RAPID EYE MOVEMENT SLEEP DEPRIVATION INDUCES VASCULAR ENDOTHELIAL DYSFUNCTION IN RATS: ROLES OF OXIDATIVE STRESS-MEDIATED NITRIC OXIDE SIGNALLING AND VITAMIN C SUPPLEMENTATION

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2024

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by

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Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

August 2024

ACKNOWLEDGEMENTS

First of all, I am very thankful to the Most Merciful for giving me the strength and guidance to finish my research project and thesis. I would like to take this opportunity to express my sincere gratitude and appreciation to my supervisor, Dr. Liza Noordin, who convincingly guided and encouraged me to get things done and made sure that I was on the right track, even when the road got tough. Without her persistent help and continuous motivation, the objectives of this study would not have been achieved. Many thanks are also dedicated to my co-supervisors, Associate Prof. Dr. Wan Amir Nizam Wan Ahmad and Associate Prof. Dr. Anani Aila Mat Zin, who willingly took time out of their hectic schedule to guide young learners like me on what I should do when I am in the dark. Their expertise was appreciated and inspired me to think outside of the box.

I would like to acknowledge the financial support from the Research Universiti Grant (RU) (USM/1001/PPSP/8012316). I am also forever indebted to all staff from the Animal Research and Service Centre (USM Health Campus), Central Research Laboratory and Physiology Laboratory (School of Medical Sciences), Scanning Electron Microscope Laboratory and Biomedicine Laboratory (School of Health Sciences) for allowing me to work in their laboratories and persistently assist me in many aspects of my studies. My greatest gratitude goes to my friends and lab mates, Shafreena and Ridzuan for their kindness in supporting me over the last three years. Last but not least, I wish to acknowledge my parents, Nawi Mezah and Zamaniah Abdullah, and siblings for their endless love and support. I would not have survived the challenges of this PhD without them.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

^{o}C	degree Celsius
µg/mL	microgram per millilitre
µL	microlitre
µM	micromolar
4-HNA	4-hydroxyl nonenal
72-h REMsd	REM sleep-deprived rats for 72-h
AASM	American Academy of Sleep Medicine
ABTS	2,2'-Azinobis-(3-Ethylbenzothiazoline-6-sulfonic acid
ACh	acetylcholine
ACTH	adrenocorticotropic hormone
ADMA	asymmetric dimethylarginine
AKT	protein kinase B
AMPK	AMP-activated protein kinase
Ang II	angiotensin II
ANOVA	one-way analysis of variance
APS	ammonium persulphate
ARASC	Animal Research and Service Centre
AREs	antioxidant response elements
AWA	waking phase
Bad	B-cell lymphoma-2 gene-associated promoter
bFGF	basic fibroblast growth factor
BH4	tetrahydrobiopterin
BK_{Ca}	Ca^{2+}-activated K^{+} channel

BMI	body mass index
BSA	bovine serum albumin
BW	body weight
BWg	body weight gain
Ca^{2+}	calcium ion
$CaCl.2H_2O$	calcium chloride dehydrate
CaM	calmodulin
Caspase-9	cysteine-aspartic protease
CAT	catalase
cGMP	cyclic guanosine monophosphate
CH_2	methylene
cm	centimetre
CO_2	carbon dioxide
CPD	critical point dryer
CRP	C-reactive protein
CVD	cardiovascular disease
DBP	diastolic blood pressure
dH_2O	distilled water
DPX	distyrene plasticiser xylene
EC	endothelial cell
ECL	enhance chemiluminescence
EDCFs	endothelium-derived contractile factors
EDHFs	endothelium-derived hyperpolarisation factors
EDRFs	endothelium-derived relaxing factors
ELISA	enzyme-linked immunosorbent assay

eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
ET-1	endothelin-1
Fc	food consumption
Fe^{2+}	ferrous iron
FMC	free moving control
g/day per $kg^{0.67}$	grams per day per kilogram of body weight to the power of 0.67
GPCR	G protein-coupled receptor
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GSK-3β	glycogen synthase kinase3β
GSSG	oxidised glutathione
GTP	guanosine triphosphate
H&E	haematoxylin & eosin
H_2O_2	hydrogen peroxide
H_2S	hydrogen sulphide
HPA	hypothalamic-pituitary-adrenal
HRP	horseradish peroxidase
HUVECs	human umbilical vein endothelial cells
ICAM	intercellular cell adhesion molecule
ICAM-1	intercellular adhesion molecule-1
IGF	insulin-like growth factor
IK_{ca}	intermediate-conductance K_{Ca}
IL	interleukin

IL-1β	interleukin-1 beta
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
IP	intraperitoneal
K_{Ca} channels	calcium-dependent potassium channels
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
KHS	Kreb's Henseleit solution
L•	lipid radical
LAM-1	leucocyte adhesion molecule
L-NAME	N^G-nitro-L-arginine-methyl ester
L-NMMA	N^G-monomethtyl-L-arginine
$LOO^{\bullet}$	lipid peroxyl radical
LPS	lipopolysaccharide
MDA	malondialdehyde
mg	milligram
mg/kg	milligram per kilogram
mg/mL	milligram per millilitre
$MgSO_4.7H_2O$	magnesium sulphate heptahydrate
mL	millilitre
MLC	myosin light chain
mM	millimolar
mmHg	millimetre of mercury
mmol/L	millimoles per litre
mTOR	mammalian target of rapamycin

