ROLE OF OXIDATIVE STRESS IN REM SLEEP DEPRIVATION-INDUCED ENDOTHELIAL DYSFUNCTION IN RAT MODEL

NUR SYAFIQAH BINTI MOHMED NOR

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by

NUR SYAFIQAH BINTI MOHMED NOR

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any other masters at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research and promotional purposes.

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(NUR SYAFIQAH BINTI MOHMED NOR)

Date: ……16/9/2020……

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LIST OF ACRONYM, ABREVIATIONS AND SYMBOLS

%	Percentage
°C	Degree Celcius
µm	Micrometer
µM	Micromolar
× g	Gravity
AASM	American Academy of Sleep Medicine
ADMA	Endogenous nitric oxide synthase inhibitor
ACh	Acetylcholine
AKT	Protein kinase B
Ang II	Angiotensin II
ANOVA	Analysis of variances
AP-1	Activator protein-1
ARASC	Animal Research and Service Centre
BP	Blood pressure
BCA	Bicinchoninic acid
BH4	Tetrahydrobiopterin
BW	Body weight
BWg	Body weight gain
CAT	Catalase
cGMP	Cyclic guanosine monophosphate
cm	Centimeter
CO_3	Carbonate
CRP	C-reactive protein

COVID-19	Coronavirus disease
CSD	Chronic sleep deprivation
CTRL	Control
Cu	Copper
Cu^{+}	Cuprous
Cu^{2+}	Cupric
CVD	Cardiovascular disease
ECG	Electrocardiography
ECL	Enhanced Chemiluminescence
EDRF	Endothelium-Derived Relaxing Factor
EEG	Electroencephalography
EMG	Electromyogram
EOG	Electrooculogram
Egr-1	Early growth response protein 1
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin 1
Fc	Food consumption
FAD	Flavin adenine dinucleotide
FMC	Free moving control
FMN	Flavin mononucleotide
g	Gram
GATA6	GATA-binding protein 6
GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Oxidised glutathione

H_2O_2	Hydrogen peroxide
HO_2	Hydroperoxyl
HOCL	Hypochlorous acid
IACUC	Institutional Animal Care and Use Committee
ICAM-1	Intracellular cell adhesion molecule-1
ICSD	International Classification of Sleep Disorder
IL-1	Interleukin-1
IP	Intraperitoneal
kg	Kilogram
KLF2	Kruppel-like factor 2
MCO	Movement Control Order
MCP-1	Macrophage chemoattractant peptide-1
mg	milligram
mL	milliliter
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-Kb	*Nuclear factor kappa B*
NIBP	Non-invasive Blood Pressure
NO	Nitric oxide
NREM	Non-rapid eye movement
O_2	Oxygen
O_2^-	Superoxide
O_3	Ozone
OH	Hydroxyl
$ONOO^-$	Peroxynitrite
PAF	Platelet-activating factor

PAI-1	Plasminogen activator inhibitor
PBS	Phosphate buffer saline
p-eNOS	Phosphorylated eNOS
PI3K	Phosphatidylinositol 3-kinase
PVDF	Polyvinylidene difluoride
REM	Rapid eye movement
REMsd	Rapid eye movement sleep deprivation
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SD	Sprague-Dawley
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error mean
sGC	Soluble guanylyl cyclase
SOD	Superoxide dismutase
SP-1	Specificity protein 1
SR	Sleep recovery
SWS	Slow-wave sleep
TAC	Total antioxidant capacity
TBS	Tween buffer solution
TC	Tank controol
TM	Thrombomodulin
TNF-α	Tumour necrosis factor-alpha
TXA2	Thromboxane A2
USM	Universiti Sains Malaysia

VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growh factor
vWF	Von Willebrand factor

PERANAN STRES OKSIDATIF DALAM DISFUNGSI ENDOTELIUM ARUHAN KEKURANGAN TIDUR REM DALAM MODEL TIKUS

ABSTRAK

Kekurangan tidur mempunyai kaitan dengan disfungsi endotelium, iaitu tanda awal penyakit kardiovaskular. Patogenesis disfungsi endotelium akibat kurang tidur masih kurang difahami sehingga kini. Objektif kajian ini adalah untuk mengenal pasti faktor kemungkinan yang terlibat dalam mekanisme disfungsi endotelium akibat kurang tidur, khususnya semasa fasa REM. Eksperimen untuk objektif pertama sahaja yang dapat dijalankan disebabkan oleh Perintah Kawalan Pergerakan (PKP) oleh Kerajaan Persekutuan Malaysia akibat wabak penyakit koronavirus (COVID-19). Lapan (8) ekor tikus Sprague-Dawley (SD) jantan dibahagikan sama rata kepada 4 kumpulan; tikus kawalan bebas-gerak (FMC), tikus kurang tidur REM 72-jam (REMsd), tikus kawalan tangki (TC) dan tikus pemulihan tidur 72-jam selepas kurang tidur REM 72-jam (SR). Tidur REM dikurangkan dengan menggunakan teknik pasu terbalik. Tiada perbezaan yang signifikan dalam paras pengambilan makanan, peningkatan berat badan dan tekanan darah sistolik antara kumpulan kajian. Paras ekspresi protein eNOS dan eNOS fosforilasi (p-eNOS), dan paras penanda stres oksidatif dalam aorta; kapasiti antioksidan total (TAC), katalase (CAT), dan superoksid dismutase (SOD) tidak diukur disebabkan oleh kekangan masa. Berdasarkan anggapan bahawa kekurangan tidur REM mengaruh disfungsi endotelium, dijangkakan berlaku penurunan paras p-eNOS yang signifikan dalam kumpulan REMsd berbanding kumpulan lain. Stres oksidatif dianggap sebagai faktor yang mungkin terlibat dalam mekanisme disfungsi endotelium, oleh itu penurunan paras antioksidan yang signifikan dijangka berlaku

dalam tisu aorta. Sebagai kesimpulan, kekurangan tidur REM dijangka mempunyai kaitan dengan disfungsi endotelium yang mungkin diaruh oleh stres oksidatif.

