependent contraction. Although vascular oxidative stress has been improved in calcitriol-supplemented rats, hyperglycaemia in diabetics is still favouring the cyclooxygenase pathway that enhance vasoconstriction.

5.1.8 Microvascular Smooth Muscle Function

Besides the impairment in endothelial cells, impairment might also occur in VSMC in some disease states. This is generally termed as vascular dysfunction, the impairment in both endothelial and smooth muscle cells (Vanhoutte *et al.*, 2017). In order to evaluate whether vitamin D deficiency alters the function of VSMC in normal and diabetic rats respectively, mesenteric arteries without endothelium were stimulated with sodium nitroprusside and salbutamol to study endothelium-independent relaxation and phenylephrine for endothelium-independent contraction.

5.1.8(a) Sodium Nitroprusside-induced Endothelium-independent Relaxation

Normal Rats

With the removal of endothelium, NO is unable to be produced by eNOS. The shortage of endogenous NO can be bypassed with exogenous NO donor. SNP acts as an endothelium-independent exogenous NO donor to activate soluble guanylyl cyclase in VSMC to catalyse the production of cGMP, initiating subsequent mechanisms responsible for vasodilation (Rapoport and Murad, 1983).

In the present study, SNP-induced endothelium-independent relaxation was found to be preserved in mesenteric arteries of vitamin D-deficient normal rats. Although vascular endothelial function was significantly impaired in vitamin D-deficient rats, the impairment did not involve vascular smooth muscle function. Impaired endothelium-dependent vasodilation in vessels with intact SNP responsiveness indicates a reduction in NO production or bioavailability, consistent with increased oxidative stress (Tare *et al.*, 2011). This is in line with the present

finding that showed augmented oxidative stress as indicated by reduced SOD levels and reduced NO bioavailability as indicated by lower protein expression of eNOS.

However, calcitriol supplementation in vitamin D-deficient normal rats showed the trend of reduced SNP-induced endothelium-independent relaxation compared to vitamin D-deficient rats and the relaxation was significantly lower than the controls. This is likely due to high calcium levels found in calcitriol-supplemented rats; high calcium levels have been suggested as one of the possible mechanisms leading to the functional abnormality of VSMC (Kapustin and Shanahan, 2012).

Diabetic Rats

The reduction in responses to endothelium-dependent vasodilators can be due to the concomitant release of endothelium-derived vasoconstrictors or to the reduced or abnormal responsiveness of VSMC to NO or vasodilator stimuli. In this study, the response of VSMC to SNP was significantly attenuated in vitamin D-deficient diabetic rats compared to diabetic controls. This observation suggests that vitamin D deficiency combined with hyperglycaemia and oxidised lipids in diabetics promote the dysfunction of both endothelial and vascular smooth muscle cells.

The impairment in endothelium-independent relaxation via the cGMP pathway in vitamin D-deficient diabetic rats may be explained by the reduced NO bioavailability responsible for vasorelaxation (Tare *et al.*, 2011). Although NO is provided by the exogenous NO donor SNP directly into VSMC for endothelium-independent relaxation, NO might be dissociated and degraded into toxic peroxynitrite in the presence of superoxide anion, attenuated the subsequent cGMP-mediated vasorelaxation (Yeo *et al.*, 2004). Augmented vascular oxidative stress, which is mainly caused by an imbalance between the enhanced production of ROS and the

reduced activity of antioxidant enzymes, has been reported to be involved in increasing the chemical inactivation of bioactive NO (Vanhoutte, 1989).

The impairment in NO-mediated relaxation could also in part be explained by the decrease in the sensitivity of VSMC to its vasodilator stimuli. Hyperglycaemia and vitamin D deficiency in diabetic rats induce overproduction of ROS and/or diminished the activity of antioxidants in VSMC, further facilitate the dissociation of NO molecules and impair the responsiveness of VSMC, leading to the attenuated endothelium-independent relaxation. This is in line with the finding by Okon *et al.* (2000) that showed the sensitivity of VSMC to NO was decreased in internal mammary arteries from diabetic patients, which was evident from the impaired relaxation to SNP. VDR deficiency on VSMC may also be one of the possible mechanisms that alter the vascular smooth muscle responses to NO (Wu-Wong *et al.*, 2007), whereby the crosstalk between vascular EC and VSMC was affected.

Impaired endothelium-independent responses via the cGMP pathway may arise from several mechanisms such as abnormality in the cGMP signalling mechanism and PKG activity and reduction in smooth muscle guanylyl cyclase (Lamireau *et al.*, 2002). However, the present observation was in contrast with the previous study that demonstrated preservation of smooth muscle response to SNP in subcutaneous arteries of pregnant women with T1DM compared to controls (Ang *et al.*, 2002). The difference in finding is likely due to the non-deficient vitamin D levels in the subjects. However, calcitriol-supplementation failed to ameliorate the impairment in SNP-induced endothelium-independent relaxation of vitamin D-deficient diabetic rats.

5.1.8(b) Salbutamol-induced Endothelium-independent Relaxation

Normal Rats

Salbutamol exerts its role as a vasodilator (an exogenous prostacyclin) by binding to β_2-adrenergic receptors on VSMC to activate adenylyl cyclase. Activated adenylyl cyclase catalyses the production of cAMP responsible for vasodilation. The cAMP signalling pathway is also the pathway used by endothelium-derived prostacyclin to produce relaxation in normal condition, whereby prostacyclin binds to IP receptors on VSMC (Lim *et al.*, 2002). The removal of endothelium attenuates the production of prostacyclin, while providing the vitamin D-deficient rats with salbutamol, propagates the cAMP signalling pathway for vasodilation.

In the present study, endothelium-independent relaxation of mesenteric arterial smooth muscle to salbutamol was comparable between vitamin D-deficient rats and controls. Similarly, calcitriol supplementation did not show any effect on SB-induced relaxation. These observations suggest that vitamin D deficiency and supplementation do not affect the function of VSMC through the cAMP pathway in normal rats. Ten-weeks duration of having a vitamin D deficiency in normal rats did not produce adverse effect on the blood vessels through this pathway.

Diabetic Rats

Similarly, endothelium-independent relaxation of mesenteric arterial smooth muscle to salbutamol was comparable in both vitamin D-deficient and supplemented diabetic rats. These observations suggest that even in diabetic models, vitamin D deficiency and supplementation do not affect the function of smooth muscle through

the cAMP signalling pathway. This is likely due to the presence of β_2-adrenergic receptors on VSMC is not altered by hyperglycaemia and vitamin D deficiency.

5.1.8(c) Endothelium-independent Contraction

Normal Rats

Phenylephrine (PE) acts as a standard vasoconstrictor commonly used to induce endothelium-independent contraction. PE binds to α_1-adrenergic receptors on VSMC, increasing the concentrations of intracellular calcium ions in VSMC to evoke subsequent vasoconstriction. In the present study, vitamin D-deficient rats showed similar endothelium-independent contraction in their mesenteric arterial smooth muscle as controls. Calcitriol supplementation to vitamin D-deficient rats did not show any effect on PE-induced contraction. These findings suggest that 10-weeks duration of having a vitamin D deficiency and four weeks of calcitriol supplementation do not alter the contractile function of VSMC in normal rats.

Diabetic Rats

Likewise, the contraction of VSMC to PE in rats' mesenteric arteries is not affected in diabetic rats, regardless of their vitamin D status. Removal of endothelium did not affect the contractile responses of mesenteric arteries to phenylephrine.

5.2 Human Study

There were difficulties in obtaining sufficient and viable human tissue samples to perform functional and detailed mechanistic studies compared to animal samples. During the recruitment, we had successfully recruited 50 patients at the beginning. However, after the assessment for inclusion and exclusion criteria, only 23 patients

had been accepted for this study. Since the samples have to be fresh to sustain the viability of the vessels for functional studies, the challenges arose in obtaining suitable and viable samples during surgery. Nevertheless, adequate human tissue samples were obtained to study the effects of vitamin D deficiency on the levels of oxidative stress parameters (SOD and MDA levels). This allows the current project to assess if there are similarities in the findings of microvascular tissue oxidative stress parameters between animal diabetic models and human diabetic models.

5.2.1 Background Characteristics

Lower limb subcutaneous arteries were harvested and used in conducting this human study. Subcutaneous arteries in diabetic patients and mesenteric arteries in rats are classified as small resistance arteries with the vessel size of less than 200 μm, thus termed microcirculation.

In this study, we found that 13 out of 23 recruited diabetic patients had vitamin D deficiency. This is supported by growing evidence indicating higher prevalence of vitamin D deficiency in diabetic population. In Malaysia, the prevalence of vitamin D deficiency in normal population has been reported as 16-33% (Chin *et al.*, 2014), whereas the prevalence is more pronounced in the diabetic population, which is 43% (Munisamy *et al.*, 2016). Diabetic patients had lower vitamin D levels (serum 25(OH)D < 50 nmol/L) compared to non-diabetics. Likewise, lower vitamin D levels incur higher risks of developing diabetes (Scragg *et al.*, 2004; Di Cesar *et al.*, 2006).

All patients in this study have more than 10 years history of diabetes. A longer duration of diabetes of at least 10 years was preferred in this study. It is likely that with this duration, all patients will be having diabetic vasculopathy. All of them were on insulin therapy or taking oral hypoglycaemic agents for diabetes treatment. More than

80% of diabetic patients in both study groups (DNP: 80.00% vs DDP: 84.62%) have hypertension and taking anti-hypertensive drugs which include calcium channel blocker, angiotensin II receptor blocker and angiotensin-converting enzyme inhibitor. Likewise, 70.00% of group DNP and 53.85% of group DDP also have hypercholesterolemia and taking lipid-lowering drugs from the statin groups which include simvastatin and atorvastatin. Due to concerns that certain medications might exert effects on oxidative stress levels, statistical analysis (Chi-square test) was performed to assess if there is any significant difference in concurrent medications between these study groups. The statistical outcome showed that there was no significant difference in concurrent medications between these groups (Appendix E).

As shown in Table 4.5, there were no significant differences in the background characteristics such as gender, body mass index (BMI), blood pressures, total cholesterol, fasting blood glucose (FBG), glycated haemoglobin and calcium levels, between diabetic patients with vitamin D deficiency and non-deficiency. However, this study found that diabetic patients with vitamin D deficiency are significantly younger than those with vitamin D non-deficiency. It is possibly that the younger subjects have a more sedentary lifestyle, lack of physical and outdoor activity and low exposure of sunlight compared to those older subjects with a more active lifestyle. Younger subjects may spend more time on social media and electronic gadgets, or they are working more comfortably in the office without the need of much physical efforts. This might well explain the observation that showed higher BMI in subjects from group DDP compared to group NDP, although it is not statistically significant.

Previous findings reported a negative relationship between age and SOD levels in normal and diabetic populations (Bolzan *et al.*, 1997; Ozbay and Dulgar, 2002).

Similarly, a positive relationship between age and MDA levels was reported in the literature (Hassina *et al.*, 2012). Since age is markedly different between two study groups and has been reported to be closely associated with oxidative stress levels, statistical adjustment of age as the covariate was performed for our results of oxidative stress parameters using ANCOVA analysis.

5.2.2 Microvascular Tissue Oxidative Stress Status

5.2.2(a) Superoxide Dismutase Levels

Vitamin D deficiency in diabetics amplifies the overproduction of free radicals and ROS (see Section 2.7). SOD as the antioxidant enzyme plays its vascular protective role in scavenging the excess ROS. A decrease in SOD levels in conjunction with reduced ROS levels might represent the effectiveness of the antioxidant defence system to counteract with the increased levels of ROS.

In the present study, there was a significant difference in SOD levels between vitamin D-deficient and vitamin D non-deficient diabetic patients with independent t-test. SOD levels were significantly lower in the subcutaneous arteries of diabetic patients who are vitamin D-deficient. However, after adjusting for age as the covariate in the analysis, the difference in SOD levels between two groups was no longer statistically significant, although group DDP still showed a lower trend of SOD levels (p = 0.072) compared to group NDP. A decrease in SOD levels presumably as the compensatory response for its antioxidant activities to combat with excess ROS induced by hyperglycaemia coupled with vitamin D deficiency. Vitamin D deficiency was also reported to reduce the amount of antioxidant enzymes and diminish the antioxidant activities (Nikooyeh *et al.*, 2014; Shab-Bidar *et al.*, 2015).