NaCl	sodium chloride
NADPH	dinucleotide phosphate
$NaHCO_3$	sodium hydrogen carbonate
NIBP	non-invasive blood pressure
nM	nanomolar
nm	nanometres
nNOS	neuronal nitric oxide synthase
$NO\cdot$	nitric oxide
$NO_2\cdot$	nitrogen dioxide
NOS	nitric oxide synthase
NREM	non-rapid eye movement
Nrf2	nuclear factor-E2-related factor 2
NTP	national toxicology program
$O_2^{\cdot -}$	superoxide anion
OD	absorbance/ optical density
$OH\cdot$	hydroxyl
ONOO-	peroxynitrite
ONOOH	peroxynitrous acid
Ox-LDL	oxidised low-density lipoprotein
PAF	platelet-activating factor
PAI-1	plasminogen activator inhibitor-1
p-AKT	phosphorylated-AKT
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PECAM-1	platelet cell adhesion molecule-1

p-eNOS	phosphorylated eNOS
PGI_2	prostacyclin
Phe	phenylephrine
PI3K	phosphatidylinositol 3-kinase
PIP3	phosphatidylinositol-(3,4,5)-trisphosphate
PKG	cGMP-dependent protein kinases
PSS	power sample size
PUFAs	polyunsaturated fatty acids
PVDF	polyvinylidene difluoride
RAAS	renin-angiotensin-aldosterone system
REM	rapid eye movement
REMsd	rapid eye movement sleep deprivation
RNS	reactive nitrogen species
$RO^{\cdot}$	alkoxy radical
$ROO^{\cdot}$	peroxyl radical
ROS	reactive oxygen species
RVC	REM sleep deprived-rats for 72-h that pretreated with vitamin C
SBP	systolic blood pressure
SCN	suprachiasmatic nuclei
SD	Sprague–Dawley
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	scanning electron microscope
sGC	soluble guanylyl cyclase
SK_{Ca}	and small-conductance K_{Ca}

SOD	superoxide dismutase
SR	sleep recovery
SWS	slow-wave sleep
TA	tunica adventitia
TAC	total antioxidant capacity
TBST	tris-buffered saline tween-20
TC	tank control
TEMED	tetramethylenediamine
TF	tissue factor
TGF	transforming growth factor
TGF-β	transforming growth factor-β
TI	tunica intima
TM	thrombomodulin
TM	tunica media
TNF	tumour necrosis factor
TNF-α	tumour necrosis factor-α
tPA	tissue plasminogen activator
TXA_2	thromboxane A_2
U/mL	units per millilitre
VCAM	vascular cell adhesion molecule
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VSMCs	vascular smooth muscle cells
vWF	von Willebrand factor
WHO	World Health Organisation

LIST OF APPENDICES

KEKURANGAN TIDUR GERAK MATA CEPAT MENGARUH DISFUNGSI ENDOTELIUM VASKULAR DALAM TIKUS: PERANAN STRES OKSIDATIF-PENGANTARA ISYARAT NITRIT OKSIDA DAN SUPLEMENTASI VITAMIN C

ABSTRAK

Kekurangan tidur REM (REMsd) mempunyai kaitan dengan stres oksidatif, faktor utama dalam mekanisma disfungsi endotelium. Disfungsi endotelium telah dikaitkan dengan kekurangan ketersediaan biologi nitrit oksida ($NO^{\cdot}$). Sehingga kini, mekanisma disfungsi endotelium dalam REMsd kurang difahami. Kajian ini bertujuan untuk menilai kesan REMsd ke atas tekanan darah sistolik (SBP), fungsi endotelial vaskular, ekspresi protein eNOS, p-AKT, dan total AKT, paras penanda stres oksidatif dan penanda antioksidan, dan perubahan histopatologi dalam aorta. Kesan baik vitamin C juga telah dinilai. Tiga puluh lima (35) ekor tikus Sprague-Dawley (SD) jantan dibahagikan sama rata kepada 5 kumpulan (n=7): Tikus bergerak bebas dan kawalan (FMC), tikus REMsd selama 72 jam (72-h REMsd), tikus kawalan sangkar (TC), 72 jam pemulihan tidur selepas REMsd selama 72 jam (SR), REMsd selama 72 jam pra-rawat dengan vitamin C (RVC). Dalam kumpulan RVC, tikus diberikan vitamin C secara oral selama 4 minggu (100 mg/kg/hari) sebelum kajian dimulakan. Teknik pasu terbalik telah digunakan untuk merangsang REMsd dan telah disahkan dengan penurunan signifikan dalam pertambahan berat badan walaupun terdapat peningkatan signifikan dalam pengambilan makanan. Aorta toraks menurun diisolasi untuk kajian fungsi *in vitro* (miograf), pengukuran ekspresi protein eNOS, p-AKT, dan total AKT, dan pengukuran penanda stres oksidatif malondialdehid (MDA) dan penanda antioksidan termasuk aktiviti superoksid dismutase (SOD), katalase (CAT), glutation (GSH), dan total kapasiti antioksidan (TAC). Histopatologi aorta dinilai

