EFFECTS OF VITAMIN C ON OXIDATIVE STRESS INDUCED ENDOTHELIAL DYSFUNCTION IN RAPID EYE MOVEMENT (REM) SLEEP DEPRIVATION RAT MODEL

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by

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LIST OF SYMBOLS, ABBREVIATIONS

%	percentage
^{o}C	degree Celcius
AASM	American Academy of Sleep Medicine
ACh	acetylcholine
ADP	adenosine di-phosphate
Ang	angiotensin
ARASC	Animal Research and Service Centre
ATP	adenosine tri-phosphate
BCA	bicinchoninic acid
bFGF	basic fibroblast growth factor
BH4	tetrahydrobiopterin
BK	bradykinin
BW	body weight
BWg	body weight gain
Ca^{2+}	calcium ion
CaM	calmodulin
CAT	catalase
cm	centimeter
cGMP	cyclic guanosine monophosphate
CRP	C-reactive protein
CVD	cardiovascular disease
CVS	cardiovascular system
EDCF	endothelium-derived contractile factor

EDHF	endothelium-derived hyperpolarisation factor
EDRF	endothelium derived relaxing factors
EEG	electroencephalography
ELISA	enzyme-linked immunosorbent assay
EMG	electromyography
eNOS	endothelial nitric oxide synthase
EOG	electro-oculography
ET-1	endothelin-1
Fc	food consumption
FMC	free moving control
fMRI	functional magnetic resonance imaging
FVC	free moving control pretreated with vitamin C
GR	glutathione reductase
GSH	glutathione
GPx/ GSH-Px	glutathione peroxidase
GSSG	oxidised glutathione
GST	glutathione transferase
GTP	guanosine-triphosphate
H&E	Haematoxylin & Eosin
H_2O_2	hydrogen peroxide
HDL	high density lipoprotein
HO	heme-oxygenase
$HO_2^{\cdot}$	hydroperoxyl
HOCl	hypochlorous acid
ICAM-1	intercellular cell adhesion molecule-1

IGF	insulin-like growth factor
IL	interleukin
IP	intraperitoneal
KHS	Kreb's Henseleit solution
LAM-1	leucocyte adhesion molecule-1
LDL	low density lipoprotein
MAPK	mitogen-activated protein kinase
MDA	malondialdehyde
MCP-1	monocyte chemoattractant protein-1
NAC	N-acetylcysteine
NADPH	nicotinamide adenine dinucleotide phosphate
NO/ NO·	nitric oxide
NREM	non-rapid eye movement
$O_2^{\cdot -}$	superoxide anion
O_3	ozone
OD	optical density
$OH^{\cdot}$	hydroxyl
ONOO-	peroxynitrite
PAF	platelet-activating factor
PAI-1	plasminogen activator inhibitor-1
PDGF	platelet-derived growth factor
PE	phenylephrine
PECAM-1	platelet cell adhesion molecule-1
PI	plasmin inhibitor
PSG	polysomnogram

PVAT	perivascular adipose tissue
PVDF	polyvinylidene difluoride
REM	rapid eye movement
REMsd	rapid eye movement sleep deprivation
RNS	reactive nitrogen species
ROS	reactive oxygen species
RVC	REM sleep deprivation pretreated with vitamin C
SD	Sprague–Dawley
S.E.M	scanning electron microscope
sGC	soluble guanylyl cyclase
SOD	superoxide dismutase
SWS	slow-wave sleep
TAC	total antioxidant capacity
TC	tank control
TF	tissue factor
TG	triglyceride
TGF-β	transforming growth factor-β
TM	thrombomodulin
TNF-α	tumor necrosis factor-α
TXA_2	thromboxane A_2
VEGF	vascular endothelial growth factor
vWF	von Willebrand factor
VSMC	vascular smooth muscle cells
VCAM-1	vascular cell adhesion molecule-1
WHO	World Health Organisation

KESAN VITAMIN C KE ATAS DISFUNGSI ENDOTELIUM ARUHAN STRES OKSIDATIF DALAM MODEL TIKUS KEKURANGAN TIDUR REM

ABSTRAK

Kurang tidur telah dikenalpasti sebagai faktor risiko untuk penyakit kardiovaskular. Disfungsi endotelium merupakan tanda awal penyakit kardiovaskular. Sehingga kini, patogenesis disfungsi endotelium akibat kurang tidur masih kurang difahami. Objektif kajian ini adalah untuk menilai hubungan antara masalah kurang tidur terutamanya semasa fasa REM, dan disfungsi endotelium. Kesan vitamin C terhadap kesan buruk akibat kekurangan tidur REM turut dinilai. Empat puluh (40) ekor tikus Sprague-Dawley jantan dibahagikan sama rata kepada 5 kumpulan: kumpulan kawalan bebas-gerak (FMC), kumpulan kurang tidur REM 72 jam (REMsd), REMsd yang dirawat-awal vitamin C (RVC), FMC yang dirawat-awal vitamin C (FVC) dan kumpulan kawalan tangki (TC). Tidur REM dikurangkan dengan menggunakan teknik pasu terbalik. Vitamin C (100 mg/kg) diberikan secara oral selama 4 minggu sebelum tempoh adaptasi. Terdapat penurunan dalam pertambahan berat badan secara signifikan meskipun dalam masa yang sama pengambilan makanan meningkat secara signifikan dalam kumpulan REMsd berbanding kumpulan FMC. Dalam kajian fungsi endotelium *in vitro,* kumpulan REMsd menunjukkan respon vasodilatasi endotelium-dependen terhadap asetilkolina paling rendah berbanding kumpulan lain. Ekspresi protein eNOS yang menggunakan 'Western blot' menurun secara signifikan dalam kumpulan REMsd berbanding kumpulan FMC. Aktiviti glutation reduktase (GR) dan superoksid dismutase (SOD), dan kapasiti antioksidan total (TAC) dalam kumpulan REMsd menurun secara