The present finding is in line with the previous studies that reported diminished SOD levels and activities in the liver tissue of diabetic rats (Lucchesi *et al.*, 2013), as well as skin tissue, organs and blood samples of diabetic subjects (Shukla *et al.*, 2012; Kim, 2013; Kayama *et al.*, 2015). Reduced SOD levels were presumably due to the elevated ROS production and the enhanced oxidative stress induced by hyperglycaemia in diabetics. However, there is also contradictory finding that reported SOD levels in hepatic tissue remained unchanged in diabetic subjects (Kumawat *et al.*, 2013). The study also reported that the levels of catalase (another important antioxidant enzyme) were significantly increased in diabetic subjects compared to controls as the compensatory mechanism. The overall antioxidant status of diabetic subjects therefore remained unchanged.

SOD levels have been reported to relate with age in diabetic population; elderly diabetics normally have lower SOD levels (Ozbay and Dulger, 2002). Vitamin D deficiency has also been reported to diminish SOD levels in the diabetics. Nevertheless, diabetic patients with vitamin D deficiency who are relatively younger in this study showed reduced SOD levels. This contradictory finding might be explained by the duration of having diabetes, besides being vitamin D-deficient. The younger age of diabetic patients in our study does not necessarily represent a shorter disease duration. All subjects have more than 10 years of diabetes duration, regardless of their age. Disease duration might be more important than age in contributing to oxidative stress in diabetes.

5.2.2(b) Malondialdehyde Levels

Chronic hyperglycaemia in diabetes coupled with vitamin D deficiency induces excess formation of ROS production, propagating free radical chain reactions.

Enhanced ROS levels impair the antioxidant defence mechanism by attenuating the antioxidant activities of antioxidant enzymes. Hence, the effectiveness of endogenous antioxidant defence system to scavenge the on-going overproduction of free radicals might be reduced due to the reduced levels of antioxidants, exacerbating the imbalance condition in oxidative stress status. The built-up of oxidative stress brings detrimental effects to our body system, such as lipid peroxidative injury. MDA levels indicate the degree of lipid peroxidation induced by excess ROS. Higher MDA levels represent higher levels of ROS.

In the present study, MDA levels were significantly higher in diabetic patients with vitamin D deficiency compared to non-deficient group. Reduced SOD levels in conjunction with augmented MDA levels in vitamin D-deficient diabetic patients found in the present study might well indicate augmented oxidative stress. Elevated TBARS and MDA levels reported in plasma, serum, and skin tissues in diabetics indicated that peroxidative injury has involved in the development of diabetes complications (Moussa, 2008; Bandeira *et al.*, 2012).

There is limited evidence reported on the effects of vitamin D deficiency on MDA levels in diabetes microcirculation. However, previous studies reported MDA levels were lower in vitamin D-deficient asymptomatic subjects and patients with other CVD such as hypertension and obesity (Yesilbursa *et al.*, 2005; Hamidreza *et al.*, 2014; Wang *et al.*, 2015), presumably due to the alterations in metabolic states that induces excess mitochondrial production of free radicals, which is unable to be detoxified by antioxidants. The propagating free radical chain reactions ultimately lead to lipid peroxidation as lipid is more susceptible to oxidative damage.

CHAPTER 6

CONCLUSION

6.1 Summary

6.1.1 Animal Study

All objectives of the animal study were fulfilled. In summary, the present animal study demonstrates that: (i) Vitamin D deficiency impaired ACh-induced endothelium-dependent relaxation in isolated mesenteric arteries of both normal and diabetic rats; (ii) Endothelium-dependent contraction was augmented in vitamin D-deficient diabetic rats; (iii) Vitamin D deficiency in diabetic rats attenuated NO-mediated cGMP pathway but cAMP pathway was preserved; (iv) Lower SOD levels and protein expression of eNOS were observed in vitamin D-deficient normal and diabetic rats; (v) Vitamin D deficiency in diabetic rats enhanced MDA levels, indicating peroxidative injury.

Endothelial dysfunction in vitamin D-deficient rats is associated with reduced protein expression of eNOS which mediates NO production and augmented oxidative stress levels; the imbalance between the excess production of ROS and reduced antioxidant activities. Both mechanisms contribute to the reduced bioavailability of NO, the potent vasodilator that modulates vascular tone, leading to the impairment in NO-mediated vasodilation via cGMP pathway. However, relaxation of VSMC to salbutamol and contraction to phenylephrine were not affected in both vitamin D-deficient normal and diabetic models.

Furthermore, the present animal study also illustrates that: (i) Calcitriol supplementation improved ACh-induced endothelium-dependent relaxation in

isolated mesenteric arteries of vitamin D-deficient diabetic but not normal rats; (ii) Augmented endothelium-dependent contraction was observed in vitamin D-deficient normal rats with calcitriol supplementation; (iii) Calcitriol supplementation to vitamin D-deficient diabetic rats improved SOD levels and protein expression of eNOS; (iv) Reduced MDA levels were found in vitamin D-deficient diabetic rats receiving calcitriol supplementation; (v) Calcitriol-supplemented vitamin D-deficient normal rats showed increased calcium levels.

Calcitriol supplementation ameliorates microvascular endothelial dysfunction (improves vasorelaxation) in diabetic rats with vitamin D deficiency by upregulating eNOS expression and improving oxidative stress status. However, calcitriol supplementation failed to show any improvement in endothelium-dependent contraction and SNP-induced endothelium-independent relaxation. Calcitriol supplementation in normal rats with vitamin D deficiency even leads to vascular dysfunction as shown by augmented endothelium-dependent contraction and reduced trend in endothelium-independent relaxation via cGMP pathway. It is possibly due to the altered calcium homeostasis induced by calcitriol leads to hypercalcaemia that contributes to the unfavourable effects seen in normal rats.

6.1.2 Human Study

All objectives of the human study were fulfilled. In summary, the present human study showed that: (i) SOD levels of vitamin D-deficient diabetic patients showed the reduced trend after adjusting for age as the covariate; (b) Higher MDA levels were observed in diabetic patients with vitamin D deficiency. These findings were consistent with the study outcome from the present animal study, where vitamin D-deficient diabetic rats showed reduced SOD and increased MDA levels, indicating

augmented vascular oxidative stress levels. Enhanced oxidative stress levels may subsequently predispose to peroxidative injury in diabetics.

6.2 Significance and Novelty of the PhD Study

Diabetes mellitus is a growing threat to public health. This disease poses a significant risk of secondary complications and cardiovascular events that lead to high mortality in diabetics. It is critical to recognise and treat this devastating disease early to delay or even prevent serious diabetes-related cardiovascular complications.

This is the first study that demonstrated vitamin D deficiency impairs vascular function in the microcirculation of diabetic rats, attributed to reduced eNOS protein expression and augmented oxidative stress. This study also discovered that calcitriol supplementation ameliorates endothelial dysfunction in vitamin D-deficient diabetic rats, by upregulating eNOS protein expression and improving oxidative stress. This study also showed that calcitriol supplementation aggravates endothelial dysfunction in vitamin D-deficient rats, possibly due to calcitriol-induced hypercalcaemia.

Realising the adverse impacts of vitamin D deficiency on diabetes mellitus and its complications, emphasis should be given in preventing or reducing the prevalence of vitamin D deficiency in the country to effectively reduce diabetes-related adverse cardiovascular events associated with low vitamin D levels. The study outcome also reinforces the need for the authorities to encourage vitamin D-deficient and diabetic individuals to optimise their vitamin D levels; this can be achieved by more sunlight exposure and increased dietary vitamin D intake. Vitamin D supplementation might be a potential strategy to reduce the adverse effects of low vitamin D levels on microvascular function in the diabetic population with vitamin D deficiency.

6.3 Study Limitations and Recommendations

6.3.1 Animal Study

The present study has a few limitations that deserve more attention and improvement in future research. Firstly, this animal study assessed the effects of vitamin D deficiency and supplementation on vascular responses in general but not studied in detail the contribution of particular vasodilators (NO, prostacyclin and EDHF). This is restricted by the dual chamber wire myograph system that allows the simultaneous study of only two arteries at one time. Considering the viability of an isolated blood vessel in the physiological solution is about 20 hours (Ang *et al.*, 2012), only endothelium-dependent and independent relaxation and contraction in control solution were examined in the study. Besides that, assessment to explain the underlying mechanisms involved only protein expression of eNOS, in view that NO is the major vasodilator that accounts for endothelium-dependent relaxation. Nevertheless, this limitation might underestimate the contribution of other vasodilators such as prostacyclin and EDHF in leading to vascular dysfunction in the microcirculation of vitamin D-deficient rats. Further evaluation on the role of specific vasodilators and vasoconstrictors on vascular responses in diabetic microcirculation is an important area of future studies.

Secondly, the dosage of calcitriol supplemented to animal models remained a great challenge. The present study showed a beneficial effect in diabetic models but an adverse outcome in normal rats even though same dosage of calcitriol was provided. This observation suggests the need for close monitoring of calcium levels and other vitamin D-associated minerals and hormones such as phosphate and parathyroid hormone from time to time.

6.3.2 Human Study

There are a few limitations in the present human study for future improvement. Firstly, there are difficulties in obtaining sufficient and suitable human tissue samples to study the vascular responses and to perform Western blot and immunohistochemistry as the animal study did. Nevertheless, this PhD project was able to conduct a matching study on assessing microvascular tissue oxidative stress levels in diabetic patients with different vitamin D status. Hence, conducting a matching study on microvascular responses in human models is an important area of research that deserves further investigation. It is able to provide a better understanding of the similarities between experimental and clinical studies on the effects of vitamin D deficiency on microvascular function in diabetic and normal conditions.

Secondly, despite our best efforts to match medications that subjects were taking during the study, there might remain some dissimilarities of some drugs between the two groups. We acknowledge the potential effects of different drugs on oxidative stress. Hence, to minimise the bias in the study outcome, statistical analysis (Chi-square test) was performed to ensure the difference in concurrent medications between both study groups is not statistically significant.

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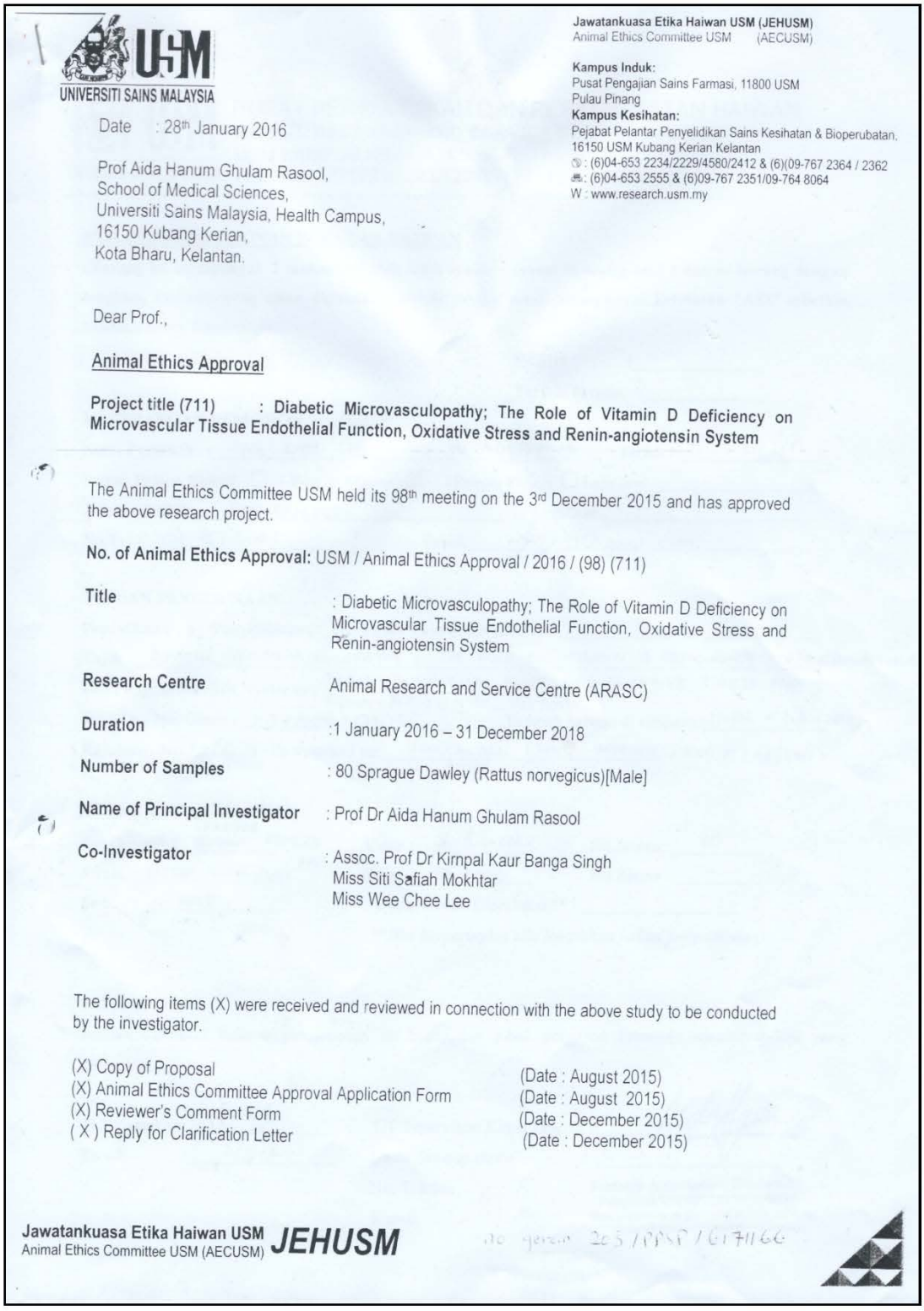
APPENDICES