ROLE OF OXIDATIVE STRESS IN REM SLEEP DEPRIVATION INDUCED ENDOTHELIAL DYSFUNCTION IN RAT MODEL

ABSTRACT

Sleep deprivation is associated with endothelial dysfunction, an early sign of cardiovascular disease. The pathogenesis underlying endothelial dysfunction in sleep deprivation remains poorly understood to date. The objective of this study was to identify the possible factors involved in the mechanism of endothelial dysfunction in sleep deprivation, in particular REM sleep phase. Only experiments for the first objective were carried out in this study as a result of the Movement Control Order (MCO) of the Federal Government of Malaysia due to the pandemic of coronavirus disease (COVID-19). Eight (8) male Sprague–Dawley rats were equally divided into four groups; free-moving control rats (FMC), 72-h REM sleep-deprived rats (REMsd), tank control rats (TC) and sleep recovery for 72 hours after 72 hours of REM sleep deprivation rats (SR). Rats were deprived of REM sleep using the inverted flowerpot technique. There wasno significant differences in the levels of food consumption, body weight gain and systolic blood pressure between the groups. The levels of protein expression of endothelial nitric oxide synthase (eNOS) and phosphorylated eNOS (p-eNOS), and the levels of oxidative stress markers in the aorta; total antioxidant capacity (TAC), catalase (CAT), and superoxide dismutase (SOD) were not measured due to time constraints. Based on the assumption that REM sleep deprivation induced endothelial dysfunction, it is expected that there will be a significant decrease in the levels of p-eNOS in the REMsd group compared to other groups. Oxidative stress has been postulated as a possible factor in the mechanism of endothelial dysfunction, thus a significant decrease in the levels of antioxidants is expected to occur in aortic tissue.

In conclusion, it is postulated that REM sleep deprivation is associated with endothelial dysfunction that may be induced by oxidative stress

CHAPTER 1

INTRODUCTION

1.1 Background of study

Sleep is crucial for an individual's mental, emotional and physiological well-being. It is vital for conserving energy, cell functioning and increasing brain protein synthesis (Siran *et al*., 2014). Sleep deprivation has become a globally emerging public health issue (Chattu *et al*., 2019), thus sleep deprivation has attracted the interest of many researchers for many years. Sleep deprivation has also been linked to the development of cardiovascular disease (CVD). CVD is the leading cause of mortality worldwide and the incidence is higher in people who had insufficient sleep (Nagai *et a*l., 2010). Previous studies demonstrated the relationship between sleep deprivation and obesity (Coughlin & Smith, 2014), hypertension (Vgontzas *et al*., 2009), atherosclerosis (May & Harrison, 2013), and diabetes (Xu *et al*., 2016), all of which are potent risk factors for CVD. It is widely accepted that CVD is preceded by endothelial dysfunction (Hadi *et al*., 2005; Jiang *et al*., 2017).

Endothelial dysfunction has been implicated in the pathophysiology of various forms of CVD; including peripheral vascular disease, stroke, heart disease and diabetes (Rajendran *et al*., 2013). Oxidative stress (Di Meo, 2016), down-regulation of endothelial nitric oxide synthase (Suganya *et al*., 2016), decreased nitric oxide bioavailability (Jiang *et al*., 2017), platelet activation (Hadi *et al*., 2005), inflammation and hypofibrinolysis (Kearney *et al*., 2017) are among the factors attributed to the pathogenesis of endothelial dysfunction.

Previous studies have demonstrated that increased reactive oxygen species (ROS) contributes to the pathogenesis of endothelial dysfunction (Laufs *et al*., 2005; Ward &

Croft, 2006; Xu *et al.*, 2020). The relationship between elevated blood pressure and an increased formation of ROS from all layers of the vascular wall has been reported (Schulz *et al.*, 2011). Tengku Adnan *et al.* (2017) demonstrated in an *in vitro* functional study that descending thoracic aorta isolated from REMsd rat had impaired acetylcholine (ACh)-induced endothelium-dependent relaxation compared to the aorta isolated from normal control rat, indicating the presence of endothelial dysfunction in REM sleep deprived rats.

Over the years, researchers have invested much effort to determine the adverse effects of REM sleep deprivation on health. Effects of REM sleep deprivation on behaviour (Hanlon *et al.*, 2005), memory (Wiesner, 2015) and lipid peroxidation (Thamaraiselvi *et al.*, 2012) have been reported previously. Sleep deprivation enhances generation of free radicals (Mahmoudi *et al.*, 2017) and assumed to be removed during sleep (Reimund, 1994). Although the endothelium provides antiproliferative and anti-inflammatory actions and protects against oxidative stress (Giannitsi *et al.*, 2019), the exact mechanism of endothelial dysfunction in REM sleep deprivation is not well clarified.

Increased oxidative stress in the vasculature is not restricted to the endothelium and has also been demonstrated to occur within the smooth muscle cell layer and the adventitia (Schulz *et al.*, 2011). Therefore, the adverse effects of REM sleep deprivation need to be thoroughly explored. It is necessary to determine the mechanism that increased risk of CVD in REM sleep deprivation.

1.2 Rationale of study

Endothelial dysfunction is known to precede CVD. REM sleep deprivation is associated with increased oxidative stress, but its effects on the central nervous system received much attention. It is therefore necessary to determine whether REM sleep deprivation has a deleterious effect on the aorta. Furthermore, increased ROS can be arise from all layers of the vascular wall. This study would also determine whether the changes following REM sleep deprivation are reverting to normal by sleep recovery.

1.3 Scope of study

This study was conducted to evaluate the effects of REM sleep deprivation on the aorta in REM sleep deprived animal model, focusing on the expression of endothelial nitric oxide (eNOS) protein and oxidative stress markers as potential factors for vascular dysfunction. The animal model was used as a substitute for human subjects; the animal model used in this study was young adult male Sprague-Dawley rat aged 8-10 weeks. The REM sleep deprivation model was based on the inverted flowerpot technique. The animal had been sleep deprived for 72 hours, reflecting human suffering from 3 months of REM sleep deprivation (Sengupta, 2013).

1.4 Objectives of study

1.4.1 General objective

To evaluate the effects of REM sleep deprivation on the aorta in sleep-deprived animal model.

1.4.2 Specific objectives

1. To determine the effects of REM sleep deprivation on physical changes; food consumption, body weight gain and systolic blood pressure in experimental groups.
2. To determine the effects of REM sleep deprivation on protein expression of endothelial nitric oxide synthase (eNOS) and phosphorylated-endothelial nitric oxide synthase (p-eNOS) in the aorta of experimental groups.
3. To determine the effects of REM sleep deprivation on the levels of oxidative stress markers in the aortic tissue of experimental groups.