signifikan berbanding kumpulan FMC. Paras malondialdehid dalam plasma adalah tidak signifikan antara semua kumpulan. Terdapat peningkatan secara signifikan dalam paras plasma fibrinogen dan perencat plasminogen aktivator-1 (PAI-1), manakala penurunan secara signifikan dalam paras tisu aktivator plasminogen (tPA) dalam kumpulan REMsd berbanding kumpulan FMC, sebagai indikasi pengaktifan proses lata koagulasi dalam kumpulan REMsd. Morfologi endotelium adalah normal dalam semua kumpulan dinilai menggunakan teknik pewarnaan hematoxylin dan eosin. Walaubagaimanapun, dalam imbasan mikroskop electron, hanya endotelium daripada kumpulan REMsd menunjukkan ciri endotelium rosak. Vitamin C mengurangkan kesan buruk kekurangan tidur REM dengan mengekalkan fungsi endothelium, memulihkan ekspresi eNOS, meningkatkan aktiviti SOD dan melindungi endotelium daripada rosak. Vitamin C juga membantu dalam menghalang penurunan aktiviti GR dan TAC, dan juga perubahan kepada faktor koagulasi akibat kekurangan tidur REM. Sebagai kesimpulan, penemuan di atas memberi bukti kukuh bahawa disfungsi endotelium terjadi dalam kekurangan tidur REM. Suplimentasi dengan vitamin C memberi kesan baik untuk menghalang disfungsi endotelium aruhan stres oksidatif dalam keadaan kekurangan tidur REM.

EFFECTS OF VITAMIN C ON OXIDATIVE STRESS INDUCED ENDOTHELIAL DYSFUNCTION IN RAPID EYE MOVEMENT (REM) SLEEP DEPRIVATION RAT MODEL

ABSTRACT

Sleep deprivation has been identified as a risk factor for cardiovascular disease. Endothelial dysfunction is an early sign of cardiovascular disease. To date, the pathogenesis of endothelial dysfunction in sleep deprivation remains poorly understood. The objectives of this study were to assess the relationship between sleep deprivation in particular REM sleep phase, and endothelial dysfunction. The effects of vitamin C on the adverse effects of REM sleep deprivation were also evaluated. Forty (40) male Sprague–Dawley (SD) rats were equally divided into 5 groups: free-moving control rats (FMC), 72-h REM sleep-deprived rats (REMsd), REMsd pretreated with vitamin C (RVC), FMC pretreated with vitamin C (FVC) and tank control rats (TC). Rats were deprived of REM sleep using the inverted flowerpot technique. Vitamin C (100 mg/kg) was administered orally for 4 weeks before the adaptation period. There was a significant reduction of body weight gain despite a significant increase in food consumption in REMsd compared to FMC group. In *in vitro* functional study, REMsd group showed the lowest endothelium-dependent vasodilator responses to acetylcholine (ACh) compared to other groups. eNOS expression determined by Western blot was significantly lower in REMsd compared to FMC group. Glutathione reductase (GR) and superoxide dismutase (SOD) activities, and total antioxidant capacity (TAC) were significantly lower in REMsd compared to FMC group. The plasma levels of malondialdehyde (MDA) were not significantly different between the groups. A significant increase in plasma levels of

fibrinogen and plasminogen activator inhibitor-1 (PAI-1), and decreased tissue plasminogen activator (tPA) level were observed in REMsd compared to FMC, which indicate an activation of coagulation cascade in REMsd group. The endothelium morphology is normal in all groups when assessed by hematoxylin and eosin staining. However, in scanning electron microscope, the endothelium of REMsd rat only showed features of endothelial damage. Vitamin C reduced the adverse effects of REM sleep deprivation by preserving the endothelial function, restoring the eNOS expression, increasing the SOD activity and protecting the endothelium from damage. Vitamin C also helps in preventing the reduction of GR activity and TAC, and changes to the coagulation factors during REM sleep deprivation. In conclusion, the above findings provide convincing evidence for the development of endothelial dysfunction in REM sleep deprivation. Supplementation of vitamin C has beneficial effects against oxidative stress induced endothelial dysfunction in REM sleep deprivation.

CHAPTER 1

INTRODUCTION

1.1 Background

Sleep comprises of two phases; non-rapid eye movement (non-REM) and rapid eye movement (REM) sleep. Good quality of sleep is crucial for human health and well-being regardless of age and gender. Sleep is vital for conserving energy, cell functioning and increasing brain protein synthesis (Siran *et al.*, 2014). Sleep deprivation has become an emerging public health issue globally (Tufik *et al.*, 2009). Unquestionably, sleep deprivation has attracted the interest of many researchers for many years. It is a stressor that affects physical and biochemical changes.

There is growing evidence that sleep deprivation is associated with cardiovascular related diseases such as hypertension (Vgontzas *et al*., 2009), atherosclerosis (Miller, 2011) and diabetes (Xu *et al*., 2016). Cardiovascular disease (CVD) caused 31% of deaths globally, which is approximately 17.5 million deaths per year (Gracia *et al*., 2017). CVD is the leading cause of mortality worldwide as reported by the World Health Organisation (WHO) (Psota *et al*., 2018) and the incidence is higher in people who had sleep problems (Nagai *et al*., 2010). For example, shift workers have a higher incidence of coronary artery disease due to the disruption of normal circadian rhythm that affects their sleep duration (Havakuk *et al*., 2018). Sleep deprivation is not only associated with a wide range of deleterious health consequences, but also leads to perception impairment, difficulties in concentration, vision disturbances, slower reactions and poor memory (Orzeł-Gryglewska, 2010).

For many years, oxidative stress has been implicated in the pathogenesis of various diseases. More recently, it has become apparent that oxidative stress plays a major role in the initiation and progression of cardiovascular diseases such as hypertension, coronary artery disease, chronic heart failure and peripheral artery disease (Gracia *et al*., 2017). It is noteworthy that sleep deprivation enhances generation of free radicals (Mahmoudi *et al*., 2017) and sleep removes free radicals or reactive oxygen species (ROS) that are produced during wakefulness (Reimund, 1994). Thus, sleep has a protective role against oxidative stress (Gopalakrishnan *et al*., 2004).