APPENDIX A: ANIMAL ETHICS APPROVAL



UNIVERSITI SAINS MALAYSIA

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

Kampus Induk:
Pusat Pengajian Sains Farmasi, 11800 USM
Pulau Pinang
Kampus Kesihatan:
Pejabat Pelantar Penyelidikan Sains Kesihatan & Bioperubatan,
16150 USM Kubang Kerian Kelantan
: (6)04-653 2234/2229/4580/2412 & (6)09-767 2364 / 2362
: (6)04-653 2555 & (6)09-767 2351/09-764 8064
W : www.research.usm.my

Date : 28th January 2016

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

Dear Prof.,

Animal Ethics Approval

Project title (711) : Diabetic Microvasculopathy; The Role of Vitamin D Deficiency on Microvascular Tissue Endothelial Function, Oxidative Stress and Renin-angiotensin System

The Animal Ethics Committee USM held its 98th meeting on the 3rd December 2015 and has approved the above research project.

No. of Animal Ethics Approval: USM / Animal Ethics Approval / 2016 / (98) (711)

Title	: Diabetic Microvasculopathy; The Role of Vitamin D Deficiency on Microvascular Tissue Endothelial Function, Oxidative Stress and Renin-angiotensin System
Research Centre	Animal Research and Service Centre (ARASC)
Duration	:1 January 2016 – 31 December 2018
Number of Samples	: 80 Sprague Dawley (Rattus norvegicus)[Male]
Name of Principal Investigator	: Prof Dr Aida Hanum Ghulam Rasool
Co-Investigator	: Assoc. Prof Dr Kirnpal Kaur Banga Singh Miss Siti Safiah Mokhtar Miss Wee Chee Lee

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 : August 2015)
(X) Animal Ethics Committee Approval Application Form	(Date : August 2015)
(X) Reviewer's Comment Form	(Date : December 2015)
(X) Reply for Clarification Letter	(Date : December 2015)

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

Jawatankuasa Etika Haiwan USM (JKEH-USM)
Animal Ethics Committee USM (AECUSM)
Kampus Induk:
Pusat Pengajian Sains Farmasi, 11800 USM
Pulau Pinang

Kampus Kesihatan:
Pusat Inisiatif Penyelidikan (Sains Klinikal & Kesihatan)
USM Kampus Kesihatan,
16150, Kubang Kerian, Kelantan
Tel: (6)04-653 2234/2229/4580/2412 & (6) 09-767 2364 / 2352
aks: (6) 04-653 2555 & (6) 09-767 2351
W: www.research.usm.my

Tarikh: 20 Disember 2016

Prof Aida Hanum Ghulam Rasool,
Lecturer
School of Medical Sciences
Universiti Sains Malaysia
16150 Kubang Kerian
Kota Bharu, Kelantan

Prof.,

KELULUSAN PERMOHONAN PERUBAHAN "DIABETIC MICROVASCULOPATHY; THE ROLE OF VITAMIN D DEFICIENCY ON MICROVASCULAR TISSUE ENDOTHELIAL FUNCTION, OXIDATIVE STRESS AND RENIN-ANGIOTENSIN SYSTEM"- USM / Animal Ethics Approval / 2016 /(98) (711)

Dengan segala hormatnya merujuk perkara tersebut, saya dengan ini meluluskan permohonan pihak tuan sebagaimana berikut :

PERKARA	KELULUSAN SEDIA ADA	KELULUSAN PERMOHONAN BARU
TAJUK	Diabetic Microvasculopathy; The Role of Vitamin D Deficiency on Microvascular Tissue Endothelial Function, Oxidative Stress and Renin-angiotensin System	Diabetic Microvasculopathy; The Role of Vitamin D Deficiency on Microvascular Tissue Endothelial Function, Oxidative Stress and Renin-angiotensin System
TEMPOH	1 January 2016 – 31 December 2018	1 January 2016 – 31 December 2018
PERTAMBAHAN BILANGAN SAMPEL	80 Sprague Dawley (Rattus norvegicus)[Male]	13 (2 Drop out) Sprague Dawley (Rattus norvegicus)[Male]
KETUA PENYELIDIK	Prof Dr Aida Hanum Ghulam Rasool	Prof Dr Aida Hanum Ghulam Rasool
PEMBANTU PENYELIDIK	Assoc. Prof Dr Kirnpal Kaur Banga Singh Miss Siti Safiah Mokhtar Miss Wee Chee Lee	Assoc. Prof Dr Kirnpal Kaur Banga Singh Miss Siti Safiah Mokhtar Miss Wee Chee Lee

Sekian,terima kasih.

"BERKHIDMAT UNTUK NEGARA"
'Memastikan Kelestarian Hari Esok'

(PROF.DR.HJ. MUNAVVAR ZUBAID ABDUL SATTAR)
Pengerusi Jawatankuasa Etika Haiwan USM

APPENDIX B: HUMAN ETHICS APPROVAL

Jawatankuasa Etika Penyelidikan Manusia USM (JEPeM)
Human Research Ethics Committee USM (HREC)

Universiti Sains Malaysia
Kampus Kesihatan,
16150 Kubang Kerian,
Kelantan. Malaysia.
T: 609 - 767 3000 *samb.* 2354/2362
F: 609 - 767 2351
E: jepem@usm.my
www.jepem.kk.usm.my

3rd March 2016

Dr. Sahran Yahaya
Department of Orthopaedic
School of Medical Sciences
Universiti Sains Malaysia
16150 Kubang Kerian, Kelantan.

JEPeM Code : USM/JEPeM/15100337
Protocol Title : The Effect of Vitamin D Deficiency on Microvascular Endothelial Function in Diabetic Patients.

Dear Dr.,

We wish to inform you that your study protocol has been reviewed and is hereby granted approval for implementation by the Jawatankuasa Etika Penyelidikan Manusia Universiti Sains Malaysia (JEPeM-USM). Your study has been assigned study protocol code **USM/JEPeM/15100337**, which should be used for all communication to the JEPeM-USM related to this study. This ethical clearance is valid from **March 2016** until **February 2017.**

The following researchers also involve in this study:

1. Prof. Dr. Aida Hanum Ghulam Rasool

The following documents have been approved for use in the study.

1. Research Proposal

In addition to the abovementioned documents, the following technical document was included in the review on which this approval was based:

1. Patient Information Sheet and Consent Form (Malay version)

Attached document is the list of members of JEPeM-USM present during the full board meeting reviewing your protocol.

While the study is in progress, we request you to submit to us the following documents:

1. Application for renewal of ethical approval 60 days before the expiration date of this approval through submission of **JEPeM-USM FORM 3(B) 2014: Continuing Review Application Form.** Subsequently this need to be done yearly as long as the research goes on.
2. Any changes in the protocol, especially those that may adversely affect the safety of the participants during the conduct of the trial including changes in personnel, must be submitted or reported using **JEPeM-USM FORM 3(A) 2014: Study Protocol Amendment Submission Form.**
3. Revisions in the informed consent form using the **JEPeM-USM FORM 3(A) 2014: Study Protocol Amendment Submission Form.**
4. Reports of adverse events including from other study sites (national, international) using the **JEPeM-USM FORM 3(G) 2014: Adverse Events Report.**
5. Notice of early termination of the study and reasons for such using **JEPeM-USM FORM 3(E) 2014.**
6. Any event which may have ethical significance.
7. Any information which is needed by the JEPeM-USM to do ongoing review.
8. Notice of time of completion of the study using **JEPeM-USM FORM 3(C) 2014: Final Report Form.**

UNIVERSITI SAINS MALAYSIA
JAWATANKUASA ETIKA PENYELIDIKAN MANUSIA

Jawatankuasa Etika
Penyelidikan Manusia USM (JEPeM)
Human Research Ethics Committee USM (HREC)

Universiti Sains Malaysia
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F : (6)09-767 2351
E : jepem@usm.my
L : www.jepem.kk.usm.my
www.usm.my

20th June 2017

Dr. Sahran Yahaya
Department of Orthopaedics
School of Medical Sciences
Universiti Sains Malaysia
16150 Kubang Kerian, Kelantan

JEPeM USM Code: USM/JEPeM/15100337

Study Protocol Title: The Effect of Vitamin D Deficiency on Microvascular Endothelial Function in Diabetic Patients.

Dear Dr:

We wish to inform you that the Jawatankuasa Etika Penyelidikan Manusia, Universiti Sains Malaysia (JEPeM-USM) acknowledged receipt of Continuing Review Application dated 23rd May 2017.

Upon review of JEPeM-USM Form 3(B) 2015: Continuing Review Application Form, the committee's decision for the **EXTENSION OF APPROVAL IS APPROVED (start from 1st March 2017 till 28th February 2018)**. The report is noted and has been included in the protocol file.

Principle Investigator (PI) should be aware and concern about the ethical expiration of the study in the future.

* Please note that JEPeM-USM will not consider the late submission of Continuing Review from the same Principle Investigator (PI) in the future.

Thank you for your continuing compliance with the requirements of the JEPeM-USM.

"ENSURING A SUSTAINABLE TOMORROW"

Very truly yours,

(PROF. DR. HANS AMIN VAN ROSTENBERGHE)
Chairperson
Jawatankuasa Etika Penyelidikan (Manusia), JEPeM
Universiti Sains Malaysia

c.c Secretary
Jawatankuasa Etika Penyelidikan (Manusia), JEPeM
Universiti Sains Malaysia

Jawatankuasa Etika
Penyelidikan Manusia USM (JEPeM)
Human Research Ethics Committee USM (HREC)

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20th March 2018

GIS 9195667

Dr. Sahran Yahaya
Department of Orthopaedics
School of Medical Sciences
Universiti Sains Malaysia
16150 Kubang Kerian, Kelantan.

JEPeM USM Code: USM/JEPeM/15100337

Study Protocol Title: The Effect of Vitamin D Deficiency on Microvascular Endothelial Function in Diabetic Patients.

Dear Dr:

We wish to inform you that the Jawatankuasa Etika Penyelidikan Manusia, Universiti Sains Malaysia (JEPeM-USM) acknowledged receipt of Continuing Review Application dated 25th February 2018.

Upon review of JEPeM-USM Form 3(B) 2017: Continuing Review Application Form, the committee's decision for the **EXTENSION OF APPROVAL IS APPROVED (start from 1st March 2018 till 28th February 2019)**. The report is noted and has been included in the protocol file.

Thank you for your continuing compliance with the requirements of the JEPeM-USM.

Thank you.