menggunakan pewarnaan haematoxylin dan eosin (H&E) dan imbasan mikroskop elektron (SEM). Kumpulan 72-h REMsd menunjukkan peningkatan signifikan pada SBP berbanding kumpulan lain. Dalam kajian fungsi *in vitro*, terdapat hiperkontraktiliti aorta dan vasorelaksasi berperantara endotelium yang terjejas dalam kumpulan 72-h REMsd. Paras ekspresi protein eNOS, p-AKT, dan total AKT menurun secara signifikan dalam kumpulan 72-h REMsd berbanding kumpulan FMC. Terdapat peningkatan yang signifikan pada paras MDA dan penurunan signifikan pada paras SOD, CAT, GSH dan TAC dalam kumpulan 72-h REMsd berbanding kumpulan lain, yang menjadikan stres oksidatif. Dalam kumpulan 72-h REMsd, pewarnaan H&E menunjukkan kerosakan pada endotelium dan struktur dibawahnya, manakala kajian SEM mengesahkan lagi kerosakan tersebut dengan kehadiran rangkaian fibrin yang padat. Keputusan dalam kumpulan SR dan RVC menunjukkan tiada perbezaan signifikan berbanding kumpulan FMC dan TC. Oleh itu, adalah dicadangkan bahawa disfungsi endothelium aruhan REMsd disebabkan oleh penurunan ketersediaan biologi $NO^{\cdot}$ yang terhasil daripada penurunan dalam ekspresi protein eNOS. Peroksidasi lipid aruhan stres oksidatif bertanggungjawab terhadap kerosakan endotelium dalam kumpulan 72-h REMsd, yang dibuktikan oleh peningkatan paras MDA. Stres oksidatif dicadangkan sebagai perantara untuk disfungsi dan kerosakan endotelium yang juga mengganggu laluan isyarat fosfatidilinositol 3-kinase (PI3K)/AKT/eNOS. Pemulihan tidur telah mengembalikan perubahan akibat REMsd kepada nilai dasar. Suplementasi vitamin C adalah bermanfaat dalam melindungi endotelium dalam mengatasi kesan buruk REMsd.

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ABSTRACT

Rapid eye movement (REM) sleep deprivation (REMsd) is associated with oxidative stress, the primary factor in the mechanism of endothelial dysfunction. Endothelial dysfunction has been attributed to a reduction in nitric oxide ($NO^{\cdot}$) bioavailability. To date, the mechanism of endothelial dysfunction in REMsd is poorly understood. This study aimed to evaluate the effects of REMsd on systolic blood pressure (SBP), vascular endothelial function, protein expression of endothelial nitric oxide synthase (eNOS), phosphorylated-AKT (p-AKT) and total AKT, levels of oxidative stress marker and antioxidants, and histopathological changes in the aorta. The beneficial effects of vitamin C were also evaluated. Thirty-five (35) adult male Sprague-Dawley rats were equally divided into 5 groups (n=7): free-moving control rats (FMC), REM sleep-deprived rats for 72-h (72-h REMsd), tank control rats (TC), sleep recovered for 72-h after 72-h of REMsd (SR), and 72-h REMsd pretreated with vitamin C (RVC). In the RVC group, rats were administered vitamin C orally for 4 weeks (100 mg/kg/day) before the study commenced. The inverted flowerpot technique was utilised to develop REMsd and was validated by a significant decrease in body weight gain despite a significant increase in food consumption. The descending thoracic aorta was isolated for *in vitro* functional study (myograph), measurement of protein expression of eNOS, p-AKT, and total AKT, and measurement of oxidative stress marker malondialdehyde (MDA) and antioxidant markers, including superoxide dismutase (SOD) activity, catalase (CAT), glutathione