Due to this, numerous studies have been conducted to explore the association between oxidative stress and sleep deprivation. A significant imbalance of oxidant-antioxidant levels has been demonstrated in the hippocampus (Alzoubi *et al*., 2012), hypothalamus and thalamus (D'Almeida *et al*., 1998), and brainstem of sleep deprived rats (Ramanathan *et al*., 2002). Researchers have put in much effort over the years to determine the effects of REM sleep deprivation on health as this phase serves many vital physiological functions. In addition to rapid eye movement, other characteristics of REM sleep include vivid dreams, loss of muscle tone, increased brain metabolism and memory consolidation (Sharma & Kavuru, 2010; Guyton & Hall, 2011; Wiesner *et al*., 2015). Effects of REM sleep deprivation on memory (Wiesner *et al*., 2015), pain-related gene expression (Siran *et al*., 2014), behaviour (Hanlon *et al*., 2015) and lipid peroxidation (Thamaraiselvi *et al*., 2012) have been done previously.

The endothelium, a simple monolayer in the blood vessel is able to respond to various physical and chemical signals by producing numerous factors that regulate

vascular tone, smooth muscle cell proliferation, cellular adhesion and vessel wall inflammation (Deanfield *et al*., 2007). Alterations of endothelium and the vasculature play a major role in the pathogenesis of various diseases (Rajendran *et al*., 2013). Endothelial dysfunction is widely accepted as the early changes in the pathophysiology of cardiovascular disease (Jiang *et al*., 2017). Various mechanisms have been implicated in endothelial dysfunction including oxidative stress (Abas *et al*., 2015; Di Meo, 2016), decreased nitric oxide bioavailability (Jiang *et al*., 2017), down-regulation of endothelial nitric oxide synthase (Suganya *et al*., 2016), inflammation (Kearney *et al*., 2017), hyperglycaemia (Suganya *et al*., 2016) and hypofibrinolysis (Kearney *et al*., 2017). However, whether the endothelium is affected during sleep deprivation needs to be clarified.

Extensive research has been done on the potential role of endogenous (naturally generated *in situ*) and exogenous (externally supplied) antioxidants in repairing and preventing damages due to oxidative stress. Vitamin C also known as ascorbic acid is an example of exogenous antioxidant. Besides protecting blood vessel (May & Harrison, 2013), vitamin C has been reported to have antioxidant, anti-carcinogenic, immunomodulator and anti-atherogenic (Pham-Huy *et al*., 2008). Hence, many researchers have focused on the vitamin C research in the context of their importance to human health and disease prevention.

1.2 Justification of study

The link between oxidative stress and sleep deprivation is being increasingly recognised; however, most of the previous studies were focused on the adverse effects on the central nervous system especially the brain. Thus, additional research

is necessary to determine the mechanism that increased risk of CVD in sleep deprivation. To date, not a single study has looked at the association between endothelial dysfunction and oxidative stress that is induced by REM sleep deprivation, which could be an important contributor to cardiovascular disease. Although vitamin C has been shown to protect blood vessel, whether it can protect the endothelial dysfunction during sleep deprivation needs to be evaluated.

1.3 Objective

1.3.1 General objective

To evaluate the effects of REM sleep deprivation on the endothelium and the protective effects of vitamin C in REM sleep deprived animal model**.**

1.3.2 Specific objectives

1. To determine the effects of REM sleep deprivation on vascular endothelial function in REM sleep deprived rat pretreated with vitamin C
2. To determine the effects of REM sleep deprivation on levels of tissue endothelial nitric oxide synthase (eNOS) protein expression in REM sleep deprived rat pretreated with vitamin C
3. To determine the effects of REM sleep deprivation on plasma levels of oxidative stress markers in REM sleep deprived rat pretreated with vitamin C
4. To determine the effects of REM sleep deprivation on plasma levels of coagulation factors in REM sleep deprived rat pretreated with vitamin C
5. To determine the effects of REM sleep deprivation on histological changes of descending thoracic aorta in in REM sleep deprived rat pretreated with vitamin C

1.4 Hypothesis

The hypothesis of the present study are as follows:

1. REM sleep deprivation significantly increases food consumption but reduces body weight gain
2. REM sleep deprivation significantly impairs vascular endothelial function
3. REM sleep deprivation significantly reduces levels of endothelial nitric oxide synthase (eNOS) protein expression in the blood vessel
4. REM sleep deprivation significantly disrupts the oxidant/antioxidant balance in the plasma
5. REM sleep deprivation significantly disrupts plasma levels of coagulation factors
6. REM sleep deprivation significantly alters histology of descending thoracic aorta
7. Vitamin C significantly reduces the effects of REM sleep deprivation

1.5 Significance of the study

This study contributes to the growing body of literature regarding the mechanism of increased CVD in sleep deprivation. Therefore, the goal of this study is to determine the mechanism of endothelial dysfunction following REM sleep deprivation and to explore factors that are involved in the process. It may also discover the role of antioxidant vitamin C in reducing the deleterious effects of REM sleep deprivation.

CHAPTER 2

LITERATURE REVIEW

2.1 Sleep

Sleep is a fundamental requirement for living individuals. It influences one's emotional wellbeing and mental health (Marks & Landaira, 2015). Sleep is regulated by circadian timing systems in the hypothalamus (Alkadhi *et al*., 2013). It has a complex physiologic process that is influenced by many internal and environmental factors such as light, temperature and the bedroom environment. The functions of sleep include memory consolidation, energy restoration, cognitive homeostasis and thermoregulation (Cipolli *et al*., 2006; Diekelmann & Born, 2010). During sleep, many physiological processes occur such as digestion, cell repair and growth in humans. Although sleep plays a vital role in good health, due to the ever increase demands of modern life, sleep has been neglected and increasingly recognised as a major public health problem.

Sleep can be described as an active state, characterised by decrease awareness and responsiveness that is promptly reversible (Carskadon & Dement, 2011). Loss of behavioural control and consciousness has been proposed as the most distinctive features of sleep (Diekelmann & Born, 2010). Sleep is divided into two broad categories; non-rapid eye movement (NREM) and rapid eye movement (REM) sleep that alternate cyclically across a sleep episode (Carley & Farabi, 2016). NREM sleep is further divided into three stages; N1 (Stage 1), N2 (Stage 2) and N3 (Stage 3). Previously, N3 consists of Stage 3 and 4, however the American Academy of Sleep Medicine (AASM) in 2007, combined both of the stages as they have similar

electroencephalography (EEG) waves, slow-wave sleep (Carskadon & Dement, 2011). N1 is a state of transition from being conscious to falling asleep that takes about five to ten minutes. N2 stage lasts about ten to twenty-five minutes in which heart rate and brain waves become slower. In addition, the eye stops from making any movement and the muscle is more relax compared to N1 stage. Meanwhile, N3 stage takes about twenty to forty minutes. During this stage, heart rate, respiratory rate, blood pressure and body temperature are at their lowest levels. This is the stage where sleep walking, sleep talking, nightmares and night terrors take place.