"ENSURING A SUSTAINABLE TOMORROW"

Very truly yours,

(ASSOC. PROF. DR. AZLAN HUSIN)
Deputy Chairperson
Jawatankuasa Etika Penyelidikan (Manusia), JEPeM
Universiti Sains Malaysia

c.c Secretary
Jawatankuasa Etika Penyelidikan (Manusia), JEPeM
Universiti Sains Malaysia

CERTIFIED BY: National Pharmaceutical Regulatory Agency (NPRA) | Forum for Ethical Review Committees in Asia & Western Pacific Region

APPENDIX C: INFORMED CONSENT FORM

LAMPIRAN A

MAKLUMAT KAJIAN

Tajuk Kajian: **KESAN KEKURANGAN VITAMIN D KE ATAS FUNGSI SALUR DARAH KECIL DI KALANGAN PESAKIT DIABETES**

Nama Penyelidik: Dr. Sahran Yahaya

Prof. Dr. Aida Hanum Ghulam Rasool

No. Pendaftaran MMC: MMC 47033

MMC 31313

PENGENALAN

Sedia dimaklumkan bahawa lebih daripada 3.2 juta rakyat Malaysia dilaporkan menghidapi penyakit kencing manis pada tahun 2014, dan dijangka akan mencecah sebanyak 4.5 juta penyakit kencing manis pada tahun 2020. Diketahui umum bahawa penyakit kencing manis akan merosakkan fungsi salur darah, dan seterusnya akan menyumbang kepada masalah kardiovaskular (sistem darah dan jantung). Komplikasi sebegini memerlukan rawatan yang berpanjangan dan juga melibatkan perbelanjaan yang besar.

Paras vitamin D yang rendah turut dilaporkan berhubungkait dengan kegagalan fungsi salur darah kecil di kalangan pesakit diabetes. Oleh sedemikian, anda dipelawa untuk menyertai satu kajian penyelidikan secara sukarela. Kajian ini adalah untuk menyelidik kesan kekurangan vitamin D ke atas fungsi salur darah kecil di kalangan pesakit diabetes (kencing manis).

Dianggarkan seramai 50 pesakit kencing manis akan menyertai kajian ini. Paras vitamin D para peserta akan diukur untuk mengenalpasti golongan pesakit, sama ada dalam golongan yang mempunyai paras vitamin D yang normal ataupun golongan yang mengalami masalah kekurangan vitamin D.

Sebelum anda bersetuju untuk menyertai kajian penyelidikan ini, adalah penting untuk anda membaca dan memahami borang ini. Sekiranya anda menyertai kajian ini, anda akan menerima satu salinan borang untuk disimpan sebagai rekod anda.

TUJUAN KAJIAN

Protokol kajian ini telah diluluskan oleh Jawatankuasa Etika Penyelidikan (Manusia), Universiti Sains Malaysia. Kajian ini bertujuan untuk menyelidik kesan kekurangan vitamin D ke atas fungsi salur darah kecil di kalangan pesakit diabetes. Kajian ini dapat mengetahui kesan kekurangan vitamin D ke atas fungsi salur darah, maka maklumat tersebut dapat membantu untuk mengenalpasti rawatan penyakit kencing manis yang berpotensi untuk membawa

manfaat kepada kesihatan salur darah. Oleh sedemikian, kerosakan salur darah dapat dicegah dengan awal dan kegagalan fungsi salur darah yang akan menyebabkan komplikasi akan diatasi.

Terdapat kemungkinannya bahawa maklumat yang dikumpulkan semasa kajian ini akan dianalisasikan oleh pihak penyelidik pada masa depan untuk kegunaan lain yang mungkin atau untuk tujuan perubatan atau saintifik yang selain daripada yang kini dicadangkan.

KELAYAKAN PENYERTAAN

Doktor yang bertanggungjawab dalam kajian ini atau salah seorang kakitangan kajian telah membincangkan kepada anda tentang kelayakan yang diperlukan untuk menyertai kajian ini. Adalah penting untuk anda berterus-terang dengan doktor dan kakitangan tersebut tentang sejarah kesihatan anda. Anda tidak seharusnya menyertai kajian ini sekiranya anda tidak memenuhi semua syarat kelayakan yang ditetapkan.

Beberapa syarat kelayakan yang telah ditetapkan untuk menyertai kajian ini, seperti: -

- Anda mesti menghidapi penyakit diabetes.
- Anda mesti berumur antara 18 hingga 60 tahun.
- Anda akan menjalani pembedahan kaki.

Anda tidak boleh menyertai kajian ini sekiranya

- Anda menghidapi masalah jantung dan hati.
- Anda mengalami tekanan darah tinggi melebihi 160/100 mmHg.
- Anda terpaksa menjalani pembedahan kaki yang berkaitan dengan tumor.
- Anda sedang mengambil vitamin D atau/dan calcium tambahan.
- Anda menghidapi penyakit diabetes kurang daripada 3 tahun.
- Anda mengalami keadaan yang tidak mengizinkan anda untuk menyertai penyelidikan ini mengikut budi bicara penyelidik.

PROSEDUR-PROSEDUR KAJIAN

Sekiranya anda bersetuju untuk menyertai kajian ini, maklumat peribadi seperti umur, jantina, berat dan tinggi badan anda akan dicatatkan. Anda perlu menjalani ukuran tekanan darah dan diminta untuk memberi maklumat tentang sejarah kesihatan anda, seperti jangka masa anda menghidapi penyakit diabetes, sebarang masalah kesihatan yang anda telah/sedang alami dan sebarang ubat/rawatan yang anda telah/sedang ambil.

Kaedah pembedahan anda memang melibatkan pengeluaran/pembedahan tisu semasa anda dibius. Untuk menyertai kajian ini, satu cebisan kecil tisu lemak yang berukuran 2 cm x 2 cm yang **TELAH** dibedah/dikeluarkan tidak akan dibuang, tetapi akan digunakan untuk tujuan penyelidikan. Maka, prosedur ini tidak akan menyakitkan anda dan tiada apa-apa risiko tambahan disebabkan tiada prosedur tambahan yang diperlukan sekiranya cebisan tisu ini diambil.

Pengambilan darah memang diperlukan untuk pembedahan yang akan anda jalankan. Dengan menyertai kajian ini, tambahan darah yang beranggaran 2 mls akan diambil serentak untuk sekali sahaja untuk mengukur paras vitamin D. Prosedur ini juga tidak akan menyakitkan anda atau mendatangkan apa-apa risiko tambahan disebabkan tiada prosedur tambahan yang diperlukan.

DARAH DAN TISU YANG DIAMBIL HANYA AKAN DIGUNAKAN UNTUK TUJUAN PENYELIDIKAN YANG DINYATAKAN DI ATAS SAHAJA. IA TIDAK AKAN DIGUNAKAN UNTUK TUJUAN-TUJUAN LAIN.

SETELAH PENYELIDIKAN DISELESAIKAN, SEMUA DARAH DAN TISU ANDA TIDAK AKAN DISIMPAN TETAPI AKAN DILUPUSKAN MENGIKUT PROSEDUR-PROSEDUR YANG SEDIA ADA.

RISIKO

Memandangkan penyelidikan ini hanya melibatkan tisu yang akan dibedah/dikeluarkan daripada tubuh anda mengikut kaedah pembedahan yang sedia ada, maka **TIDAK** ada apa-apa risiko yang mungkin terjadi ke atas tubuh anda sekiranya anda bersetuju untuk menyertai penyelidikan ini, selain daripada risiko yang sedia ada untuk pembedahan. Jika apa-apa maklumat penting yang baru dijumpai semasa kajian ini yang mungkin akan mengubah persetujuan anda untuk terus menyertai kajian ini, anda akan diberitahu dengan secepat mungkinnya.

MELAPORKAN PENGALAMAN KESIHATAN

Jika anda mengalami apa-apa kecederaan, kesan buruk, atau apa-apa pengalaman kesihatan yang luar biasa semasa kajian ini, pastikan anda memberitahu jururawat atau Dr. Sahran Yahaya (MMC: 47033) atau Prof. Dr. Aida Hanum Ghulam Rasool (MMC: 31313) di talian 09-767 6398/6123 atau 019-919 5667 dengan secepat mungkinnya. Anda boleh membuat panggilan pada bila-bila masa sahaja, siang atau malam, untuk melaporkan pengalaman sedemikian.

PENYERTAAN DALAM KAJIAN

Penyertaan anda dalam kajian ini adalah secara sukarela. Anda berhak menolak untuk menyertai kajian ini atau anda boleh menamatkan penyertaan anda pada bila-bila masa sahaja, tanpa sebarang hukuman atau kehilangan sebarang manfaat yang sepatutnya anda perolehi.

Penyertaan anda juga mungkin diberhentikan oleh doktor yang terlibat dalam kajian ini tanpa memerlukan persetujuan anda. Sekiranya anda diberhentikan untuk menyertai kajian ini, doktor yang terlibat dalam kajian ini atau salah seorang kakitangan akan berbincang dengan anda mengenai isu perubatan yang berkenaan dengan pemberhentian penyertaan anda.

MANFAAT YANG MUNGKIN [Manfaat terhadap Individu, Masyarakat, Universiti]

Sebagai peserta dalam kajian ini, anda berhak untuk mendapatkan maklumat tentang kesihatan anda daripada pemeriksaan fizikal dan ujian darah yang dilakukan dalam kajian ini tanpa kos. Sekiranya anda didapati mengalami masalah kekurangan vitamin D, anda akan dirujukkan kepada doktor perubatan untuk rawatan, sama ada perlu mengambil bekalan vitamin D tambahan.

Anda akan menerima rawatan sekiranya komplikasi penyakit dikesani. Walaupun anda tidak akan menerima sebarang pampasan untuk menyertai kajian ini, namun sebarang keperluan berkaitan dengan penyertaan anda dalam kajian ini akan diberikan.

Walaupun masih tidak ada rancangan untuk memperkembangkan produk kesihatan secara komersial daripada hasil kajian ini buat sementara ini, hasil/maklumat kajian ini diharapkan dapat membantu penyelidik untuk mengenalpasti sama ada kekurangan vitamin D akan mempengaruhi fungsi salur darah kecil yang seterusnya akan membawa kepada komplikasi penyakit diabetes. Penemuan ini diharapkan dapat memberi manfaat kepada pihak penaja iaitu USM, para penyelidik dan pesakit pada masa yang akan datang.

PERSOALAN

Sekiranya anda mempunyai sebarang soalan mengenai prosedur kajian ini, sila hubungi:

1. **Dr. Sahran Yahaya** (MMC:47033) - Penyelidik utama
   Jabatan Ortopedik

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LAMPIRAN S

Borang Keizinan Pesakit/ Subjek

(Halaman Tandatangan)

Tajuk Kajian: **KAJIAN KESAN KEKURANGAN VITAMIN D KE ATAS FUNGSI SALUR DARAH KECIL DI KALANGAN PESAKIT DIABETES**

Nama Penyelidik: Dr. Sahran Yahaya (MMC: 47033)

Prof. Dr. Aida Hanum Ghulam Rasool (MMC: 31313)

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- Semua soalan saya telah dijawab dengan memuaskan.
- Saya secara sukarela, bersetuju menyertai kajian penyelidikan ini, mematuhi segala prosedur kajian dan memberi maklumat yang diperlukan kepada doktor, para jururawat dan juga kakitangan lain yang berkaitan apabila diminta.
- Saya boleh menamatkan penyertaan saya dalam kajian ini pada bila-bila masa.
- Saya telah pun menerima satu salinan Borang Maklumat dan Keizinan Pesakit untuk simpanan peribadi saya.

Nama Pesakit (Dicetak atau Ditaip)	**Nama Singkatan & No. Pesakit**
No. Kad Pengenalan Pesakit (Baru)	**No. K/P** (Lama)
Tandatangan Pesakit atau Wakil Sah	**Tarikh** (dd/MM/yy) (Masa jika perlu)
Nama & Tandatangan Individu yang Mengendalikan Perbincangan Keizinan (Dicetak atau Ditaip)	**Tarikh** (dd/MM/yy)
Nama Saksi dan Tandatangan	**Tarikh** (dd/MM/yy)

Nota: i) Semua subjek/pesakit yang mengambil bahagian dalam projek penyelidikan ini **tidak dilindungi insuran.**

LAMPIRAN P

Borang Keizinan bagi Penerbitan Bahan yang berkaitan dengan Pesakit/ Subjek

(Halaman Tandatangan)

Tajuk Kajian: **KAJIAN KESAN KEKURANGAN VITAMIN D KE ATAS FUNGSI SALUR DARAH KECIL DI KALANGAN PESAKIT DIABETES**

Nama Penyelidik: Dr. Sahran Yahaya (MMC: 47033)
Prof. Dr. Aida Hanum Ghulam Rasool (MMC: 31313)

Untuk menyertai kajian ini, anda atau wakil sah anda mesti menandatangani mukasurat ini.