Note:

Three objectives were planned as described above during the presentation of the proposal. However, the objectives could not be achieved due to the Movement Control Order (MCO) of the Federal Government of Malaysia between 18/3/2020 and 9/6/2020 due to the pandemic of coronavirus disease (COVID-19). Since time was limited for laboratory work, only the first objective was carried out and only half of the intended animals were involved. As a result of this condition, the results of objective 1 and the expected results for Objectives 2 and 3 have been included and discussed.

1.5 Hypothesis

1. REM sleep deprivation significantly increases food consumption but reduces body weight gain
2. REM sleep deprivation significantly increases systolic blood pressure
3. REM sleep deprivation significantly reduces levels of phosphorylated-endothelial nitric oxide synthase (p-eNOS) with or without changes in eNOS in the aorta
4. REM sleep deprivation significantly disrupts oxidant/antioxidant balance of the aorta.

1.6 Significance of the study

The findings of this study will contribute to an increasing body of literature on the mechanism underlying the effects of REM sleep deprivation on the aorta. The study will also identify possible factors that may be involved in the process, which will subsequently increase systolic blood pressure following REM sleep deprivation. It may also reveal whether sleep recovery can reverse the changes following REM sleep deprivation. Preventing endothelial dysfunction by knowing the potential mechanism will be a significant step towards improving the quality of life and health of a significant number of Malaysians.

CHAPTER 2

LITERATURE REVIEW

2.1 Physiology of sleep

Sleep is undoubtedly one of the most essential requirements for the health and well-being. During sleep, human body will undergo repair mechanisms for damaged tissues and organs. It is therefore sleep deficiency will increase the risk of heart and kidney diseases, high blood pressure, diabetes, and stroke (Reutrakul & Van Cauter, 2018). The quantity and quality of sleep contributes greatly to physical and mental well-being and performance for the next day and thus sleep has a huge impact on overall quality of life (Roth & Roehrs, 2003).

Sleep consists of four different stages; rapid-eye-movement (REM) and three stages of non-rapid eye movement (NREM) sleep (Patel *et al*., 2020). A healthy young adult usually cycles through these stages on average 4 to 6 times, averaging 90 minutes in each stage. The duration of REM sleep episodes increases as the sleep progress and it occupies about 25% of the total sleep time (Roehrs & Roth, 2019). Sleep is therefore broadly categorised as two types, NREM and REM sleep. The clinical evaluation of sleep is performed through a polysomnogram, which consists of electroencephalography (EEG), electrooculogram (EOG), electrocardiography (ECG) and the electromyogram (EMG) that measures brain waves, eye movements, electrical activity of the heart and muscle movement respectively (Patel *et al*., 2020). During sleep, the brain switches periodically between these two states (Saper *et al.*, 2010), which are distinguished by specific types of brain activity (Buzsáki *et al*., 2013).

There are three defined sub-states of NREM including N1 (Stage 1), N2 (Stage 2), and N3 (Stage 3) that consists about 75-80% of total time spent in sleep (Kishi *et*

al., 2011). N1 stage is a transition role in sleep-stage cycling and at this stage the average individual's sleep episode begins (Carskadon & Dement, 2005). During this short period, the heartbeat, breathing and eye movements slow while muscles relax with occasional twitches. In addition, the breathing rate is regular and tends to last about 1 to 5 minutes, consisting of around 5% of the total cycle (Patel *et al.*, 2020). In N2 sleep, it requires more intense stimuli than stage 1 to awaken. N2 stage is a period of light sleep before entering deeper sleep that lasts about ten to twenty-five minutes where the heartbeat, breathing and brain waves activity slow.

Majority of sleep is spent in N2 stage and consists about 50% of total sleep (Malik *et* al., 2018). N3 is the period of deep sleep that takes about twenty to forty minutes. During this stage, the heart rate, respiratory rate, blood pressure and body temperature are at their lowest levels. The brain waves become even slower, muscles are relaxed, and is the most difficult to awaken from (Carskadon & Dement, 2005). American Academy of Sleep Medicine (AASM) combined Stages 3 and 4 into one stage, referred as N3 as they have similar EEG waves, slow-wave sleep (Keenan & Hirshkowitz, 2011).

REM sleep, which is also known as 'paradoxical sleep' or 'active sleep' is characterised by wake-like EEG patterns (Mishra & Colgin, 2019) and low-amplitude oscillatory brain activity (Rasch & Born, 2013). According to National Institute of Neurological Disorders & Stroke (2017), in REM sleep, the eyes move rapidly from side to side behind closed eyelids, breathing becomes faster and irregular, and the heart rate and blood pressure increase to near waking levels. During REM sleep period, the brain is as active as in waking (Chow *et al.*, 2013). Bergel *et al.* (2018) recently shows that REM sleep is associated with increased blood flow to numerous brain areas which provides new insights into REM study. Besides, the muscles become temporarily

paralysed, which prevents a person from acting out during dreams (McCarter *et al.*, 2012).

Figure 2.1 shows the progression of sleep stages across a single night in young adult. A healthy adult usually has four or five basic repetitive sleep cycles throughout the night (Huang *et al.*, 2020). NREM is predominant during the early part and decreases in intensity and duration across the sleep period, whereas REM sleep becomes more deeper and longer towards the end of the sleep period (Rasch & Born, 2013).