(GSH), and total antioxidant capacity (TAC). The histopathology of the aorta was evaluated by haematoxylin and eosin (H&E) staining and scanning electron microscope (SEM). The 72-h REMsd group demonstrated a significant increase in SBP compared to other groups. In the *in vitro* functional study, there was hypercontractility of the aorta and impaired endothelium-dependent vasorelaxation in the 72-h REMsd group. The levels of protein expression of eNOS, p-AKT, and AKT were significantly decreased in the 72-h REMsd group compared to the FMC group. There were significantly increased levels of MDA, and decreased levels of SOD, CAT, GSH, and TAC in the 72-h REMsd group compared to other groups, which produce oxidative stress. In the 72-h REMsd group, H&E staining showed endothelial damage and its underlying structures, while the SEM study further confirmed the damage by the presence of dense fibrin networks. The results in the SR and RVC groups were not significantly different compared to the FMC and TC groups. Thus, it is suggested that REMsd-induced endothelial dysfunction due to decreased $NO^{\cdot}$ availability resulting from the decrease in eNOS protein expression. Oxidative stress-induced lipid peroxidation is responsible for endothelial damage in the 72-h REMsd group, which was evidenced by increased MDA levels. Oxidative stress is suggestive to be the mediator for endothelial dysfunction and damage that also disrupts the phosphatidylinositol 3-kinase (PI3K)/ AKT/eNOS signalling pathway. Sleep recovery reverts the changes following REMsd to the baseline values. Supplementation of vitamin C is beneficial in protecting the endothelium against the adverse effects of REMsd.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Sleep is essential for maintaining life and quality of mental and physical health (Hashizume *et al.,* 2024). Sleep is a natural physiological process that occurs in all living creatures and is divided into two stages; nonrapid eye movement (NREM) sleep and rapid eye movement (REM) sleep (Yan *et al.,* 2024). Sleep has a profound impact on neurodevelopment, synaptic plasticity, memory consolidation, metabolic function, and the immune system (Miletinova & Buskova, 2021). The American Academy of Sleep Medicine (AASM) recommends seven to nine hours of sleep per night for healthy adults aged 18 to 64 years to function at their best (Watson *et al.,* 2015). Sleep deprivation mostly refers to quantitative or qualitative changes in normal sleep and appears to be inextricably related to the modern technology world and way of life. Sleep deprivation is commonly associated with mood disturbance, poor judgment, stress, and anxiety (Aidy *et al.,* 2022) and has also been associated with a risk of cardiovascular disease (CVD) (Cherubini *et al.,* 2021). As a result, sleep deprivation has emerged as a global public health concern (Chattu *et al.,* 2019).

CVD is the leading cause of death worldwide and has been a burden to many developing countries, including Malaysia, for decades. According to the World Health Organisation (WHO), 30,000 people die from CVD every day which accounts for more than 40% of noncommunicable disease fatalities each year (Yang *et al.,* 2023). Despite several actions and precautions that have been taken to prevent and manage CVD, it remains the major cause of morbidity and mortality, with 23.6 million deaths predicted

by 2030 (Firus Khan *et al.,* 2022). CVD has become the leading cause of death in developed countries (Higano, 2020), and the risk increases with sleep deprivation (Nagai *et al.,* 2010; Cappuccio & Miller, 2017). The molecular mechanism underlying this phenomenon is poorly understood, although previous research has shown a link between sleep deprivation and obesity (Rathod *et al.,* 2018), atherosclerosis (Wang *et al.,* 2022), and diabetes (Samy *et al.,* 2021), all of which are major risk factors for CVD (Kadoya *et al.,* 2019).

Endothelial dysfunction is known to precede CVD (Jiang *et al.,* 2017; Cherubini *et al.,* 2021), and it has been linked to the development of a variety of diseases such as atherosclerosis, hypertension, ageing, stroke, heart disease, diabetes, and obesity (Sun *et al.,* 2020). Hypertension is a significant modifiable risk factor for CVD and has become one of the leading causes of morbidity and mortality worldwide (Brouwers *et al.,* 2021). The prevalence of hypertension is rising as the world's population of ageing and obesity increases, and it is predicted that one-third of the worldwide population will suffer from hypertension by 2025 (Isayeva *et al.,* 2022). Furthermore, patients with sleep problems have a 21% higher risk of developing hypertension compared with those who have quality sleep (Isayeva *et al.,* 2022).

Endothelial dysfunction is described as an imbalance between endothelium-derived relaxing factors (EDRFs) and endothelium-derived contracting factors (EDCFs) (Wang *et al.,* 2022). Oxidative stress, down-regulation of endothelial nitric oxide synthase (eNOS), and decreased nitric oxide ($NO^{\cdot}$) bioavailability are among the mechanisms attributed to the pathogenesis of endothelial dysfunction (Huang *et al.,* 2018). In addition, the phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (AKT) (Liang *et al.,* 2017; Lin *et al.,* 2020), nuclear factor-E2-related factor 2 (Nrf2)/ antioxidant response elements (AREs) (Keum & Choi, 2014), and AMP-activated

protein kinase (AMPK) (Head *et al.,* 2015) signalling pathways have been implicated in the regulation of eNOS activity and $NO^{\cdot}$ generation.

The adverse effects of REM sleep deprivation (REMsd) on health have been demonstrated. These effects include systemic lipid peroxidation (Thamaraiselvi *et al.,* 2012), altered gene expression (Siran *et al.,* 2014), pain behaviour changes (Mohd Shafie *et al.,* 2022), and memory impairment (Wiesner, 2015). A link between REMsd and oxidative stress in various organs was reported, including the thalamus (Mohd Shafie *et al.,* 2022), the hippocampus (Alzoubi *et al.,* 2012; Konakanchi *et al.,* 2022), submandibular glands (Lasisi *et al.,* 2021), the liver and pancreas (Hernandez Santiago *et al.,* 2021). REMsd results in decreased antioxidant enzymes, including catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) in the hippocampus (Alzoubi *et al.,* 2012). Sleep has antioxidative properties because it eliminates free radicals or reactive oxygen species (ROS) created during awake (Reimund, 1994). Hence, the link between sleep deprivation, particularly REMsd, and oxidative stress should be explored.