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- Bahan yang akan diterbitkan dalam penerbitan mingguan/bulanan/dwi bulanan/suku tahunan/ dwi tahunan merupakan satu penyebaran yang luas dan tersebar ke seluruh dunia. Kebanyakan penerbitan ini akan tersebar kepada doktor-doktor dan juga bukan doktor termasuk ahli sains dan ahli jurnal.
- Bahan tersebut juga akan dilampirkan pada laman web jurnal di seluruh dunia. Sesetengah laman web ini bebas dikunjungi oleh semua orang.
- Bahan tersebut juga akan digunakan sebagai penerbitan tempatan dan disampaikan oleh ramai doktor dan ahli sains di seluruh dunia.
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Saya juga memberi keizinan bahawa bahan tersebut boleh digunakan sebagai penerbitan lain yang diminta oleh penerbit dengan kriteria berikut:

- Bahan tersebut tidak akan digunakan untuk pengiklanan atau bahan untuk membungkus.
- Bahan tersebut tidak akan digunakan di luar konteks - contohnya: Gambar tidak akan digunakan untuk menggambarkan sesuatu artikel yang tidak berkaitan dengan subjek dalam foto tersebut.

Nama Pesakit (Dicetak atau Ditaip) **Nama Singkatan atau No. Pesakit**

No. Kad Pengenalan Pesakit **T/tangan Pesakit** **Tarikh** (dd/MM/yy)

Nama&Tandatangan Individu yang Mengendalikan Perbincangan Keizinan **Tarikh** (dd/MM/yy)

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APPENDIX D: CASE REPORT FORM

1- Subject's Data

1. Name : _____
2. Date of Birth : _____/_____/_____
3. Age : _____
4. Gender : Male / Female
5. I/C Number : _____

   R/N : _____
6. Race : _____
7. Contact Number : _____
8. Weight (kg) : _____
9. Height (cm) : _____
10. BMI (kg/m^2) : _____
11. Concurrent medications :

    _____

    _____

    _____

    _____
12. Other medical problems :

    _____

    _____

    _____
13. Duration of Diabetes : _____

2- Patient's Characteristics

1. Systolic Blood Pressure (mmHg) : _____
2. Diastolic Blood Pressure (mmHg) : _____
3. Total Cholesterol (mmol/L) : _____
4. Triglyceride (mmol/L) : _____
5. HDL Cholesterol (mmol/L) : _____
6. LDL Cholesterol (mmol/L) : _____
7. Serum Creatinine (µmol/L) : _____
8. Fasting Blood Glucose (mmol/L) : _____
9. HbA_{1C} (%) : _____

3- Samples

1. Sites of sample : _____
2. Sample taken by : _____
3. Date taken : _____
4. Time taken : _____
5. Time of experiment : _____
6. Diagnosis : _____
7. Types of operation procedure : _____

APPENDIX E: CHI-SQUARE TEST FOR CONCURRENT MEDICATIONS OF DIABETIC PATIENTS

Parameters	Group DNP (n = 10)	Group DDP (n = 13)	X^2 (df)	*p* value
Insulin	6 (60.00)	10 (76.92)	0.765 (1)	0.382
Oral Hypoglycaemic Agent	7 (70.00)	7 (53.85)	0.619 (1)	0.431
Angiotensin-converting enzyme (ACE) Inhibitor	3 (30.00)	2 (15.38)	0.306 (1)	0.400
Angiotensin II Receptor Blocker	2 (20.00)	3 (23.08)	0.031 (1)	0.859
Calcium Channel Blocker	5 (50.00)	8 (61.54)	0.720 (1)	0.580
Beta-blockers	1 (10.00)	3 (23.08)	0.673 (1)	0.412
Lipid Lowering Drug	8 (80.00)	8 (61.54)	0.910 (1)	0.340

Categorical data were expressed in number (%).

[DNP: vitamin D non-deficient diabetic patients; DDP: vitamin D-deficient diabetic patients]

APPENDIX F: PUBLISHED PAPERS AND PRESENTED ABSTRACTS

Review Paper

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C M B
Review

Vitamin D status and oxidative stress in diabetes mellitus

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Abstract: Diabetes mellitus is an epidemic that is gaining global concern. Chronic hyperglycemia in diabetes induces the excess production of free radicals. The deleterious effects of excess free radicals are encountered by endogenous antioxidant defense system. Imbalance between free radicals production and antioxidants defense mechanisms leads to a condition known as "oxidative stress". Diabetes mellitus is associated with augmented oxidative stress that induced micro- and macrovascular complications, which presents a significant risk for cardiovascular events. Low vitamin D levels in the body have also been reported to be associated with the pathogenesis of diabetes and enhanced oxidative stress. The article is to review available literature and summarize the relationship between oxidative stress and vitamin D levels in diabetes. We also review the effects of vitamin D analogs supplementation in improving oxidative stress in diabetics.

Key words: Vitamin D; Oxidative stress; Diabetes mellitus; Deficiency; Supplementation; Cardiovascular risk; Biomarkers.

Introduction

Diabetes mellitus

Diabetes mellitus (DM) is a metabolic disorder, characterized by elevated blood glucose levels over a prolonged period, resulting from defective insulin production and/or insulin action (1). There are three major types of DM:

1. Type I diabetes (T1DM) is due to immune-mediated destruction of pancreatic β-cells that leads to insufficient insulin production;
2. Type II diabetes (T2DM) is due to cellular insulin resistance, whereby the targeted tissues are unable to properly respond to insulin; and
3. Gestational diabetes (GDM), which is glucose intolerance during pregnancy (2).

The worldwide prevalence of DM increases tremendously during the past decades. International Diabetes Federation (IDF) reported that the global prevalence of DM has increased from 285 million (6.6%) in year 2010 (3) to 425 million (8.8%) in year 2017. The figure is estimated to exceed 629 million (9.9%) in year 2045. IDF has also reported that diabetes causes death of 4 million individuals globally (10.7% of global all-cause mortality), and the treatment cost for diabetes constituted 727 billion USD (12.5% of global health expenditure) in year 2017 (4).

In Malaysia, the prevalence of DM increases from 11.6% in year 2006 (5) to 16.9% (3.5 million diabetic patients) in year 2017 (4). It is predicted to rise up to 21.6% in year 2020 with 4.5 million individuals having diabetes (6). Annual expenditure for diabetes treatment in Malaysia has been reported to be as high as RM20.9 billion in year 2015 (7). This constitutes a huge portion of the Malaysian national healthcare budget. In short, high prevalence of DM consumes large amount of global expenditure and will adversely impact on the nation's economy.

Long term augmented blood glucose level, known as chronic hyperglycemia, exposes an individual with DM to the significant risk of specific diabetes-related micro- and macrovascular complications. Microvascular complications include diabetic nephropathy (kidney damage), diabetic neuropathy (neural damage) and diabetic retinopathy (eye damage). On the other hand, macrovascular complications comprise of coronary heart disease, peripheral vascular disease and cerebrovascular disease (stroke) (8).

Both micro- and macrovascular complications contribute significantly to the rates of morbidity and mortality in DM (9), with approximately 65-75% of diabetic deaths being due to cardiovascular diseases (CVD) (10-11). Hence, the prevention of diabetic-related complications becomes the main objective in managing DM (9).

Vascular oxidative stress, in addition to chronic hyperglycemia is reported to be a key contributor in the pathogenesis of DM and its secondary complications (12). In DM, augmented oxidative stress is due to the enhanced free radical-generating process (13-14) and/or impaired capacity of the antioxidant defense system to scavenge the excess free radicals which are induced by chronic hyperglycemia (15).

Vitamin D deficiency was reported to be closely associated with enhanced vascular oxidative stress and increased risk of major CVD in diabetic populations (16). This review aims to explore the association between vitamin D levels and oxidative stress in diabetes. The

possible beneficial effects of vitamin D supplementation in correcting any vitamin D insufficiency leading to improvement in vascular oxidative stress, and the ability to reduce risk of diabetes and cardiovascular complications are also discussed.

Oxidative stress

Oxidative stress is a condition caused by the overproduction of reactive species, known as pro-oxidants and the incapability of the antioxidant defense system to scavenge these species (17). Highly reactive species are normally generated as a by-product of aerobic metabolism in the body (18). These reactive species at a certain amount is often necessary to maintain normal metabolic processes (19). However, the excess in radical species produced is likely to bring deleterious effects to the human body (20); thus they need to be sufficiently removed by the body's antioxidant defense system to maintain the homeostasis of the body system (15).

In diabetes, chronic hyperglycemia induces excessive formation of these reactive species that might diminish antioxidant activity, leading to the domination of oxidative stress (17,21). Oxidative stress then further propagates the production of more reactive species, subsequently causing the development of pathological conditions in DM and its secondary complications (12).

Highly reactive species and free radicals

In general, highly reactive species can be classified into reactive oxygen species (ROS) and reactive nitrogen species (RNS). Both species are divided into free radical or non-radical species. Free radical ROS comprise of superoxide anion ($\bullet O_2^-$), peroxyl ($\bullet RO_2$), hydroxyl radical ($\bullet OH$) and hydroperoxyl ($\bullet HRO_2^-$) while non-radical ROS include hydrochlorous acid (HOCl) and hydrogen peroxide (H_2O_2). Free radical RNS include nitric oxide ($\bullet NO$) and nitrogen dioxide ($\bullet NO_2^-$) whereas non-radical RNS comprise of nitrous oxide (HNO_2), peroxynitrite ($ONOO^-$), and alkyl peroxynitrates ($RONOO$) (22-23). The classification of highly reactive species is listed in Figure 1.

Free radicals are defined as any chemical entities that contain unpaired electrons in an atomic orbital. They are relatively unstable and highly reactive with short half-life (24). They behave as oxidants or reductants because

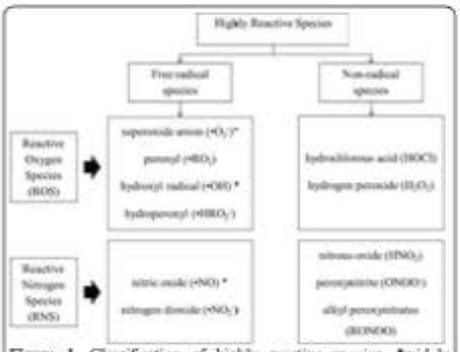

Figure 1. Classification of highly reactive species. *widely addressed in diabetes-induced cardiovascular complications.

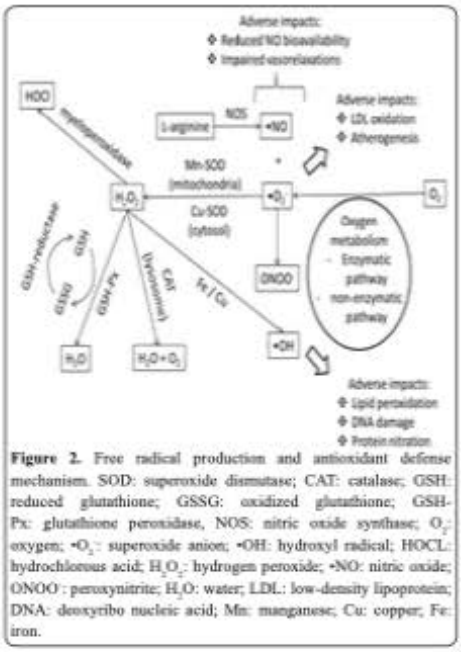

Figure 2. Free radical production and antioxidant defense mechanism. SOD: superoxide dismutase; CAT: catalase; GSH: reduced glutathione; GSSG: oxidized glutathione; GSH-Px: glutathione peroxidase, NOS: nitric oxide synthase; O_2: oxygen; $\bullet O_2^-$: superoxide anion; $\bullet OH$: hydroxyl radical; HOCL: hydrochlorous acid; H_2O_2: hydrogen peroxide; $\bullet NO$: nitric oxide; $ONOO^-$: peroxynitrite; H_2O: water; LDL: low-density lipoprotein; DNA: deoxyribo nucleic acid; Mn: manganese; Cu: copper; Fe: iron.

they tend to either accept an electron from or donate an electron to other molecules to achieve stable electron configuration (25).

In diabetes-induced cardiovascular complications, $\bullet O_2^-$, $\bullet OH$ and $\bullet NO$ are among the free radicals that are widely addressed in the literature (26). $\bullet O_2^-$ is produced by one electron reduction of molecular oxygen (O_2) in oxygen metabolism (Figure 2). It is the initial source that propagates the free radical chain reaction. Hydrogen peroxide, H_2O_2 which is produced from $\bullet O_2^-$ during dismutation, in the presence of transition elements such as copper and iron, might be converted to $\bullet OH$ (27). $\bullet OH$ is highly reactive and the most potent oxidant to attack most biological molecules, further propagating the chain reaction. $\bullet O_2^-$ might also react rapidly with $\bullet NO$ to produce cytotoxic $ONOO^-$. This pathological modification impairs the role of $\bullet NO$ to act as the mediator of vascular tone and inhibits its anti-proliferative property, leading to the pathogenesis of vascular dysfunction (22).