During REM sleep, both eyes move rapidly and this phase is usually associated with active dreaming where the person's dream seems as in reality world. The heart rate and respiratory rate are irregular which indicate a dream stage. However, the muscle tone throughout the body is depressed and the person is difficult to be aroused by sensory stimuli. REM sleep that occupies about 25% of the total sleep time, occur every 90 to 120 minutes throughout the night and the duration increases as the sleep progress (Shrivastava *et al*., 2014). The optimum amount of sleep is eight hours and healthy adults usually have four to five sleep cycles per night.

Physiologically, polysomnogram (PSG) is the gold standard for assessment of sleep and wake states in the laboratory (Carley & Farabi, 2016). The PSG monitors many body functions during sleep including brain (electroencephalography; EEG), eye movements (electro-oculography; EOG), muscle activity or skeletal muscle activation (electromyography; EMG) and heart rhythm (Electrocardiography; ECG). The output from the parameters measured is recorded simultaneously on a graph by a computer, which is known as hypnogram as shown in Figure 2.1.

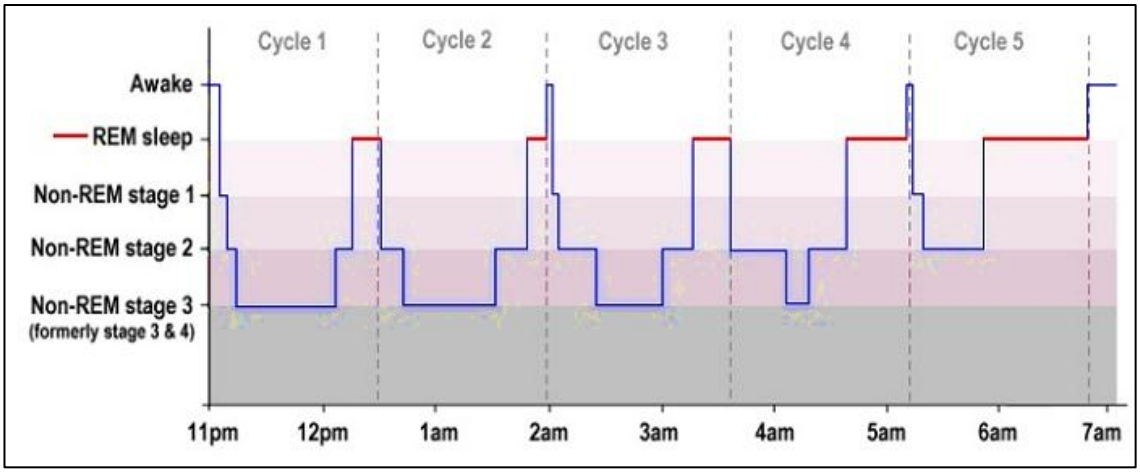

Figure 2.1: A typical hypnogram of sleep cycles in adult. One sleep cycle consists of REM sleep and NREM sleep (Stage 1, 2 and 3). The duration of REM sleep is increased as sleep progress. Adapted from Carley & Farabi, 2016.

2.2 Physiological changes during sleep deprivation

Sleep deprivation can be described as the partial or near-complete removal of sleep in an organism that results in various deleterious effects on the human body (Chen & Kushida, 2005). Sleep deprivation, which includes insufficient duration, irregular timing of sleep, poor sleep quality, and sleep/circadian disorders, is highly prevalent in modern society (Laposky *et al*., 2016). It affects a large part of the general population. Sleep deprivation is widely known as a stressor that affects physical and biochemical changes in human that subsequently results in health consequences. The symptoms of sleep deprivation may include tiredness, headache, burning eyes, nausea, blurred vision and joint pain (Chen & Kushida, 2005). Increasing awareness of the effects of sleep deprivation on health, safety, productivity and quality of life has led the researchers to explore the pathophysiology of sleep deprivation as a cause of various diseases including cardiovascular diseases.

The physiologic mechanisms whereby sleep deprivation adversely affects the human life are poorly understood. Research on sleep deprivation using animals and human has been started since 1890s with the aimed to understand the function of sleep (Chen & Kushida, 2005). A considerable body of clinical evidence revealed the relationship between sleep deprivation and obesity (Coughlin & Smith, 2014), hypertension (Vgontzas *et al*., 2009), atherosclerosis (Miller *et al.*, 2011) and diabetes (Gottlieb *et al*., 2005), all of which are potent risk factors for cardiovascular diseases. Besides, sleep deprivation impairs energy homoeostasis, immune system, hormonal regulation and inflammatory responses (Mahmoudi *et al*., 2017). Sleep modulates neuroendocrine function and glucose metabolism (Beccuti & Pannain, 2011), thus sleep deprivation results in glucose intolerance that responsible for the

development of type 2 Diabetes mellitus (Xu *et al*., 2016). Sleep deprivation increased risk for suicidal behaviours, including suicidal ideation, suicide attempts, and death by suicide (Bernert *et al*., 2014), which indicates sleep influences mental health (Marks & Landaira, 2015). There is no doubt that sleep deprivation has a major impact on health and wellbeing and needs to be explored thoroughly.

The physiological activities are different between NREM sleep and REM sleep. Selective deprivation of REM sleep has been largely focused by many researchers since the discovery of REM sleep phase in 1953 (Shepard *et al*., 2005). It is interesting to note that the pattern of EEG during REM sleep is similar to wakefulness which indicates that the brain is as active as during awake (Guyton & Hall, 2011). Although the precise physiological significance of REM sleep remains one of the great challenges of sleep medicine, prolonged REM sleep deprivation is known to be fatal (Mallick & Gulyani, 1996). Thus, it has attracted much attention to the researchers to study REM sleep deprivation extensively.