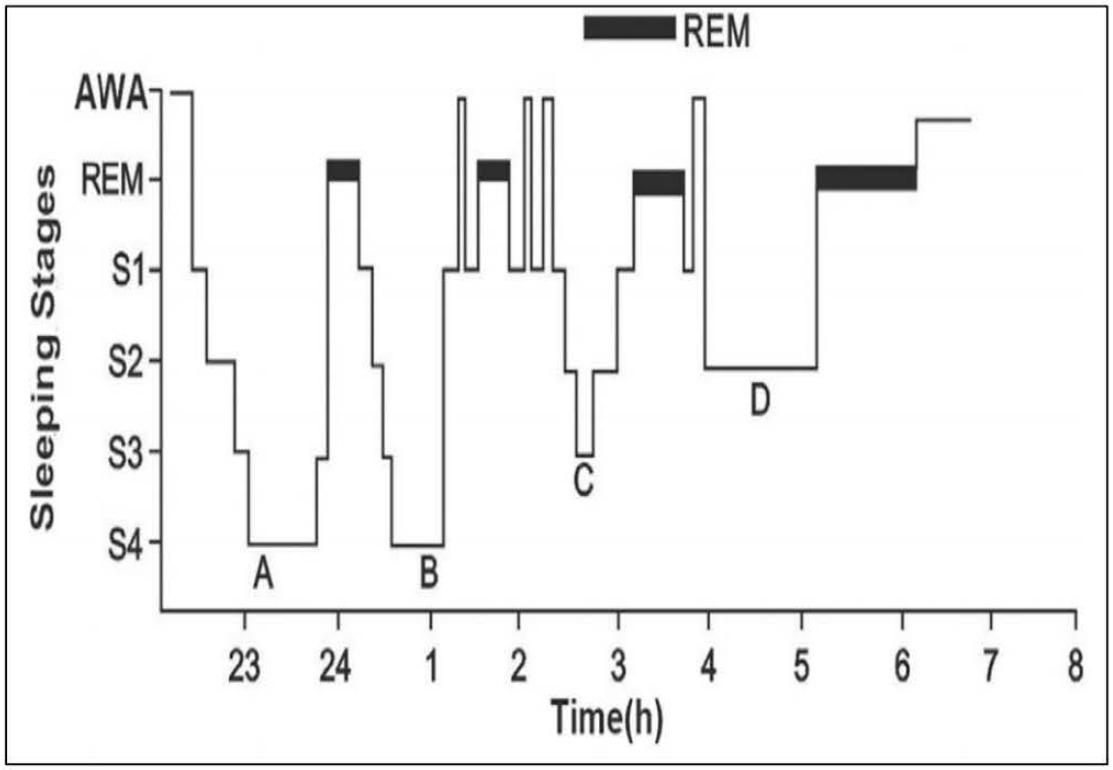

Figure 2.1: A hypnogram of sleep cycles that consists of NREM and REM stages across an 8-h sleep period. NREM sleep consists of three stages, known as S1 (Stage 1) or N1, S2 (Stage 2) or N2 and a combination of S3 (Stage 3) and S4 (Stage 4) or N3 (Huang *et al.*, 2020).

Note:

Abbreviations: REM; rapid eye movement, AWA; awake

2.2 Pathophysiology of sleep deprivation

Sleep consumes approximately one third of a person's life (Elliott *et al.*, 2014). On average, most adults need about seven to eight hours of sleep each night to feel alert and well rested. Sleep deprivation can result from poor quality of sleep and/or reduced sleep duration. International Classification of Sleep Disorders (ICSD-3) has defined sleep deprivation as a curtailed sleep pattern that has persisted for at least three months for most days of the week, along with complaints of sleepiness during the days (Chattu *et al.*, 2019). Numerous studies reported that sleep deprivation is an important risk factor in obesity, type 2 Diabetes and CVD (Miller *et al.*, 2013). Sleep deprivation is associated with various acute and chronic diseases and results in increased mortality and morbidity for CVD (Liu & Chen, 2019). Sleep deprivation is associated with increased cardiovascular morbidity and mortality in epidemiologic and case-control studies (Mullington *et al.*, 2009), including myocardial infarction and heart failure (Janszky *et al.*, 2012).

Figures 2.2 describes the association between sleep deprivation and CVD. Briefly, sleep deprivation increased reactive oxygen species (ROS) and decreased nitric oxide (NO) that leads to endothelial dysfunction. Metabolic disorder may arise from sleep deprivation due to disruption in glucose and lipid metabolism. Autonomic nervous system is also activated following sleep deprivation as it stimulates sympathetic activity and decreases parasympathetic activity. Sleep deprivation also results in immune cell subpopulation dysfunction and leads to immunity impairment.