Metabolic abnormalities of DM induce mitochondrial overproduction of $\bullet O_2^-$, leading to uncontrolled elevation of free radicals (28). Free radicals attack important macromolecules in the body, leading to cellular damage and homeostatic disruption (29). Free radicals also lead to foam cell formation and atherosclerotic plaque by stimulating the low-density lipoprotein (LDL) oxidation (30), causing it to be taken up by scavenger receptors of macrophages (26). Besides the uncontrolled propagation of free radical chain reaction, free radicals also involve in the alteration of enzymatic antioxidant defense system by impairing glutathione metabolism and thus reducing the levels of antioxidant

(1). The reduced level of antioxidant makes the cell and tissue more prone to oxidative stress, causing oxidative damage, further exacerbating diabetes complications (31).

Antioxidant defense system and antioxidants

Antioxidant defense system plays a pivotal role to scavenge excess radical species and neutralize the toxicity arising from the elevated amount of reactive species. The system is generally divided into endogenous and exogenous antioxidants (32). Enzymatic endogenous antioxidants include superoxide dismutase, catalase and glutathione (oxidized/reduced) while exogenous antioxidants can be acquired from diet and supplements. The antioxidant defense mechanism is simplified in Figure 2. Vitamin C and vitamin E are among the most classic naturally occurring antioxidants that regulate oxidative stress in the pathogenesis of diabetes-related vascular complications.

Superoxide dismutase (SOD)

SOD is an important antioxidant enzyme in the regulation of oxidative stress in DM (16). It acts as a first line defense against reactive species to reduce oxidative stress and subsequently reduce the risk of cellular and histological injury (33). The different forms of SOD, manganese superoxide dismutase (Mn-SOD) in the mitochondria and copper superoxide dismutase (Cu-SOD) in the cytosol catalyze the dismutation of $\bullet O_2^-$ into less toxic O_2 and H_2O_2 (27). Alteration in the metabolic state of DM leads to diminished activity and level of SOD in diabetic organs, tissue and blood (34-35). Reduced SOD level elevates the production of reactive species, leading to augmented vascular oxidative stress and increased CVD risk in diabetics.

Catalase (CAT)

CAT is another important enzyme that metabolizes H_2O_2 into H_2O and O_2 in lysosome (26). Chronic hyperglycemia induces the augmented formation of H_2O_2 and down-regulates the gene expression of CAT (36), leading to the decreased production of this enzyme. As the major regulator in H_2O_2 metabolism, CAT enzyme deficiency causes cell injury mediated by the accumulation of H_2O_2 in diabetic model.

Glutathione (GSH)

GSH acts as direct scavenger and co-substrate for the enzyme glutathione peroxidase (GSH-Px). GSH-Px is one of the antioxidant enzymes in H_2O_2 metabolism that metabolizes H_2O_2 into H_2O and O_2 in mitochondria (26). Reduced GSH-Px expression has been reported in most diabetic models (37-38).

Vitamins

It is well established that vitamin C and E are non-enzymatic exogenous antioxidants against oxidative stress in the prevention of diabetes and its complications. These antioxidant vitamins neutralize free radicals directly and also interact in recycling processes to regenerate reduced forms of vitamins for further antioxidant actions (39).

The molecular structure of vitamin C offers electron donating and accepting potential to be involved in redox reaction, thus it acts as a reducing agent in free radical-mediated oxidation processes to efficiently scavenge free oxygen radicals (40). Vitamin E is a fat-soluble vitamin that can protect cell membrane from oxidative damage by discontinuing a potentially destructive series of oxidative chain reactions on the structural and functional components of cells and vessel walls (41). Vitamin E has proved to be effective in preventing glucose -induced lipid peroxidation and other free radical-driven oxidative events. It can also prevent LDL oxidation and reduce oxidized LDL uptake which will subsequently lead to foam cell and atherosclerotic plaque formation, by downregulating the protein expression of scavenger receptor (42).

The supplementation of vitamin E (alone or in combination) produces a significant impact on the parameters of antioxidant status, particularly in plasma antioxidant capacity and enzyme concentrations. Unlike vitamin E, vitamin C contributes in neutralizing the radical form of other antioxidants to regain their antioxidant ability. Hence, intervention using vitamin C did not show impactful outcome compared to those using vitamin E. However, intervention using the combination of vitamin C and E showed promising results in increasing GSH and SOD activity (43-44).

Considerable efforts have been put into studying the antioxidant action of vitamin D in the past decade. Vitamin D may be regarded as an antioxidant in terms of their homologous structure to cholesterol. Wiseman *et al.* (1993) demonstrated that vitamin D acts as a membrane antioxidant in view of its ability to inhibit iron-induced lipid peroxidation of brain liposomes (45). Moreover, vitamin D has been reported to attenuate oxidative stress by up-regulating antioxidant enzymes and suppressing elevated lipid peroxidation (37,46).

Oxidative stress biomarkers

The reactions of free radicals and antioxidants occur instantaneously; hence it becomes a major problem to perform direct measurement on oxidative stress (47). Thus, the indirect way to evaluate oxidative stress is through observable biomarkers (48). The effectiveness of antioxidant defense system to counteract elevated amounts of free radicals can be measured by the levels of the endogenous antioxidant enzymes. Meanwhile, excess production of free radicals can be determined by measuring the products produced as the result of oxidative damage caused by these species.

Oxidative damage comprises of cellular protein glycation, membrane lipid peroxidation and the damage to nucleic acids (49). Among the oxidative damage, augmented lipid peroxidation has been reported to be closely associated with chronic hyperglycemia in DM (50). DM alters the lipid profile of the cells by removing hydrogen from lipids to produce $\bullet HRO_2^-$ when attacked by $\bullet RO_2$, further propagating the free radical pathway (29). Furthermore, the natural presence of multiple bonds in polyunsaturated fatty acids in cell membrane makes them more susceptible to free radicals for lipid peroxidation.

$\bullet HRO_2^-$ can exert direct toxicity on the cells; it can be degraded to $\bullet OH$, or to react with transition metals (such as iron or copper) to form stable aldehydes (30), such as malondialdehyde (MDA) to consequently damage cell

membranes (51). A stable MDA has been documented as a primary biomarker (52) to evaluate lipid peroxidation, mostly studied with thiobarbituric acid reactive substances (TBARS) assays (53). Elevated TBARS and MDA levels in plasma, serum, and other tissues in diabetic patients suggest that peroxidative injury may be involved in the development of diabetes complications (12,50).

Vitamin D

Vitamin D is classically known for its role as an important hormone in mineral homeostasis and maintenance of musculoskeletal health (54). However, vitamin D also possesses antioxidant properties as potent as, or even better than the classical antioxidant vitamin E (45,55-57). Furthermore, vitamin D has also been discovered to be a potent hormone that exerts significant biological actions, such as induction of cell differentiation, reduction in inflammation and immunomodulation (58).

Vitamin D is a fat-soluble vitamin, whereby more than 90% are obtainable by cutaneous production from sunlight exposure while only approximately 10-20% is obtained by dietary intake. There are two major forms of vitamin D, which differ chemically only in their side chains. Ergocalciferol (vitamin D_2) is synthesized by ultraviolet irradiation of plant sterols (ergosterol) and invertebrates while cholecalciferol (vitamin D_3) is photosynthesized endogenously when solar ultraviolet B radiation with wavelength of 280-320 nm strikes human epidermis. Irradiation stimulates non-enzymatic photolytic conversion of pro-vitamin D (7-dehydrocholesterol) to pre-vitamin D, thereafter undergoes thermal isomerization into vitamin D_3 (54). Vitamin D_3 is also naturally present in food especially from animal sources, such as oily fish, fortified dairy products and animal fats.

Vitamin D is biologically inert and needs to be biologically activated via two hydroxylation processes in the body before being utilized for biological actions. Vitamin D is absorbed in the small intestines, transported via the lacteal system and conveyed via the lymphatic system into the venous circulation (59). Vitamin D is then bound to vitamin D-binding protein (DBP), and is transported to the liver via the blood circulation in this bound form (60). In the liver, hepatic cells transform vitamin D into 25-hydroxyvitamin D, 25(OH)D (calcidiol) in the presence of vitamin D 25-hydroxylase enzyme. In the kidney, 25(OH)D is then metabolized either intrarenally by 25(OH)D-1α-hydroxylase enzyme or intracellularly at extra-renal sites in a variety of cells/tissues to the physiologically active vitamin D, 1,25-dihydroxyvitamin D, $1,25(OH)_2D$ (calcitriol) (59,61). $1,25(OH)_2D$ entered the bloodstream to act as the primary steroid hormone in mineral and skeleton homeostasis. The process of vitamin D synthesis and metabolism is illustrated in Figure 3.

Vitamin D status and supplementation

Instead of $1,25(OH)_2D$, serum 25(OH)D is the most common determinant of vitamin D level in the body (62). This is in view of its longer circulating half-life of 15 days (63); it is also the primary circulating form of vitamin D (64). The level of serum 25(OH)D reflects the precise storage amount of vitamin D (both by solar-activated cutaneous production and long period dietary intake) in the human body. The status of vitamin D (deficiency, insufficiency and sufficiency) in the body can be defined after measurement of serum 25(OH)D. The Endocrine Society defines vitamin D insufficiency as serum 25(OH)D between 50–74 nmol/L, serum 25(OH)D of less than 50 nmol/L as vitamin D deficiency (65) while vitamin D sufficiency as serum 25(OH)D of at least 75 nmol/L. The optimal range of 75-125 nmol/L is needed to optimize intestinal calcium absorption and to cover all physiological functions of vitamin D (66).

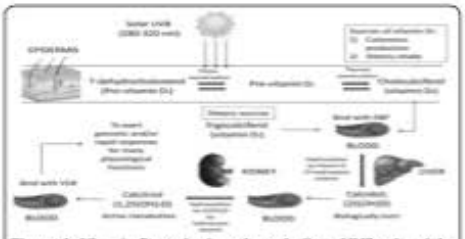

Figure 3. Vitamin D synthesis and metabolism. UVB: ultraviolet B radiation; DBP: vitamin D-binding protein; 25(OH)D: 25-hydroxyvitamin D; $1,25(OH)_2D$: 1,25-dihydroxyvitamin D; VDR: vitamin D receptor.

Hypervitaminosis D

Hypervitaminosis D, or commonly known as vitamin D toxicity, occurs when excess pharmacologic doses of vitamin D is taken, translating to a large increase in circulating 25(OH)D concentration, that is beyond DBP binding capacity. Excessive sun exposure does not lead to hypervitaminosis D as pre-vitamin D_3 will undergo photo-degradation to inactive sterols in the skin (54). The main clinical consequences of vitamin D toxicity are hypercalcemia and other symptoms including hypercalciuria, ectopic calcifications, hyperphosphatemia, kidney stones, polyuria and polydipsia, hypertension, anorexia, nausea, vomiting and constipation (62). Although it is uncommon, hypervitaminosis D has been reported in multiple age groups and from multiple causes, including manufacturing errors (67), errors in milk fortification (68), incorrect dosing from liquid preparations (69) and ingestion of mega doses of vitamin D supplements (70). There are studies which had reported an increase in cancer incident rates and mortality risks at both low and high levels of serum 25(OH)D (71-72). Nimesh *et al.* (2015) reported that a one-year old child developed acute hypertension and severe hypercalcemia due to vitamin D toxicity after high doses of oral calcitriol supplementation (70). Gallagher (2016) also showed that the administration of annual bolus doses of vitamin D at concentration of 300,000 IU or 500,000 IU resulted in the increased risk of falls and fractures (73). Therefore, until further evidence is available, a reasonable upper limit for 25(OH)D level at 125 nmol/L (50 ng/mL) is suggested in elderly individuals (73), infants (74) and healthy young adults (75).