In human, special equipment is needed to assess REM sleep such as EEG (detects electrical activity in brain), EMG (evaluates the health of muscles and nerves), EOG (record eye movements) or functional magnetic resonance imaging (fMRI) (Brown, 2012). fMRI is a neuroimaging technique that measures brain activity by measuring changes in blood flow. Previous study using fMRI demonstrated REM sleep deprivation enhanced emotional reactivity that indicates REM sleep regulates the neural substrates for emotional responsiveness in human (Rosales-Lagarde *et al*., 2012). The use of EEG to compare the brain activity during sleep deprivation and undisturbed sleep has been done for many years (Dijk *et al.*, 1997; Endo *et al.*,

1998). Meanwhile, the EMG in REM sleep phase showed a lower muscular activity when compared to during awake state (Estrada *et al.,* 2006).

Sleep deprivation in rats has been shown to be associated with significant weight loss despite hyperphagia (Hipolide *et al.,* 2006; Thamaraiselvi *et al.,* 2012; Siran *et al.,* 2014), decreased levels of anabolic hormones (Everson & Crowley, 2004) and increased of metabolic rate (Koban & Swinson, 2005). Moreover, previous studies observed brain cells impairment (Inoue *et al.,* 1995), aggressive behaviour (Gulyani & Mallick, 1995), elevated plasma catecholamines (Rechtschaffen & Bergmann, 1995), increased neuronal metabolic activity and damage of muscular tone (Villafuerte *et al.,* 2015). Thus, it is clear that REM sleep deprivation alters behavioural, physiological, cellular functioning and responsiveness (Mallick & Gulyani, 1996).

Sleep has antioxidative role as it removes free radicals or reactive oxygen species (ROS) that are produced during wakefulness (Reimund, 1994; Villafuerte *et al.,* 2015). Therefore, the association between REM sleep deprivation and oxidative stress has been investigated extensively. Published data on the effects of REM sleep deprivation on oxidative stress parameters in rats have been inconsistent. Most recently, it has been proposed that sleep deprivation exacerbates generation of free radicals (Mahmoudi *et al*., 2017). Previous studies have shown a significant reduction in antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in the hippocampus (Alzoubi *et al*., 2012) following REM sleep deprivation.

In addition, a significant decreased of SOD activity in the hippocampus and brainstem has been demonstrated (Ramanathan *et al*., 2002). However, no association of sleep deprivation and oxidative stress has also been reported. This is supported by a finding that sleep deprivation did not affect oxidant production, antioxidant enzyme activity, lipid peroxidation or protein oxidation in brain, liver and skeletal muscle in a rat model (Gopalakrishnan *et al*., 2004).

2.3 REM sleep deprivation paradigms

REM sleep deprivation has been the most common type of sleep-state selective deprivation in animals. Animal-based research is widely used to elucidate the pathophysiological changes in rats following REM sleep deprivation. For many years, animal models that have been used include dogs, cats, mouse and rats; the rat being the most extensively studied to date (Chen & Kushida, 2005). Among the various techniques that are available, the single platform (inverted flowerpot) and multiple platforms are widely used due to their simplicity and low cost (Mahmoudi *et al*., 2017).

There are various techniques have been utilised in the study of REM sleep deprivation in rats:

i. Classic single platform

This technique is also known as water tank, platform or pedestal technique procedure (Gulyani *et al*., 2000; Landis, 2005). This is the most popular and effective method of selective REM sleep deprivation in rats (Gulyani *et al*., 2000; May *et al*., 2005; Lungato *et al*., 2013; Siran *et al*., 2014). During the intervention, a rat is placed in a tank or chamber containing a small platform; often an inverted flower pot, about 4.5-

8 cm diameter. The tank is filled with water and the height of the platform is 1 cm above a pool of water. During REM sleep, as animals lose their muscle tone, they fall off from the platform into the water, and wake up instantly. Interestingly, this method has been shown to deprive about 90-99% of REM sleep in rats (May *et al*., 2005). REM sleep deprivation study using inverted flower pot technique has been done extensively to study memory (Alzoubi *et al*., 2012), appetitive behaviour (Hanlon *et al*., 2005), lipid peroxidation (Thamaraiselvi *et al*., 2012) and pain-related gene expression (Siran *et al*., 2014).

ii. Multiple platform technique

This technique uses a single tank or chamber with multiple small platforms (Alzoubi *et al*., 2012; Hirotsu *et al*., 2013; Lima *et al*., 2014). Multiple rats are placed in the tank such as 5 rats with 7 platforms or10 rats with 18 platforms filled with water at the level of 1 cm below the platform upper surface (Landis, 2005). Another example, 15 narrow cylindrical platforms are fixed to the bottom of the tank that is filled with water (Mahmoudi *et al*., 2017). The multiple platform technique can prevent immobility as it has many platforms, furthermore it can prevent social isolation as more rats can be placed at one time (Landis, 2005). However, other researchers have proposed that this technique showed increased social conflict (Suchecki *et al*., 1998).

iii. Electrical stimulation

In this technique, direct brain stimulation is introduced when the EEG and EMG showed features of REM sleep (Landis, 2005). However, this technique is not commonly used by the researchers.

iv. Pendulum or swing technique

This technique involved a slowly rotating apparatus that consists of three separate compartments that resembled a swing (Landis, 2005). During the deprivation, the

apparatus continuously oscillated back and forth producing awakenings. The speed of oscillations is adjusted to increase the number of awakenings, which prevent the REM sleep to occur.

v. Cold ambient environment

Cold temperature of 0 °C and -10 °C for 1-2 days reduce REM sleep in rat. The loss of REM sleep is nearly complete at -10 °C compared to 0 °C (Amici *et al*., 1998).

vi. Disk-over-water

In this setting, a rat is placed on a disk (Gulyani *et al*., 2000; Ramanathan *et al*., 2002). When the rat shows signs of falling asleep, the disk begins to slowly rotate, at a few revolutions per minute. Rat is connected to polygraph for continuous recording of EEG and this polygraph is linked to a computer programmed to trigger rotation of the disks depending on the type of deprivation that is desired. To keep pace with the disk, the rat must walk or it will be carried into a pool of water.