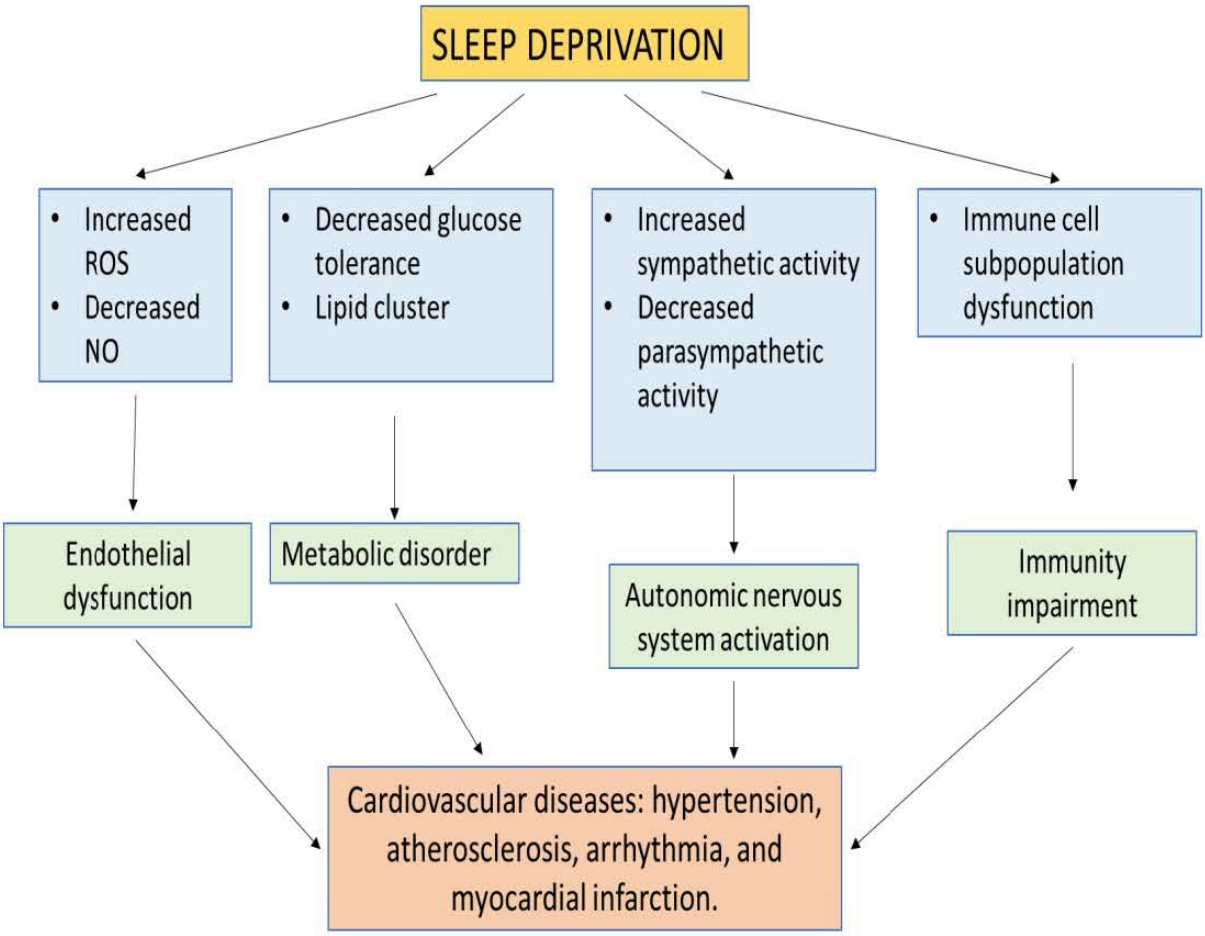

Figure 2.2 Effects of sleep deprivation on cardiovascular diseases. Adapted from Liu & Chen (2019).

The deleterious effects of sleep deprivation will ultimately cause CVDs including hypertension, atherosclerosis, arrhythmia, and myocardial infarction. Previous studies reported that both short (less than 7 hours) and long (more than 8 hours) sleep are associated with increased risk of CVD and stroke (Buxton & Marcelli, 2010). Disruption of sleep is known to interfere with learning and memory, among others, through oxidative stress (Silva *et al.*, 2004). The production of excessive ROS due to uncontrolled metabolic processes under stressful conditions can lead to a state, where maintenance and recovery to normal physiological levels become difficult (Pandey & Kar, 2018). This can ultimately lead to cell death and tissue disintegration.

REM sleep deprivation causes a state of high caloric ingestion due to hyperphagia without weight gaining (Siran *et al.*, 2014; Tengku Adnan et al., 2017). In addition, REM sleep deprivation increased metabolic rate (Koban & Swinson, 2005) and increased plasma catecholamines (Rechtschaffen & Bergmann, 1995). Metabolic disorders caused by sleep deprivation may contribute to metabolism syndrome and increase risk of CVD whereby short sleep duration i.e. 7 hours or less, is linked to hypercholesterolemia (Gangwisch *et al.*, 2010).

The majority of REM sleep deprivation research has focused on its effects on memory and related processes (Walker *et al.*, 2002). A previous study using *in vivo* imaging and optogenetics demonstrated that the activity of dentate gyrus adult-born neurons during REM sleep is essential for contextual fear memory consolidation (Kumar *et al.*, 2020). Using the same techniques, Boyce *et al.* (2016) found that neural activity occurring specifically during REM sleep is critical for normal memory consolidation. Prolonged loss of REM sleep can alter blood-brain barrier functions and can be fatal (Baumann *et al.*, 2005). Reports also suggested that REM sleep

deprivation can cause apoptosis of neuronal cells (Somarajan *et al*., 2016) and muscle, and cardiac injuries (Mejri *et al.*, 2017).

2.3 Structure and functions of blood vessel

Blood vessels are the conduits or channels through which blood is distributed to body tissues. Blood vessels play a key role that include transporting nutrients, oxygen, metabolic wastes and maintenance of homeostasis throughout the body (Ng *et al*., 2018). Figure 2.3 shows diagram of a blood vessel that comprised of three layers. The innermost layer is the tunica intima, followed by the middle layer, tunica media and the outermost layer, tunica externa that was formerly known as tunica adventitia (Ng *et al.*, 2018). Tunica externa is composed of connective tissue that contains elastin, collagen, fibroblasts, and occasionally smooth muscle cells (Lusis, 2010). Tunica media is predominantly comprising of layers of smooth muscle cells arranged circumferentially around the vessel with type I and III collagen and the elastic tissue on the external side (Berillis, 2013). Meanwhile, there are three major components in the tunica intima; the endothelium that is formed by a single layer of simple squamous epithelium, the basal lamina that consists of mainly type IV collagen and laminin, and the sub endothelial layer that consists mainly of loose connective tissue.

Blood vessels are classified based on their structure and function as either arteries, capillaries or veins. Compared to veins, the arteries have a thicker tunica media and a thinner tunica adventitia. There are three types of arteries that are divided according to the size and characteristics of their tunica media; the large (or elastic arteries), the medium (or muscular arteries) and small arteries and arterioles (Ng *et al*., 2018). Aorta, the structure used in this study is an example of a large artery.