Discussion

Vitamin D status and diabetes mellitus

In the development of adverse cardiovascular events,

especially in DM, low vitamin D level is suggested as one of the risk factors (59). This is supported by statistics showing high prevalence of vitamin D insufficiency and deficiency occurring in the diabetic population; 91.1% in India (76), 73.6% in Saudi Arabia (77) and 43% in Malaysia (78).Studies have shown that higher vitamin D levels were associated with 40% lower risk of T2DM in women while subjects that developed diabetes had lower vitamin D levels (serum 25(OH)D < 50 nmol/L) compared to non-diabetics (79). This suggested that low circulating vitamin D concentrations played a significant role in the pathogenesis of DM. This might occur via decreased pancreatic insulin release, underscored insulin resistance, reduced insulin sensitivity and deteriorated glucose tolerance (80). This is supported by Wang's study, whereby the incidence of adverse CVD was doubled in individuals with low vitamin D levels (serum 25(OH)D <37.5 nmol/L) in a 6-year-period study (16).

Moderate or severe vitamin D deficiency also increases the activities of glutathione-dependent enzymes (16), resulted in overproduction of ROS in diabetics (81). Vitamin D supplementation might be an alternative way to improve the vitamin D levels in the body, when there is insufficient cutaneous production or dietary intake. Vitamin D_3 supplementation is 87% more effective in raising serum 25(OH)D level compared to vitamin D_2 as it is converted 500% faster to calcitriol, the biologically active hormone (82). Vitamin D_3 is also the natural form of vitamin D produced by our body which is more potent in raising and maintaining vitamin D concentrations in the circulatory system longer, and produces 2-3 fold greater storage of vitamin D. Besides, the action of vitamin D_3 is approximately three times more effective because it binds with high affinity to DBP in plasma, thus it can stay longer in the circulation (59).

Vitamin D status and oxidative stress

In some studies involving human and animal models of diabetes or vitamin D deficiency, supplementation with vitamin D showed reduction in the levels of oxidative stress (37-38,83-84). Calcitriol supplementation had been shown to improve SOD activities and reduce ROS production in renal arteries of hypertensive patients as well (81).

In diabetic rats, high level of serum TBARS was significantly reduced nearly to control values after the treatment with vitamin D_3 (83). In asymptomatic vitamin D-deficient subjects, supplementation with vitamin D_3 (300,000 IU monthly for 3 months) significantly decreased serum TBARS similar to that of basal TBARS in control group (84). In subjects with T2DM, treatment with vitamin D_3-fortified doogh (500 IU twice a week for 12 weeks) showed reduction in MDA and increased GSH levels (37). However, treatment with vitamin D_3 capsules (50,000 IU twice) did not affect the biomarker of oxidative stress (MDA and GSH levels) in women with GDM (85). Similarly, Efterkhari *et al.* (2014) reported that there was no significant change in MDA levels in T2DM subjects after receiving calcitriol supplementation (0.5 µg/day for 12 weeks) (86). Another study in T2DM vitamin D-deficient patients reported that vitamin D_3 treatment (5,000 IU/day for 12 weeks) did not significantly affect SOD levels (87).

The difference in findings between these studies might be due to: 1) vitamin D dosage and study duration; 2) the status of vitamin D in the chosen diabetes population studies; and 3) the type of vitamin D analog used in the study. No improvement to oxidative stress markers was observed in the study by Yiu *et al.* (2013) despite increase in serum 25(OH)D levels. The vitamin D_3 dose given for only a short duration of treatment (12 weeks) may not be adequate to sufficiently increase the already low baseline serum vitamin D levels to therapeutic levels in their study subjects (87). No effect of vitamin D_3 supplementation on oxidative stress markers in women with GDM might be caused by insufficient dosage of vitamin D_3 given to the study subjects, which was 50,000 IU per capsule given twice throughout the study period of 6 weeks (85). The type of vitamin D analog used for the treatment was also a critical indicator. Vitamin D_3 performs better than vitamin D_2 as an effective supplement because non-active vitamin D metabolites might face impairment in their conversion to the active metabolite, especially in patients with kidney problems. This results in reduction of serum vitamin D concentrations in the circulation that is able to improve oxidative stress in diabetics. A limitation to the interpretation of the effect of vitamin D supplementation on oxidative stress is, some articles did not report baseline levels of vitamin D and oxidative stress biomarkers of enrolled patients before supplementation. These parameters should be measured to display better outcome on the beneficial effect of vitamin D supplementation, and also to eliminate the impact of confounding variables that might interfere with the results.

A study reported on the potential of vitamin D_3 supplementation either as a preventive measure or therapeutic strategy in diabetic rats. Diabetic rats received 5000 IU/kg bw/day vitamin D_3 supplement by gastric gavage before and after alloxan-induction respectively for 2 months as preventive and therapeutic groups of diabetes. The results showed that administration of vitamin D_3 in both groups (preventive and therapeutic groups) enhanced hepatic and renal activity of SOD, CAT and GSH-Px, as well as reduced lipid peroxidation as indicated by decreased TBARS level compared to untreated diabetic rats. However, vitamin D might play a better role in regulating oxidative stress prior to the development of diabetes as shown by the better significant improvement in the preventive group compared to the therapeutic group of diabetic rats (38). The relationship between vitamin D interventions and oxidative stress biomarkers in animal and human studies is summarized in Table 1.

Vitamin D status and atherosclerosis

Epidemiological studies demonstrate that low levels of vitamin D is a risk factor for development of atherosclerosis (88). Vitamin D deficiency correlates with endothelial dysfunction, vascular smooth muscle cell (VSMC) proliferation and migration, augmented systemic inflammation, increased intima-media thickness (IMT) and enhanced oxidative stress in the development of atherosclerosis (16,89-91).

Endothelial dysfuntion is the precursor of early atherosclerosis development, which is due to the imbalance in production of endothelium-derived relaxing and

Table 1. Relationship between vitamin D interventions and oxidative stress biomarkers in animal and human studies.

Animal model/ Population studied	Intervention/ Treatment	Summary of findings	References (historical sequences)
Animal study			
Alloxan-induced diabetic adult male Wistar rats n = 10	Calcitriol (5,000 IU/kg bw) Once daily for 4 weeks Gastric gavage Study duration: 2 months i) preventive group: 15 days before alloxan injection ii) therapeutic group: 15 days after alloxan injection	• Marked elevated of SOD, CAT and GSH-Px in hepatic and renal tissues of both treatment groups. • Activity of SOD, CAT and GSH-Px in hepatic and renal tissues was significantly higher in the preventive group than the therapeutic group. • Reduced TBARS levels in hepatic and renal tissues of both treatment groups. • Reduction in TBARS is more significant in the preventive group than the therapeutic group.	Hamden K *et al.*, 2009 (38)
STZ-induced diabetic adult male Wistar rats n = 6-8	Cholecalciferol (12 µg/kg bw) Once daily for 14 days Oral administration Study duration: 15 days	• Significant elevated levels of TBARS in liver of diabetic group were significantly reversed to near control value in treatment group. • Significant decrease in SOD and GSH-Px gene expression in liver of diabetic group were reversed nearly to control level for treatment group.	George N *et al.*, 2012 (83)
Animal model/ Population studied	**Intervention/ Treatment**	**Summary of findings**	**References (historical sequences)**
Human Study			
Asymptomatic vitamin D-deficient subjects baseline level of plasma 25(OH)D < 25 nmol/L n = 23	Vitamin D_3 (300,000 IU) Once monthly for 3 months Intramuscular injection	• Low baseline plasma 25(OH)D levels were significantly increased after treatment. • High basal TBARS levels were decreased significantly after treatment, similar to that of basal TBARS in control group.	Tarcin O *et al.*, 2009 (84)
Pregnant women with GDM baseline level of serum 25(OH)D < 75 nmol/L n = 27	Cholecalciferol capsules (50,000 IU) Twice (baseline and on 21st day) Oral administration Study duration: 6 weeks	• Serum 25(OH)D increased significantly. • Plasma TAC levels were not affected. • No significant change in total GSH. • Significant decrease in fasting plasma glucose, serum total and LDL-cholesterol concentrations.	Asemi Z *et al.*, 2013 (85)
T2DM patients baseline level of serum 25(OH)D < 75 nmol/L n = 50	Vitamin D_3 tablets (5,000 IU) Once daily for 12 weeks Oral administration	• Significant increases in serum vitamin D levels. • No significant change in SOD levels.	Yiu *et al.*, 2013 (87)
Hyperlipidemia T2DM patients unknown baseline vitamin D levels n = 35	Calcitriol capsules (0.5 µg) Once daily for 12 weeks Oral administration	• Significant reduction in basal MDA levels in both placebo and treatment group. • The alteration in MDA levels was not significant between the groups.	Eftekhari *et al.*, 2014 (86)
T2DM subjects baseline level of serum 25(OH)D < 40 nmol/L n = 50	Vitamin D_3-fortified doogh (500 IU) Twice a week for 12 weeks Oral administration	• Serum 25(OH)D increased significantly. • Reduction in serum MDA levels. • Increased GSH levels.	Shab BS *et al.*, 2015 (37)

STZ: streptozotocin; bw: body weight; SOD: superoxide dismutase; CAT: catalase; TAC: total antioxidant capacity; MDA: malondialdehyde; GSH-Px: glutathione peroxidase; TBARS: thiobarbituric acid reactive substances; T2DM: type 2 diabetes mellitus; GDM: gestational diabetes mellitus; 25(OH)D: 25-hydroxyvitamin D.

contracting factors. Vitamin D exerts direct vasoprotective effects against endothelial dysfunction by enhancing endothelial-dependent vasorelaxation and inhibiting vasocontraction (92-93). Vitamin D improves the bioavailabity of endothelial nitric oxide (NO), the potent vasorelaxing factor and inhibitor of platelet and leucocyte aggregation and adhesion. This occurs via direct enhancement of transcriptional regulator of endothelial nitric oxide synthase (eNOS) (94) and/or exert effect on phosphatidylinositol 3 kinase in endothelial cell (EC), which activates eNOS to catalyse the production of NO from L-arginine (95-96). By inducing NO production, vitamin D able to stimulate EC proliferation and inhibit apoptosis (97). Vitamin D also induces the production of prostacyclin in VSMC through the cyclooxygenase (COX) pathway, which prevents thrombus formation, cell adhesion and VSMC proliferation (98-99). Besides that, vitamin D interferes with calcium influx into the EC (100), decreases the expression of COX-2 and downregulates the expression of prostaglandin receptors to reduce the production of vasoconstrictors (101).

IMT, which is a reflection of atherosclerotic burden was reported to be negatively related to serum 25(OH)D concentration (90). There was marked increase in carotid artery IMT in T2DM patients with low vitamin D levels compared to those with higher vitamin D levels. T2DM patients who developed carotid plaque has significantly lower 25(OH)D concentration compared to T2DM patients without carotid plaque (102). Vitamin D and its analogue suppress the mechanisms that leads to increased IMT and vascular calcification by inhibiting the over-expression of multiple adhesion molecules on EC (91) and the accumulation of plaque lipid in VSMC (103).

Vitamin D also has anti-inflammatory properties. It may suppress the production and release of several pro-inflammatory (91) and increase the production of anti-inflammatory cytokines (104). Vitamin D downregulates the inflammatory process by limiting the major role of T-helper 1 in pro-atherogenic response and shifting the T-cell response from T-helper 1 to T-helper 2 to limit the pro-atherogenic response (105). Vitamin D displays anti-atherogenic properties through an endoplasmic reticulum (ER) stress-dependent mechanism. ER stress is a functional switch that controls macrophage differentiation which may have a role in atherosclerotic plaque regression in diabetics (106). Vitamin D acts as ER stress reliever to prevent foam cell formation during macrophage differentiation, reduce macrophage infiltration and migration and stimulate an anti-atherogenic macrophage phenotype, thus reducing vascular inflammation and complications in T2DM patients. Vitamin D also reverses atherogenic cholesterol metabolism deposition by preventing the progression of macrophage cholesterol uptake and promoting cholesterol egression in macrophages from T2DM patients (107).

In order to counteract the dominant effect of oxidative stress in human endothelial cells, vitamin D analogs act as a negative endocrine regulator of the renin-angiotensin system by reducing the renin synthesis (108). Vitamin D also inhibits the augmented production of ROS, especially superoxide anion (100) through the authophagic and survival pathways (109). Vitamin D also improves SOD, CAT, GSH-Px in enhancing antioxidant defense mechanism and reduces lipid peroxidation that will lead to oxidative cell damage (37-38,83-84).

Conclusion

The relationship between vitamin D levels and oxidative stress in diabetes has been quite well studied. Low vitamin D levels substantially impaired insulin and glucose metabolism which may contribute to the pathogenesis and development of DM. Chronic hyperglycemia in DM further disrupted the homeostasis between the generation of radical species and the effectiveness of enzymatic antioxidant defense system (47), predisposing to the development of diabetes-related cardiovascular incidents and its complications, leading to morbidity and mortality.