2.4 Basic structure of a blood vessel

Alteration of the vasculature lead to the development of many diseases including peripheral vascular disease, stroke, diabetes, heart disease, chronic kidney failure and venous thrombosis (Rajendran *et al*., 2013). Thus, the pathogenesis of most of the diseases involved changes to the structure of the blood vessels. Blood vessels are divided into arteries, arterioles, capillaries, venules, and veins, depending on function, location and size. The wall of arteries and veins consists of three layers, or tunics; from innermost to outermost are tunica intima (interna), tunica media and tunica externa (adventitia) (Zhao *et al*., 2015). Endothelium is a monolayer of endothelial cells that line the tunica intima, forming an interface or barrier between circulating blood in the lumen and the vessel wall (Sena *et al.,* 2013; Suganya *et al*.,

2016). Endothelium is a thin layer of simple squamous cells supported by the internal elastic lamina. The thickness of the endothelium is less than 0.2 μm and it is comprised of 1 to 6×10^{13} endothelial cells (Yau *et al*., 2015).

The tunica media is a muscular and connective tissue layer that consists of smooth muscle cells and substantial amounts of elastic fibers. The smooth muscle cells that extend circularly around the lumen regulate the diameter of the lumen. Thus, this layer is responsible for vasodilation and vasoconstriction of the blood vessels (Sandoo *et al*., 2010; Suganya *et al*., 2016). Meanwhile, the tunica externa is the outermost layer that consists of elastic and collagen fibers. This layer helps anchor the vessels to surrounding tissues by its connective elements including fibroblasts and collagen fibers. Tunica externa is composed of nerve endings, fibroblasts, collagen fibers and perivascular adipose tissue (PVAT). The diagram of a typical artery is shown in Figure 2.2.

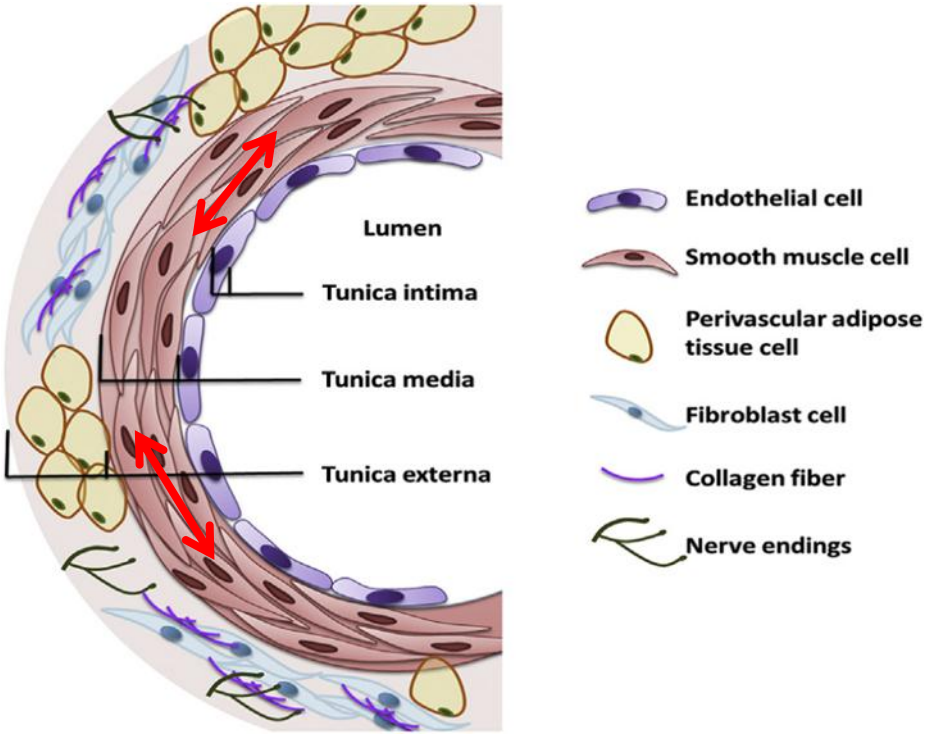

Figure 2.2: Structure of arterial wall. A typical artery consists of three layers; tunica intima, tunica media and tunica externa. The smooth muscle cells are oriented horizontal to the lumen and disposed circularly around the vessel (red double arrow). Endothelial cells (endothelium) line the innermost layer. Adapted from Zhao *et al.*, 2015.

2.5 Functions of endothelium

The endothelium that lines the interior layer of blood vessels of the entire circulatory system controls the microvascular permeability, vessel wall tone, coagulation and anticoagulation cascades, lipid homeostasis, inflammation, angiogenesis and vasculogenesis (Calvin *et al*., 2014; Suganya *et al*., 2016). The healthy endothelium is able to respond to vascular changes by production of various chemical mediators that regulate vascular tone, cellular adhesion, thromboresistance, smooth muscle cell proliferation, and vessel wall inflammation (Bauer & Sotnikova, 2010; Suganya *et al*., 2016).

The vascular tone is determined by production and release of several vasoactive molecules that relax or constrict the vessel. Endothelium derived relaxing factors (EDRF) that possess vasodilatory effects comprise of nitric oxide (NO), endothelium-derived hyperpolarisation factor (EDHF) and prostacyclin (Bauer & Sotníkova, 2010; Rajendran *et al*., 2013). Among them, the most significant EDRF is NO (Roberts & Porter, 2013). Meanwhile, examples of mediators secreted by the endothelium that have vasoconstricting effects are angiotensin II (Ang II), endothelin-1 (ET-1), thromboxane A_2 (TXA_2) and platelet-activating factor (PAF) (Sandoo *et al*., 2010).

A fine balance between anti- and prothrombotic states is maintained by the endothelium (Suganya *et al*., 2016). A healthy endothelium suppresses thrombosis by the release of thrombomodulin (TM) and tissue plasminogen activator (tPA), which are crucial for inhibiting fibrinolysis (Bauer & Sotnikova, 2010; Suganya *et al*., 2016). In contrast, when there is damage to the endothelium, endothelial cells secrete

prothrombotic molecules like von Willebrand factor, plasminogen activator inhibitor-1 (PAI-1) and thromboxane (TxA_2) that are responsible in stimulating coagulation process including platelet aggregation and adhesion (Roberts & Porter, 2013).