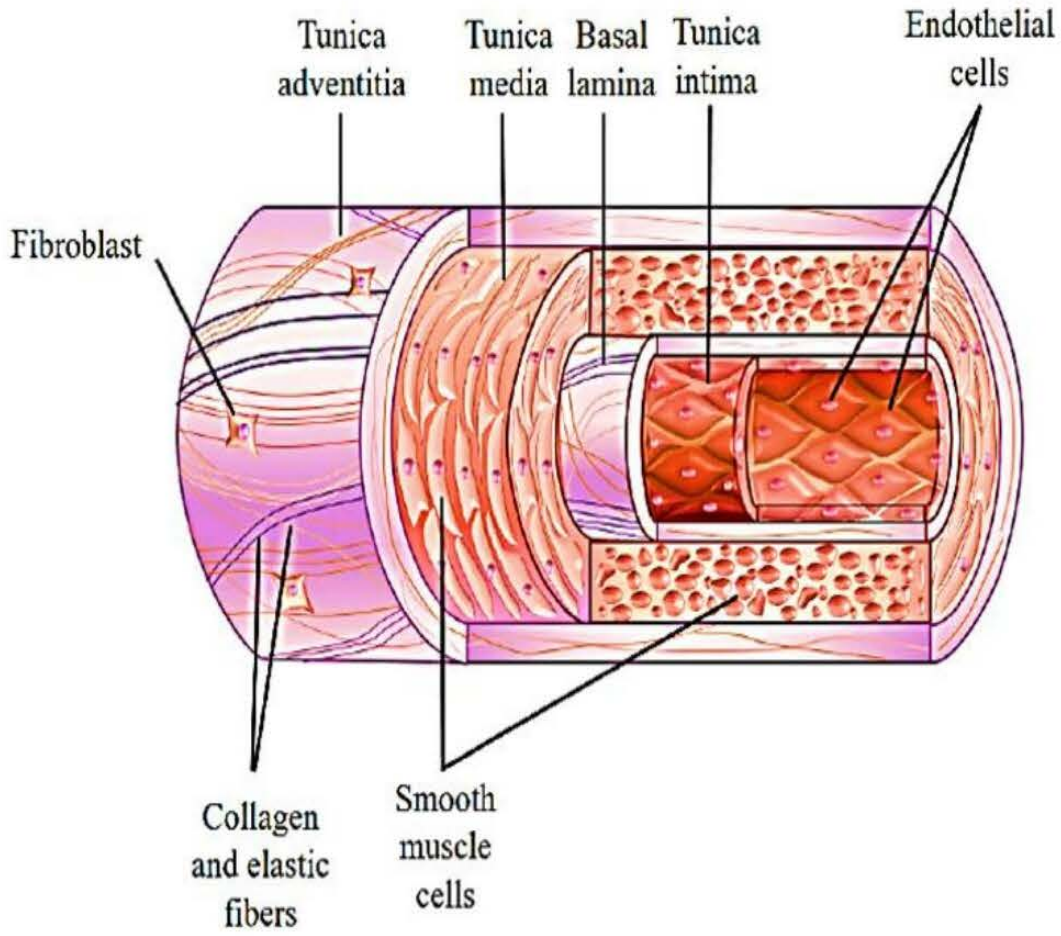

Figure 2.3 Structure of a blood vessel. It consists of three layers: tunica intima, tunica media and tunica adventitia (externa). Tunica adventitia contains fibroblast cells, collagen and elastic fibers (Ng *et al*., 2018).

Endothelium, consisting of endothelial cells, is a single layer of cells that provides the inner layer of blood vessels and the lymphatic system. Endothelium forms a semi-permeable barrier that separates the blood stream from the underlying organs and tissues. It also controls the transport of fluids, solutes and cells across the walls of the blood vessel (Cerutti & Ridley, 2017). In human, endothelium consists of approximately 10 trillion cells, contributing about 1.5% of the total body mass (Galley & Webster, 2004). Endothelium plays a major role in the maintenance of the vascular homeostasis in the ever-changing pathophysiological environment of the circulatory system (Lusis, 2010). The major functions of endothelium are maintaining vascular tone, cell adhesiveness, platelet aggregation, leucocyte trafficking, coagulation cascade, inflammation, permeability, regulation of thrombosis and fibrinolysis (Aird, 2007).

In the regulation of the vascular tone, the endothelium secretes vasodilator and vasoconstrictor mediators which are in balance under normal conditions (Lackovic *et al.,* 2011). Endothelium-Derived Relaxing Factor (EDRF) which includes nitric oxide (NO) is one of the most important vasodilator substances that plays an important role in many vasoprotective effects ranging from vasodilatation, anti-aggregatory and anti-inflammatory actions to inhibition of lipid oxidation and vascular smooth muscle proliferation (Moncada & Higgs, 2006). Endothelin-1 (ET-1), angiotensin II (Ang II), thromboxane A2 (TXA2), and platelet-activating factor (PAF) (Park & Park, 2015) are the examples of vasoconstrictor mediators. Thus, increased endothelial permeability, platelet aggregation, leucocyte adhesion and generation of cytokines may occur when there is damage to the endothelium that creates an imbalance between vasodilation and vasoconstriction mediators (Suganya *et al*., 2016). Endothelium also

forms a tight non-thrombogenic barrier that serves a protective role in preventing infections and inflammation of surrounding tissues (Ng *et al*., 2018).

2.4 Mechanism of endothelial dysfunction

It is widely accepted that endothelial dysfunction is an early event of CVD (Jiang *et al*., 2017). Endothelium is the primary target for many diseases, including diabetes mellitus, hypercholesterolaemia and chronic inflammation. These diseases promote the disruption of the endothelial protective barrier, which has increased adhesion of the endothelium to leukocytes, altered permeability of the endothelium, and increased vascular smooth muscle proliferation (Bauer & Sotnikova, 2010). Endothelial dysfunction is a pathological condition characterised by either a reduced production of NO or a predominant vascular generation of vasoconstricting and proatherosclerotic substances (Virdis *et al*., 2010). Vascular homeostasis is not maintained in endothelial dysfunction due to a shift in the normal endothelial functions, including reduced vasodilation, proinflammatory and prothrombic state (Suganya *et al*., 2016). The inability of arteries and arterioles to dilate optimally in response to a vasodilator such as acetylcholine (ACh) is an important feature of endothelial dysfunction.

Endothelial dysfunction is a major factor in the initiation of vascular complications. Numerous CVDs, including atherosclerosis, diabetes, hypertension, obesity, dyslipidaemia and smoking, have been attributed to endothelial dysfunction (Hadi *et al.,* 2005; Park & Park, 2015). Some evidence indicates that REM sleep deprivation is associated with endothelial dysfunction (Jiang *et al*., 2017; Tengku Adnan *et al*., 2017). Endothelial dysfunction has prognostic significance and serves as an early indicator of the development of various vascular diseases, including hypertension (Mendizábal *et al*., 2011). It increases the development and progression

of vascular diseases especially in diabetic and obese patients (Muniyappa and Sowers, 2013). There are two main factors involved in the pathogenesis of endothelial dysfunction i.e. nitric oxide and oxidative stress.

2.4.1 Role of nitric oxide

Endothelial dysfunction is associated with decreased NO availability, either through loss of NO production or loss of NO biological activity (Endemann & Schiffrin, 2004; Simsek *et al.*, 2010). NO is the most important compound generated by endothelial cells that influence vascular homeostasis. NO, an important gaseous free radical, is an endothelium-dependent vasodilator that is continuously produced in the tissue by endothelial nitric oxide synthase (eNOS), a key enzyme that generates NO (Virdis *et al*., 2010). Activated eNOS converts L-arginine and O_2 to L-citrulline and NO in the presence of co-factors such as tetrahydrobiopterin (BH4), flavin-mononucleotide, nicotinamide adenine dinucleotide phosphate (NADPH) and flavin adenine dinucleotide (FAD) (Wang *et al*., 2017).