Optimal control of blood glucose level might be able to slow down diabetes complications, although it is not able to completely prevent diabetes complications. Thus, evidences on the ability of antioxidants to regulate oxidative stress in diabetes are compelling and suggests potential additional treatment strategy to reduce cardiovascular risk and complications in diabetes. In view that vitamin D supplementation is able to improve many cardiovascular biomarkers in both diabetic (78, 110) and non-diabetic (111) population with vitamin D deficiency, it may be a potential measure in regulating oxidative stress underlying diabetic complications. By correcting the vitamin D levels as shown by accumulating evidences in epidemiology studies, enhanced oxidative damage and reduced antioxidant activities might be reversed or at least improved in diabetic population. Even though clinical trials conducted to date failed to provide adequate support for the implementation of vitamin D supplement in diabetes treatment, but they did show improvement on certain biomarkers for cardiovascular health. Thus, using vitamin D supplement in diabetes treatment, especially in vitamin D-deficient and insufficient patients, definitely deserves further assessment and consideration.

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Conflict of interests

None.

Authors' contribution

C. L. WEE – drafting the article
S. S. MOKHTAR – proofread and revision of the article
S. MUNISAMY – gathering information to prepare draft
S. YAHAYA – outline the design and critical revision of the article
A. H. G. RASOOL – conception and final revision/approval of the article.

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Conference (1)

Oral Presentation (1)

OA06

VITAMIN D DEFICIENCY ATTENUATES MICROVASCULAR ENDOTHELIAL FUNCTION IN MESENTERIC ARTERIES OF DIABETIC RATS

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Objective: To study the effects of vitamin D deficiency on microvascular endothelial function in streptozotocin-induced diabetic rats. Thereafter, to investigate the effects of oral calcitriol treatment on endothelial function in diabetic rats with vitamin D deficiency. **Methodology:** 30 Sprague Dawley rats were induced for diabetes with 50mg/kg streptozotocin intraperitoneally. They were randomly divided into three equal groups (n=10). Diabetic control group (DC) received normal diet, diabetic vitamin D-deficient group (DVD) received vitamin D-deficient diet, while diabetic supplementation group (DVS) received vitamin D-deficient diet and started to receive 4 weeks 0.15µg/kg calcitriol daily by oral gavage from 7th week after diabetes induction. At the end of 10 weeks, all rats were sacrificed. The mesenteric arterial rings, with and without endothelium, were studied for isometric force measurements using wire myograph. **Results:** The study showed significant reduction in endothelium-mediated relaxation to acetylcholine (ACh) and significant augmented contraction to calcium ionophore (CaI) in DVD group compared to DC group (ACh - DC: 53.48±6.316% vs DVD: 30.06±3.042%, P=0.005) (CaI – DC: 58.78±8.093% vs DVD: 97.55±6.578%, P=0.002). By treatment with calcitriol, DVS group showed significant enhanced relaxation to ACh (DVS: 48.34±2.641%, P<0.05) compared to DVD group while there was no significant reduction in contraction to CaI between these two groups. There was significant reduction in relaxation to sodium nitroprusside in DVD group compared to DC group (DC: 67.83±6.993% vs DVD: 34.42±2.986%, P=0.001), while the DVS group showed significant improvement (DVS: 52.24±5.071%, P=0.007) compared to DVD group. There were no significant difference in relaxation to salbutamol and contraction to phenylephrine among three study groups. **Conclusions:** This study showed that vitamin D deficiency in diabetic rats attenuates endothelium and smooth muscle function by reducing relaxation and enhancing contraction. Treatment with calcitriol may be the potential therapy to improve both functions in diabetics.

Conference (2)

Poster Presentation (1)

ER 05

Augmented Acetylcholine-Mediated Vasodilation In Aorta Of Diabetic Vitamin D-Deficient Rats After Treatment With Calcitriol

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INTRODUCTION:

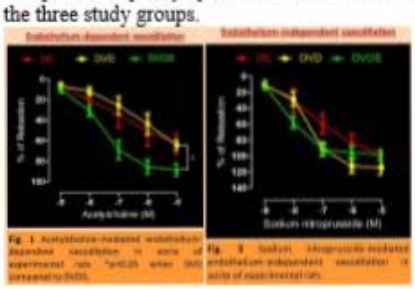
Recent evidence suggests that vitamin D-deficiency is associated with endothelial vascular dysfunction and increased risk of cardiovascular diseases. The prevalence of vitamin D-deficiency is high in diabetes mellitus (DM) patients. It is not well-known whether vitamin D supplementation in diabetic patients with vitamin D-deficiency will cause reduction in endothelial dysfunction. The aim of this study is to evaluate the effect of oral vitamin D supplementation on the aortic endothelial function of rats with vitamin D-deficiency.

MATERIALS & METHODS:

Diabetic rats (n=24) were induced with 50 mg/kg streptozotocin and were divided into three equal groups: diabetic controls that received normal diet throughout study duration (DC), diabetic vitamin D-deficient rats without supplement (DVD), and diabetic vitamin D-deficient rats with supplement (DVDS). Diabetic vitamin D-deficient rats were developed by using vitamin D-deficient diet throughout 10 weeks of study duration. The rats in DVDS received oral gavage of 0.15 µg/kg calcitriol supplement for four weeks. At the end of 10 weeks, rats were sacrificed and aortic rings with and without endothelium were studied in tissue organ baths for isometric force measurement.

RESULTS:

Body weight of rats in DVD group were significantly increased compared to baseline (Week 10; 332.38±16.28g vs. Baseline; 253.75±9.16g), whereas showed reduction in DVDS (Baseline; 272.25±12.13g vs. Week 10; 230.25±12.53g) ($p<0.05$). The endothelium-mediated vasodilation to acetylcholine was similar in aorta of DC and DVD. Acetylcholine-induced endothelium-mediated vasodilation was significantly increased in aorta of DVDS (89.05±6.093%) compared to those in DC (63.47±10.65%) and DVD groups (61.88±5.06%) ($p<0.05$). There was no significant difference in vasodilation to sodium nitroprusside and contraction to either calcium ionophore or phenylephrine between aortas of the three study groups.



DISCUSSIONS:

This study demonstrates that vitamin D-deficiency does not exacerbate endothelial and smooth muscle dysfunction in aorta of diabetic rats. Interestingly, rats that received vitamin D supplement show significant reduction in body weight. Treatment with calcitriol improves endothelium-dependent vasodilation in aorta of diabetic rats with vitamin D-deficiency.

CONCLUSION:

Vitamin D can be an adjunct therapy to reduce cardiovascular risks in diabetic patients. Further studies to support the use in clinical setting is warranted.

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Conference (3)

Oral Presentation (2)

33rd Scientific Meeting of Malaysian Society of Pharmacology and Physiology (MSPP)

co-organised with University of Malaya & Controlled Release Society Malaysian Chapter (MyCRS)

ORA022

Calcitriol Supplementation Ameliorates Microvascular Endothelial Dysfunction in Vitamin D-deficient Diabetic Rats by Upregulating eNOS Expression and Reducing Oxidative Stress

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Background: Vitamin D deficiency in diabetics contributes to endothelial dysfunction and augmented oxidative stress. Correcting vitamin D status by supplementation might be a potential intervention.
Objective: To evaluate the effects of calcitriol supplementation in ameliorating microvascular endothelial dysfunction and oxidative stress in vitamin D-deficient diabetic rats.
Methodology: Streptozotocin-induced male Sprague-Dawley rats were divided into three groups of 10 rats each. Diabetic controls (DC) received 10-weeks normal diet, vitamin D-deficient diabetics (DD) received 10-weeks vitamin D-deficient diet, while the supplemented group received 10-weeks of vitamin D-deficient diet with 4-weeks oral calcitriol supplementation (DDS) (0.15 mcg/kg daily, five times a week), starting from week 7 of diabetes induction. At the end of 10 weeks, microvascular endothelial function of mesenteric artery was assessed using wire myography. Endothelial nitric oxide synthase (eNOS) expression was determined using western blotting. Immunohistochemistry was used to detect the presence and localization of eNOS. Tissue superoxide dismutase (SOD) and malondialdehyde (MDA) levels; FBG and serum 25(OH)D levels in blood were measured.
Results: Calcitriol supplementation significantly improved the attenuated endothelium-dependent relaxation in mesenteric artery of vitamin D-deficient diabetics [R_{max} (DDS: 48.40±8.41% vs DD: 28.45±10.70%)]. However, augmented endothelium-dependent contraction and impaired sodium-nitroprusside-induced endothelium-independent relaxation in vitamin D-deficient diabetics were failed to be ameliorated by calcitriol supplementation. Besides, calcitriol supplementation in vitamin D-deficient diabetics significantly increased SOD and reduced MDA levels; eNOS expression is also increased.
Conclusion: Calcitriol supplementation ameliorates microvascular endothelial dysfunction in diabetics with vitamin D deficiency by upregulating eNOS expression and improving oxidative stress status.

Keywords: *vitamin D; diabetes mellitus; oxidative stress*

LIST OF PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

Research Papers

1. **Lee, W.C.**, Mokhtar, S.S., Munisamy, S., Yahaya, S. & Rasool A.H.G. (2018). Vitamin D status and oxidative stress in diabetes mellitus. *Cell Mol Biol*, 64(7), 60-69. (ISI-indexed).

2. **Lee, W.C.**, Mokhtar, S.S., Singh, K.K.B., Yahaya, S., Leung, S.W.S & Rasool A.H.G. (2020). Calcitriol supplementation ameliorates mirovascular endothelial dysfunction in vitamin D-deficient diabetic rats by upregulating vascular eNOS protein expression and reducing oxidative stress. *Oxid Med Cell Longev.* (ISI-indexed). (Submitted)

3. **Lee, W.C.**, Mokhtar, S.S., Singh, K.K.B., Leung, S.W.S & Rasool A.H.G. (2020). Vitamin D deficiency attenuates endothelial function by reducing antioxidant activities and vascular eNOS protein expression in rat's microcirculation. *Nutrients*. (ISI-indexed). (Submitted)

4. Siti Safiah Mokhtar, **Wee Chee Lee**, Low Jen Hou, Ahmah Khusairi Azemi, Anani Aila Mat Zin, Che Badariah Ab Aziz & Aida Hanum Ghulam Rasool. (2020). Vitamin D deficiency in diabetes alters the aortic media thickness but not its functional properties. *Braz J Pharm Sci*. (ISI-indexed). http://dx.doi.org/10.1590/s2175-979020200001181042

5. Seetha Munisamy**, Wee Chee Lee**, Siti Safiah Mokhtar & Aida Hanum Ghulam Rasool. (2018). Vitamin D, inflammation and atherosclerosis. *Trop J Pharm Res.* (ISI-indexed). (Accepted for publication on 2018)

PRESENTATIONS

1. **Wee Chee Lee**, Siti Safiah Mokhtar, Kirnpal Kaur Banga Singh & Aida Hanum Ghulam Rasool. Vitamin D supplementation ameliorates microvascular endothelial dysfunction in vitamin D-deficient diabetic rats by upregulating eNOS expression and reducing oxidative stress. Presented at the 33rd Scientific Meeting – Malaysian Society of Pharmacology & Physiology at the Everly Hotel, Putrajaya, Selangor, Malaysia on 15th to 16th July 2019 (Oral presentation).

2. **Wee Chee Lee**, Siti Safiah Mokhtar, Sahran Yahaya & Aida Hanum Ghulam Rasool. Vitamin D deficiency attenuates microvascular endothelial function in mesenteric arteries of diabetic rats. Presented at the 31st Scientific Meeting – Malaysian Society of Pharmacology & Physiology at the School of Dental

Sciences, Universiti Sains Malaysia (Health Campus), Kelantan, Malaysia on 18th to 19th August 2017 (Oral presentation).

3. Sahran Yahaya, Siti Safiah Mokhtar, **Wee Chee Lee**, Che Badariah Ab Aziz & Aida Hanum Ghulam Rasool. Augmented acetylcholine-mediated vasodilation in aorta of diabetic vitamin D-deficient rats after treatment with calcitriol. Presented at the 48th Scientific Meeting/ Annual General Meeting 2018 – Malaysian Orthopaedic Association at the Equatorial Hotel, Penang, Malaysia on 10th to 12th May 2018 (Poster presentation).