The endothelium also maintains the vascular homeostasis by controlling the vessel wall permeability (Wang *et al.*, 2015). Infection and inflammation have been demonstrated to increase vessel wall permeability as both conditions expose the endothelial cells to nuclear transcription of leukocyte-adhesion molecules such as vascular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM), P- and E- selectins. (Feletou, 2011; Roberts & Porter, 2013). A sequence of binding between adhesion molecules expressed on leukocytes and adhesion molecules expressed by the intraluminal and intercellular membranes of vascular endothelial cells mediate leukocyte adherence. Several diseases including diabetes mellitus, chronic inflammations and hypercholesterolemia promote disruption of the endothelial protective barrier. Thus, the endothelium is the primary target of many diseases. The disruption increased adhesiveness of the endothelium to leukocytes, altered permeability of the endothelium, and increased vascular smooth muscle proliferation (Bauer & Sotnikova, 2010).

In addition to the above functions, the endothelium also plays an important role in cell proliferation and differentiation. This is achieved by the secretion of various growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), transforming growth factor-β (TGF-β), and basic fibroblast growth factor (bFGF) (Suganya *et al.*, 2016). The growth factors are important in vascular development. The endothelium also

regulates the immune response whereby numerous humoral factors have been attributed in endothelial dysfunction. Increased concentration of interleukin (IL)-6, monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α) and C-reactive protein (CRP) facilitate consequences of endothelial dysfunction (Bauer & Sotnikova, 2010).

Moreover, the endothelium plays an important role in angiogenesis. Vascular endothelial growth factor (VEGF) and transforming growth factor-β (TGF-β) are among the tissue growth factors that initiate the process of angiogenesis through the activation of mitogen-activated protein kinase (MAPK) signaling (Roberts & Porter, 2013). The major functions of endothelial cells in maintaining the vascular homeostasis are shown in Figure 2.3.

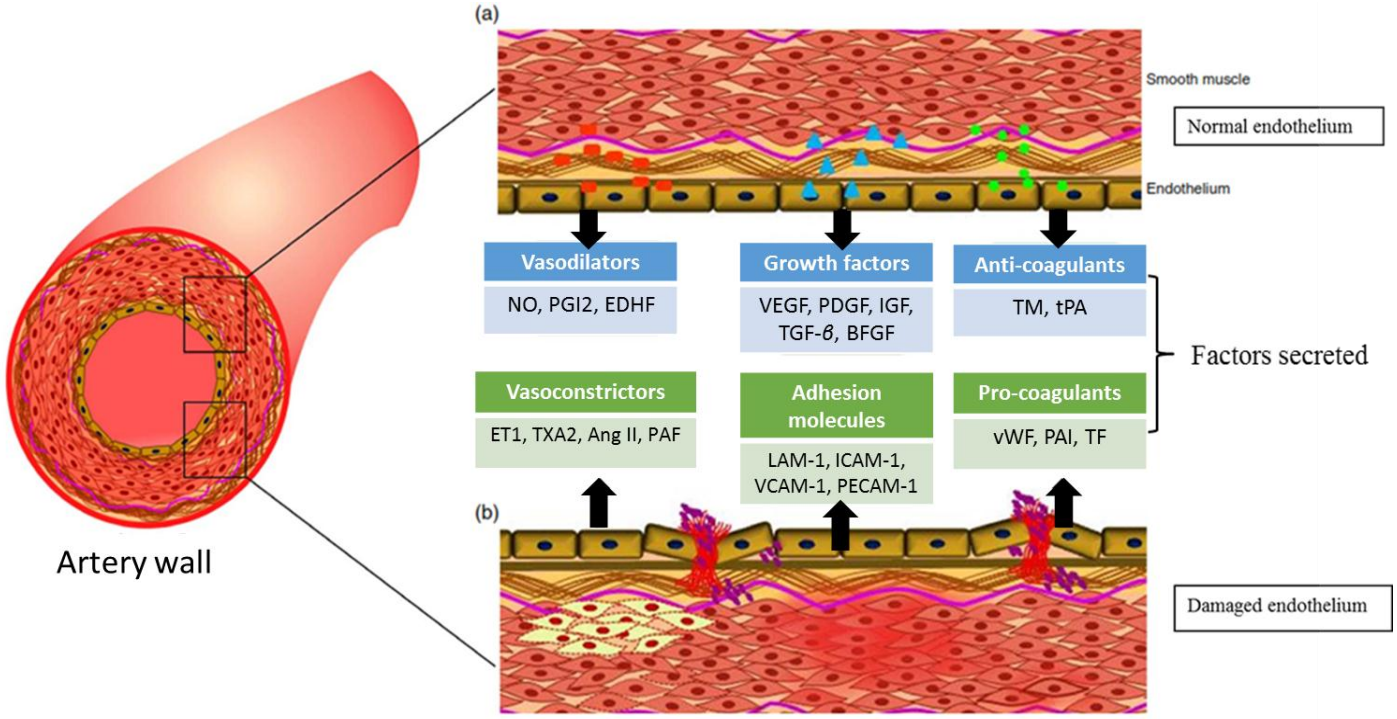

Figure 2.3: Factors secreted by the endothelium. (a) In normal condition, the endothelium secretes vasodilators, growth factors and anti-coagulants (b) In abnormal or damaged endothelium, the endothelium secretes vasoconstrictors, adhesion molecules and pro-coagulants. Adapted from Suganya *et al*., 2016.

NO; nitric oxide, $PG1_2$,; prostacyclin, EDHF; intercellular adhesion molecule-1, VEGF; vascular endothelial growth factor, PDGF; platelet-derived growth factor, IGF; insulin-like growth factor, TGF-β; transforming growth factor-β, bFGF; basic fibroblast growth factor, TM; thrombomodulin, tPA; tissue plasminogen activator,. ET-; endothelin-1, TxA_2; thromboxane A_2, Ang II; angiotensin II, PAF; platelet-activating factor, LAM-1; leucocyte adhesion molecule-1, V-CAM-1; vascular adhesion molecule-1, I-CAM; intercellular adhesion molecule 1, PECAM-1; platelet cell adhesion molecule-1, vWF; Von Willebrand factor; PAI-1, plasminogen activator inhibitor-1, TF; tissue factor

2.6 Mechanism of endothelial dysfunction

Growing evidence suggests endothelial dysfunction is an early event in cardiovascular diseases (CVD) (Brandes, 2014; Jiang *et al*., 2017). It has been implicated in many diseases such as hypertension, coronary artery disease, chronic heart failure, peripheral artery disease, diabetes and chronic renal failure (Rajendran *et al*., 2013; Sena *et al*., 2013). Endothelial dysfunction is a strong predictor for cardiovascular risk factors (Sandoo *et al*., 2010; Calvin *et al*., 2014). There is an uneven amount of production and bioavailability of endothelium-derived relaxing factors (EDRF) and endothelium-derived contractile factors (EDCFs) (Coco & de Oliveira, 2015).