Vascular homeostasis and cardiac functions are maintained by NO through its protective properties such as anti-platelet, anti-proliferative, anti-migratory, antioxidant and anti-inflammatory action in vessels (Taneja *et al*., 2019). eNOS expression is localised to endothelium and that smooth muscle cells are passively regulated by NO released, a major endothelium-derived relaxing factor (Michel & Vanhoutte, 2010). This molecule also diffuses to the vascular smooth muscle cells, activates soluble guanylyl cyclase and increases cyclic guanosine monophosphate (cGMP)-mediated vasodilation, which results in muscle relaxation by decreasing intracellular ionised calcium (Förstermann and Münzel, 2006).

Decreased endothelial NO production or availability has been implicated in the pathogenesis of a variety of vascular disorders including atherosclerosis. In atherosclerosis, the expression of leukocyte adhesion molecules (such as VCAM-1) and cytokines such as monocyte chemoattractant protein-1 become elevated, causing the endothelial phenotype shifted to a proinflammatory and prothrombotic state (Landmesser *et al.*, 2004). Previous study had suggested that one of the factors that contribute to endothelial phenotypical changes is caused by the depletion of endothelium-derived NO (Rajendran *et al.*, 2013). Figure 2.4 shows the production and functions of NO. NO provide protection against the onset of atherogenesis by inhibiting the adhesion of leukocyte to the vessel wall. In addition, NO is also involved in inhibiting platelet aggregation and adhesion, both of which protect smooth muscle cells from exposure to platelet-derived growth factors. These mechanisms may lead to the formation of fibrous plaque; therefore, NO also prevents a later step of atherogenesis. NO suppresses key processes in vascular lesion and represents the most important anti-atherogenic defense principle in the vasculature.

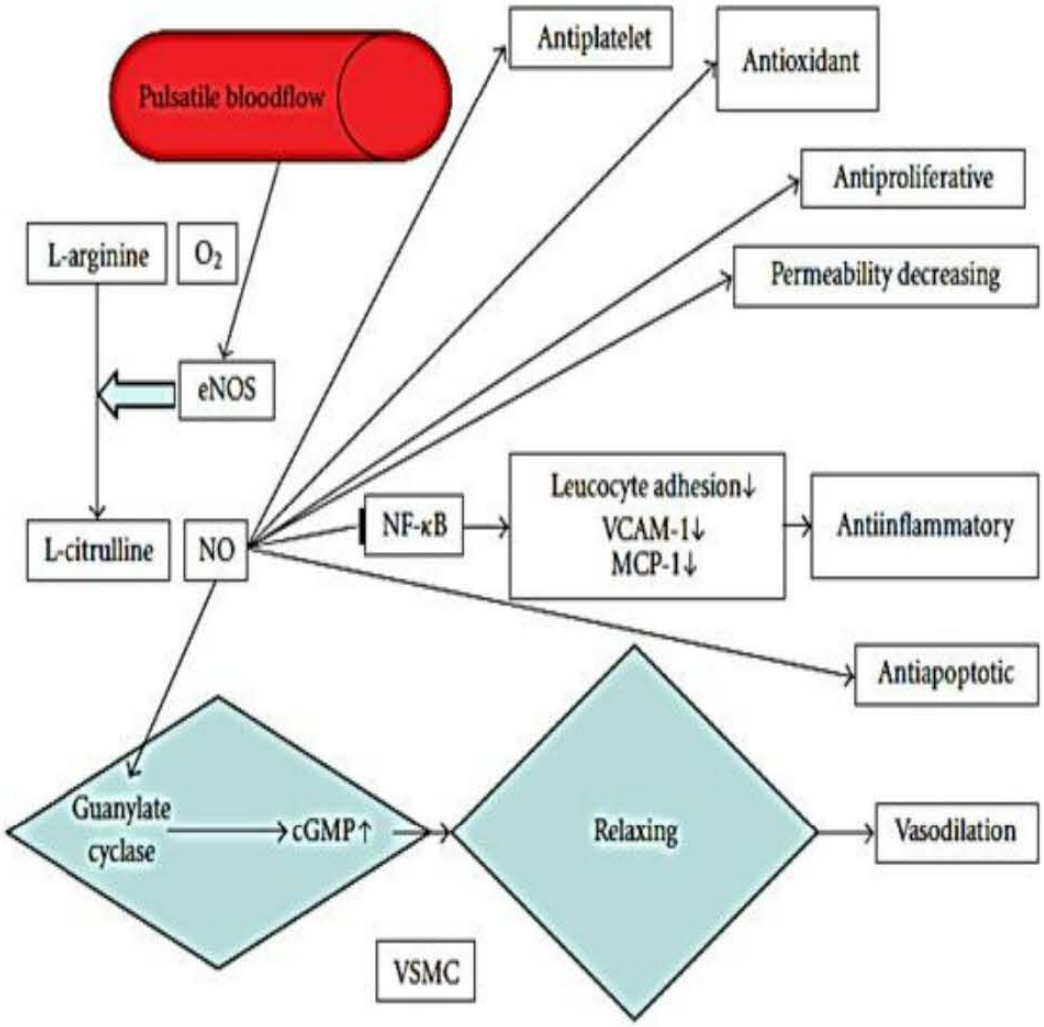

Figure 2.4 Production of nitric oxide from L-arginine by endothelial cells (Simsek *et al.*, 2010). Activated eNOS converts L-arginine and O_2 to L-citrulline and nitric oxide. The functions of nitric oxide include anti-platelet, antioxidants, anti-inflammatory and act as a vasodilator.

Note:

Abbreviations: NO; nitric oxide, O_2; oxygen, NF-kB; Nuclear factor kappa B, cGMP; Cyclic guanosine monophosphate, VCAM-1; vascular cell adhesion molecule 1, MCP-1; Monocyte chemoattractant protein-1

2.4.2 Role of oxidative stress

Oxidative stress has been implicated in the pathogenesis of cancer, atherosclerosis, diabetes, and neurodegenerative disorders (Droge, 2002). Oxidative stress is defined as a condition in which highly reactive molecules or free radicals; ROS or reactive nitrogen species (RNS) overwhelm the production of antioxidants (Villafuerte *et al.*, 2015). ROS constitute of both oxygen free radicals, such as superoxide ($O_2^{\cdot-}$), hydroxyl ($OH^{\cdot}$), hydroperoxyl ($HO_2^{\cdot}$) and carbonate ($CO_3^{\cdot}$) as well as non-radicals, such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and Ozone (O_3) (Liochev, 2013). Meanwhile, RNS free radicals include peroxynitrite ($ONOO^-$), S-nitrosoglutathione and S-nitrosothiols. In the presence of high concentrations of ROS and/or RNS, they can cause toxic effects that are associated with various pathologies including atherosclerosis, diabetes, carcinogenesis, neurodegeneration (Di Meo, 2016) or even cell death (Lum & Roebuck, 2001). Damage to nucleic acids, lipids and protein may occur due to high concentration of ROS (Valko *et al.*, 2007).