Despite oxidative stress being the primary contributor to endothelial dysfunction (Incalza *et al.,* 2018) and REMsd being associated with oxidative stress, data on REMsd-induced endothelial dysfunction and damage are scarce. Thus, there is a need to explore further whether REMsd has negative effects on vascular endothelium, which may be a risk factor for CVD. Moreover, most of the previous studies on REMsd have focussed on the nervous system. The mechanism by which sleep deprivation, particularly during the REM sleep phase, increases the risk of CVD should be explored.

Exogenous antioxidants have been widely studied for their roles in healing and preventing oxidative stress-related damage. Vitamin C, also known as L-ascorbic acid, is a nutrient that is required for a range of biological functions (Grosso *et al.,* 2013). Vitamin C is a powerful antioxidant and radical scavenger, it shields cell constituents from oxidative stress caused by ROS and free radicals (Morelli *et al.,* 2020). Furthermore, the redox potential of vitamin C allows for the maintenance of lowered levels as well as the regeneration of other antioxidants. Vitamin C also regulates the growth of endothelial cells, tightens the endothelial barrier, and helps in collagen deposition in the basement membrane of blood vessels (May *et al.,* 2013). However, little is known about the molecular mechanism of vitamin C on endothelial protection during REMsd.

1.2 Problem statement

REMsd is linked to increased oxidative stress. While numerous studies have examined the detrimental effects of oxidative stress following REMsd, the focus has primarily been on memory and behaviour. There is limited data on the harmful effects of REMsd on the endothelium, highlighting the need to understand the underlying mechanisms of endothelial dysfunction resulting from REMsd.

1.3 Justification of study

The justification for this study lies in the growing awareness of the link between oxidative stress and REMsd. Previous studies have primarily focused on the adverse effects of REMsd on the nervous system, particularly in terms of brain function in memory and behaviour. This study investigates the relationship between endothelial dysfunction and oxidative stress induced by REMsd. There have been very few studies that specifically investigated the effects of REMsd on the vascular

endothelium. There were multiple human and animal studies that have linked endothelial dysfunction to sleep deprivation, but not REMsd. As a result, more studies are needed to determine whether REMsd has detrimental effects on the vascular endothelium, potentially increasing the risk of CVD. The pathogenesis of endothelial dysfunction following REMsd needs to be explored. Furthermore, it is unclear whether the changes caused by REMsd can be reversed through sleep recovery or protected by interventions such as vitamin C supplementation. Addressing these knowledge gaps will not only contribute to our understanding of the physiological consequences of REMsd but also have significant implications for cardiovascular health and potential preventive strategies for individuals at risk.

1.4 Objectives of study

1.4.1 General objective

To determine the effects of REMsd on the vascular endothelium and the protective effects of vitamin C in REM sleep-deprived animal model

1.4.2 Specific objectives

The specific objectives of this study are stated below:

1. To determine the effects of REMsd on systolic blood pressure (SBP) in REM sleep-deprived animal model
2. To determine the effects of REMsd on vascular endothelial function in REM sleep-deprived animal model
3. To determine the effects of REMsd on protein expression of endothelial nitric oxide synthase (eNOS), phosphorylated-AKT (p-AKT), and total AKT in the aorta of REM sleep-deprived animal model

4. To determine the effects of REMsd on levels of oxidative stress marker malondialdehyde (MDA) and antioxidant markers (superoxide dismutase (SOD) activity, catalase (CAT), glutathione (GSH), and total antioxidant capacity (TAC)) in the aorta in REM sleep-deprived animal model
5. To evaluate the effects of REMsd on histopathological changes in the aorta in REM sleep-deprived animal model
6. To evaluate the beneficial effects of sleep recovery and vitamin C supplementation on SBP, vascular endothelial function, protein expression of eNOS, p-AKT, and total AKT, levels of oxidative stress marker and antioxidant markers, and histopathological changes in the aorta in REM sleep-deprived animal model

1.5 Hypotheses of study

1. REMsd significantly increases SBP and impairs vascular endothelial function
2. REMsd significantly decreases protein expression of eNOS, phosphorylated-AKT (p-AKT), and total AKT
3. REMsd significantly increases oxidative stress marker and decreases antioxidant markers
4. REMsd causes histopathological changes in the aorta
5. Sleep recovery and vitamin C supplementation significantly improve the adverse effects following REMsd

1.6 Conceptual framework

The conceptual framework of this study is shown in Figure 1.1. This conceptual framework illustrates the pathway linking REMsd to increased SBP through oxidative stress and endothelial dysfunction. REMsd induces oxidative stress, which adversely

affects the aorta. The oxidative stress leads to a decrease in eNOS protein expression, reducing $NO^{\cdot}$ production, and diminish $NO^{\cdot}$ bioavailability. These changes result in endothelial dysfunction. The framework also highlights the role of mitigating factors: the antioxidant vitamin C and sleep recovery. These factors can inhibit oxidative stress and thereby mitigate the adverse effects of REMsd, ultimately helping to prevent endothelial dysfunction and high SBP.