Most recently, endothelial dysfunction is described as a reduction in the ability of the endothelium to transmit a vasodilatory influence on blood flow (Jiang *et al*., 2017). Furthermore, endothelium is enable to maintain vascular homeostasis whereby there is a shift in the normal endothelial functions including reduced vasodilation, proinflammatory and prothrombic state (Suganya *et al*., 2016). An important feature of endothelial dysfunction is the inability of arteries and arterioles to optimally dilate in response to a vasodilator.

Numerous studies suggest a link between sleep deprivation and endothelial dysfunction in animals (Sauvet *et al*., 2014; Jiang *et al*., 2017) and human (Calvin *et al*., 2014; Kohansieh & Makaryus, 2015), however the underlying mechanism remains to be elucidated. There are several characteristics of endothelial dysfunction such as excess production of growth factors, elevated levels of reactive oxygen

species (ROS), impaired fibrinolytic ability and extreme generation of ROS (Taddei *et al*., 2003; Laughlin *et al*., 2008).

Endothelial dysfunction can also be detected when there is a decreased of endothelium-mediated vasorelaxation, augmented expression of adhesion molecules and inflammatory genes and also dysregulation of hemodynamic (Cade, 2008; Addabbo *et al*., 2009; Hirose *et al*., 2010). Endothelial dysfunction also contributes to vasospasm, vasoconstriction, excessive thrombosis and abnormal vascular proliferation (Andor, 2005). Indeed, the mechanism of endothelial dysfunction is complex and various factors have been implicated in this condition.

2.6.1. Role of oxidative stress

Free radicals and oxidants play a dual role; either harmful due to their toxic effects or helpful due to their beneficial compounds. Both are produced either from normal cell metabolisms or from external sources such as cigarette smoke, radiation, pollution or medication. In a condition whereby there is an overload of free radicals that cannot be destroyed, it leads to a phenomenon called oxidative stress (Pham-Huy, 2008). It can also be defined as a condition when highly reactive molecules or free radicals; reactive oxygen species (ROS) or reactive nitrogen species (RNS) overwhelm the production of antioxidants (Coco & Oliveira, 2015; Villafuerte *et al.*, 2015), or inadequate removal of oxidants by antioxidants (Valko *et al*., 2007). Thus, there is an imbalance in the free radicals-antioxidant equilibrium in the biological system (Pham-Huy, 2008).

ROS are a collection of chemically-reactive molecules that include both oxygen radicals and certain non-radicals or known as oxidants or oxidising agents that are easily converted into radicals (Halliwell, 2006). Free radicals ROS include superoxide anion ($O_2^{\cdot -}$), hydroxyl ($OH^{\cdot}$), hydroperoxyl ($HO_2^{\cdot}$) and carbonate ($CO_3^{\cdot}$) whereas non-radicals ROS include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and Ozone (O_3). Among them, $O_2^{\cdot -}$, H_2O_2 and $OH^{\cdot}$ have been associated with CVD (Gracia *et al*., 2017). Meanwhile, free radicals nitrogen derivatives such as peroxynitrite ($ONOO^-$), S-nitrosoglutathione and S-nitrosothiols are collectively known as RNS. Radicals are less stable with stronger reactivity than non-radical species (Pham-Huy, 2008).

At normal low levels, ROS and RNS exert beneficial effects to the biological system in the regulation of various cell activities including cell proliferation, phagocytosis, electron transport, signal transduction and gene expression (Valko *et al*., 2007). On the other hand, at higher concentrations, when they are not completely removed by the antioxidant defence system, 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). In addition, the biomolecular damage due to high concentration of ROS involves damage to nucleic acids, lipids and protein (Valko *et al*., 2007). Mitochondria respiration and uncoupling nitric oxide synthase in vascular cells may release ROS. ROS that are generated in the endothelial cells include $O_2^{\cdot -}$ and H_2O_2. In biological system, $O_2^{\cdot -}$ is short-lived and unstable due to its rapid reduction to H_2O_2 by SOD (Puac *et al*., 2014). Meanwhile, H_2O_2 has longer lifespan than $O_2^{\cdot -}$, which is relatively stable and easily diffusible within and between cells.

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). In addition, the activation of endothelial cells also generates ROS and contributes in maintaining the oxidant-rich environment. Smoking, hypertension, diabetes and dyslipidemia were shown to increase the production of ROS (Lakshmi *et al.,* 2009). For example, increased malondialdehyde (MDA) levels resulted from lipid peroxidation has been implicated in the vascular complication in diabetic patient (Ogura *et al.,* 2006; Abas *et al.,* 2015).

2.6.2 Role of nitric oxide

Nitric oxide (NO or NO·) is an important gaseous free radical derived from nitrogen (Prochazkova *et al*., 2015). NO is an endothelium-dependent vasodilator, which is continuously produced in the tissue by endothelial nitric oxide synthase (eNOS) during the metabolic conversion of amino acid L-arginine substrate to citrulline. The conversion requires co-factors such as tetrahydrobiopterin (BH_4), flavin-mononucleotide, nicotinamide adenine dinucleotide phosphate (NADPH) and flavin adenine dinucleotide (Wang *et al*., 2015).

In inactive state, eNOS is bound to the protein caveolin that is located in the cell membrane called caveolae (invaginations in cell membranes). Factors or substances that can detach eNOS from caveolin (NO agonists) include acetylcholine (ACh), bradykinin (BK), adenosine di-phosphate (ADP), adenosine tri-phosphate (ATP), substance P and thrombin (Sandoo *et al*., 2010). When the intracellular levels of calcium increase, the NO agonists displace the caveolin from calmodulin (CaM), a calcium-binding messenger protein (Davignon & Ganz, 2004). This will activate