It is hypothesised that free radicals or ROS produced during wakefulness are removed during sleep that suggested sleep has an anti-oxidative function (Reimund, 1994). Maintaining a steady state concentration of free radicals is essential for adequate functioning of aerobic organism. REM sleep deprivation alters membrane bound ATPases, membrane fluidity, calcium ion concentration and gene expression. These alterations are potentially capable of inducing changes in cellular physiology including the generation of free radicals (Mathangi *et al.*, 2012). Inhibition of NADPH oxidase has been established as a key molecular mechanism leading to reduced arterial oxidative stress and normalisation of endothelial dysfunction in mice (Durrant *et al.*, 2009). Although most ROS are byproducts of the electron transport chain, oxidants can also be produced by extra mitochondrial sources such as NADPH oxidases and

nitric-oxide synthases (NOS) (Murrant and Reid, 2001). Mitochondria respiration in vascular cells may release ROS, including $O_2^{\cdot-}$ and H_2O_2 that have short and longer lifespan respectively (Puac *et al.*, 2014).

Increased superoxide dismutase (SOD) activity was demonstrated in rats subjected to total sleep loss (3-14 days) compared to control groups (Gopalakrishnan *et al.*, 2004). ROS production has been demonstrated in the endothelial cell layer and also within the tunica media and adventitia, all of which may impair NO signalling within the vascular tissues (Sorescu *et al.*, 2002). Previous study has shown that ROS are generated at the sites of inflammation and injury with the majority are released from the activated blood leukocytes that adhere to the endothelial cell surface (Lum & Roebuck, 2001). It is proven that under physiological and pathological conditions, high levels of ROS causes redox imbalance and induce cell apoptosis (Zhang *et al.*, 2013). Li *et al.* (2016) suggests that the regulation of MAPK signalling pathway had contributed to ROS-induced apoptosis.

Several oxidative enzyme systems such as NADPH oxidase, xanthine oxidase, cyclooxygenases, lipoxygenases, myeloperoxidases, cytochrome P450 monooxygenase, uncoupled NOS, and peroxidases lead to the inactivation of NO. These enzyme systems represent critical mechanism that lead to endothelial dysfunction through an elevated level of superoxide anion ($O2^{\bullet-}$) (Ghosh *et al.*, 2017). ROS may uncouple the endothelial NOS (eNOS) catalysed reduction of molecular oxygen from the oxidation of L-arginine, resulting in the paradoxical production of the superoxide anion instead of the reducing NO. Alternatively, ROS may react with NO directly, reducing its bioavailability (Beckman & Koppenol, 2018).

2.4.3 Role of other factors

Insulin resistance may occur in hyperglycaemia, hypertension and dyslipidemia. This condition has been associated with endothelial dysfunction as insulin stimulates NO release via a signalling cascade involving activation of Phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) (PI3K-Akt) signalling pathway and phosphorylation of eNOS (Xu & Zou, 2009). In addition, the production of NO also depends on the activation of the insulin receptor substrate-1 (IRS-1) (Deqiu *et al.*, 2011). Increased plasma concentration of thrombomodulin (TM) is a marker for endothelial dysfunction in patients with Type 2 diabetes mellitus (Kubisz *et al.*, 2015).

The expression of inflammatory markers including macrophage chemoattractant peptide-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), C-reactive protein (CRP) and intercellular cell adhesion molecule-1 (ICAM-1) in the vessel wall may also be upregulated by ROS in endothelial dysfunction (Griendling & Fitzgerald, 2003). In the presence of prolonged inflammation, endothelial damage may occur that causes an increase in leucocytes accumulation at the damaged site. Inflammatory mediators such as tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) and chemokines will prevent leucocytes from adhering to the endothelium and reduce the expression of adhesion molecules (Brandes, 2014). Van Leeuwen *et al.* (2009) reported that acute sleep deprivation increased immune cells such as monocytes, neutrophils, and phagocytic cells in peripheral circulation (Mullington *et al.*, 2009). Endothelial dysfunction may also caused disruption of coagulation factors, including plasminogen activator inhibitor (PAI-1) and von Willebrand factor (vWF) that may lead to atherosclerosis (Wei *et al.,* 2013).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and reagents

The chemicals and reagents used in this study are shown in Table 3.1

Table 3.1 List of chemicals and reagents.

Chemicals and Reagents	Brands
Animal experiment	
Rat pellet	Atromin, Germany
Sodium pentobarbitone (DORMINAL 20%)	Alfasan Woerden-Holland
Normal Saline solution	Merck, USA

3.1.2 Equipment

The laboratory equipment used in this study are shown in Table 3.2

Table 3.2 List of laboratory equipment.

Laboratory equipment	Brands
Animal experiment	
Polypropylene cage	Supplied by ARASC, USM
Tank	Bought from local market
Platform (glasses and clay vases)	Bought from local market
Weighing scale for animals	Precisa Instrument AG, Switzerland
Syringe (1 mL)	Terumo, Tokyo
Needle (26' gauge)	Terumo, Tokyo
Dissecting set	World Precision Instruments, Inc.
MRBP Single Animal Tail Cuff Blood Pressure System	IITC Life Science, California

3.2 Animal and housing

Eight (8) Sprague-Dawley (SD) male rats weighing between 180-250 g and 8-10 weeks of age were used in this study. Rats were purchased from Animal Research and Service Centre (ARASC), Universiti Sains Malaysia. Animals were kept in standard polypropylene cages individually and maintained in standard laboratory conditions at 23±1 °C, 60–70% humidity with 12-h of light and dark cycle. They were maintained on standard food pellets and tap water *ad libitum*. Rats were allowed to acclimate to the experimental environment for at least three days prior to the start of the study. This study was approved by the Animal Ethics Committee USM (USM/IACUC/2018/(941)(114)) and in accordance with the institutional guidelines.