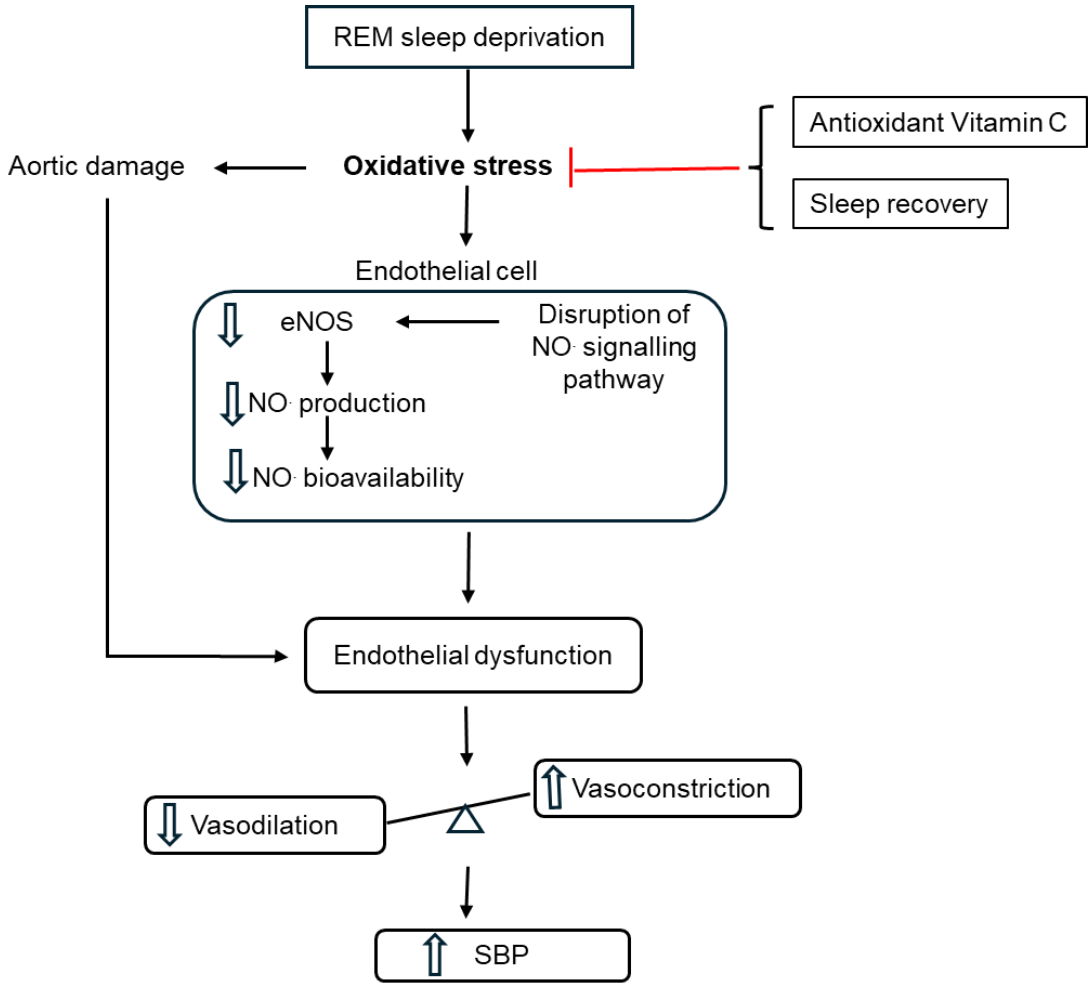

Figure 1.1: The conceptual framework of the study. REM sleep deprivation induces oxidative stress, which negatively impacts the aorta. This oxidative stress affects endothelial cells, leading to reduced expression of the endothelial nitric oxide synthase protein, diminished nitric oxide production, and decreased nitric oxide bioavailability, resulting in endothelial dysfunction. Additionally, oxidative stress can disrupt signalling pathways, further reducing nitric oxide bioavailability. Damage to the aorta also contributes to endothelial dysfunction. The overall effects of endothelial dysfunction lead to increased systolic blood pressure. Antioxidant vitamin C and sleep recovery can mitigate the adverse effects of REM sleep deprivation by inhibiting oxidative stress.

Abbreviations: eNOS; endothelial nitric oxide synthase, NO·; nitric oxide, SBP; systolic blood pressure

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of sleep

Humans spend approximately a third of their lives sleeping, and various other animals spend even more (Simon *et al.,* 2022; Yan *et al.,* 2024). Sleep plays a critical role in various functions, which include cognitive performance, memory consolidation, regulation of metabolism, pain regulation, hormonal function, and appetite regulation (Medic *et al.,* 2017; Simon *et al.,* 2022; Hashizume *et al.,* 2024). Sufficient quantity and quality of sleep minimise the likelihood of accidents and injuries resulting from drowsiness and fatigue, encompassing workplace and traffic accidents (Ramar *et al.,* 2021). The sleep system is regulated by the circadian rhythm system which is located deep within the brain hypothalamus called the 'suprachiasmatic nuclei' or SCN (Foster, 2020). Although the importance of sleep has long been acknowledged, it has been ignored and is gradually being recognised as a major public health problem.

Sleep is divided into two stages: non-REM (NREM) sleep and rapid eye movement (REM) sleep that alternate cyclically (Yan *et al.,* 2024). One sleep cycle consists of NREM and REM sleep, each associated with distinct neurological and physiological characteristics. On average, a person goes through four to six sleep cycles over eight hours of sleep in a night, as shown in Figure 2.1 (Huang *et al.,* 2020a).

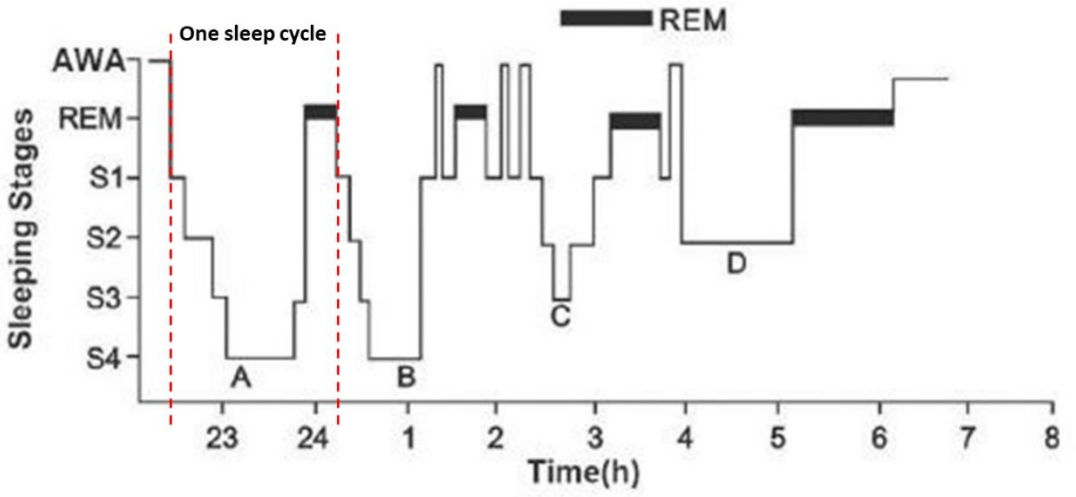

Figure 2.1: A graphical representation depicts the various stages of sleep across eight hours of sleep in a night, which include waking, REM, and NREM (S1-S4) stages. One sleep cycle is started from NREM through REM (Adapted from Huang *et al.,* 2020a). AWA; waking, REM; rapid eye movement, NREM; non-REM.

In an adult, sleep begins with the NREM stage followed by the REM stage, each lasting about 90 minutes on average. There are three stages of NREM: N1 (Stage 1; S1), N2 (Stage 2; S2), and N3 (Stage 3 and Stage 4; S3 and S4) (Patel *et al.,* 2022). N1 is a light sleep stage, a brief transition from awake to sleep that occurs at the beginning of sleep. The heart rate, respiration rate, and eye movements are slow, and the muscles are relaxed with occasional twitches during the N1 stage. Meanwhile, N2 lasts about ten to twenty-five minutes, with the body temperature, respiratory rate, heart rate, and brain wave activity dropping lower than N1. N3 is the deepest sleep stage, also known as slow-wave sleep (SWS), with the body temperature, respiratory rate, blood pressure, and heart rate at their lowest levels and lasts about twenty to forty minutes (Patel *et al.,* 2022).

Meanwhile, REM sleep, also known as “paradoxical sleep”, is an enigmatic and intriguing sleep phase (Mukai & Yamanaka, 2023). This phase constitutes approximately 25% of the overall sleep duration, occurring approximately every 90 to

120 minutes throughout the night, with its duration extending as the sleep cycle advances. It is also known as “active sleep” or “dream sleep” since it is associated with dreaming and is not regarded as restful sleep (Patel *et al.,* 2022). Besides rapid movements of the eyes beneath closed eyelids, other biological changes during this phase are muscle atonia, vivid dreaming, and irregular heart rate, blood pressure, and breathing rate, which are near waking levels (Peever *et al.,* 2017; Crisan *et al.,* 2023; Mukai & Yamanaka*,* 2023).

2.2 Sleep deprivation

For a healthy adult, the American Academy of Sleep Medicine (AASM) suggests getting seven to nine hours of sleep every night. Sleep deprivation or disruption occurs when sufficient sleep is not obtained, and humans must get enough sleep to function properly. The International Classification of Sleep describes sleep deprivation as a restricted sleep pattern on most days for at least three months together with symptoms of daytime drowsiness (Chattu *et al.,* 2019). Partial sleep deprivation in animals and humans usually results in negative physiological responses, however, total and prolonged sleep deprivation might result in mortality (Nollet *et al.,* 2020). One of the roles of sleep is to regulate the autonomic nervous system. Thus, sleep deprivation can lead to impaired autonomic function which may result in cardiovascular problems. For example, a higher nocturnal systolic blood pressure (SBP) may occur during sleep deprivation because the SBP is reduced by about 10-20% during sleep (Liew & Aung, 2021). Sleep deprivation is frequently followed by an increase in hypothalamic-pituitary-adrenal (HPA) axis activity, which results in increased circulating levels of stress hormones, namely corticosterone in rats and cortisol in humans (Nollet *et al.,* 2020). Heart disease, stroke, and high blood pressure

are long-term comorbidities linked to sleep deprivation, all of which have significant morbidity and mortality (Javaheri *et al.,* 2008).

Sleep deprivation is caused by various circumstances, including environmental factors, lifestyle, psychosocial issues, and medical illness. Drowsiness, tiredness, headache, blurred vision, nausea, and moodiness have been attributed to symptoms of sleep deprivation (Chen *et al.,* 2005). In addition, sleep deprivation causes abnormal behaviour, increases suicidal thoughts in adolescents, increases the likelihood of motor vehicle accidents, and causes mental health problems (Lee *et al.,* 2012; Marks *et al.,* 2015). Sleep deprivation has also been demonstrated to impair cognition and performance, including flexible thinking, emotional reactivity, attention or alertness, memory formation, judgement, and decision-making (Khan *et al.,* 2022). Other physiologic effects of sleep deprivation include increased reactive oxygen species (ROS) and inflammatory markers, which are shown in Figure 2.2. Unfortunately, the physiologic effects of sleep deprivation can result in both short-term consequences like stress and somatic problems, as well as long-term consequences like obesity and cardiovascular disease.

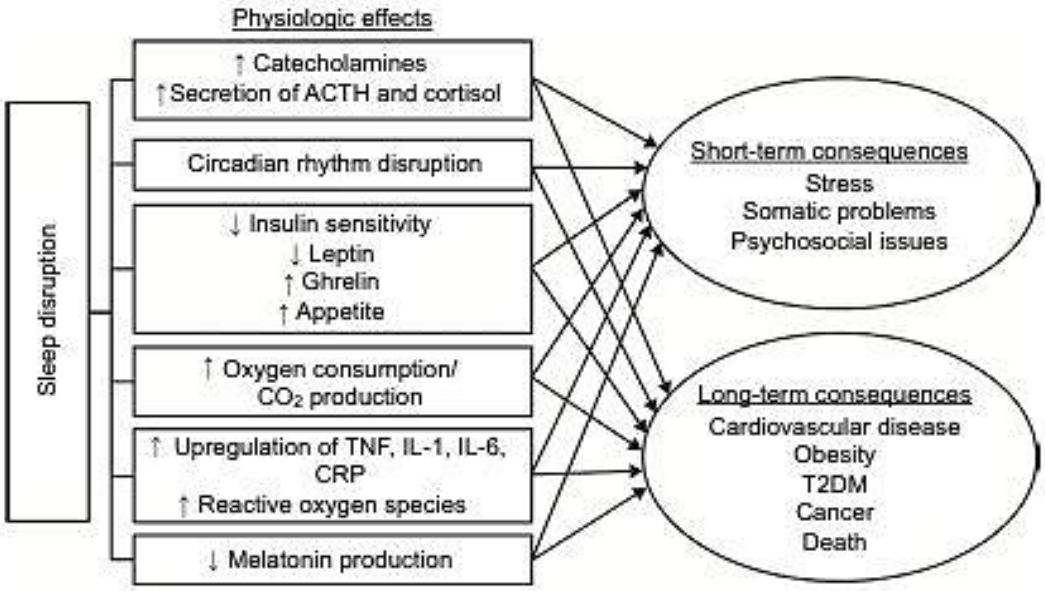

Figure 2.2: The physiologic effects of sleep deprivation that lead to short-term and long-term consequences (Medic *et al.,* 2017).

Abbreviations: ACTH; adrenocorticotropic hormone, CO_2; carbon dioxide, TNF; tumour necrosis factor, IL-1; interleukin 1, IL-6; interleukin 6, CRP; C-reactive protein

It has also been shown that REM sleep deprivation (REMsd) has detrimental effects on health. REMsd adversely affects memory consolidation (Groch *et al.,* 2015, Kim *et al.,* 2022), emotion (Goldstein & Walker, 2014; Turan *et al.,* 2021), and performance (Kim *et al.,* 2022). Besides, levels of oxidative stress markers in the thalamus (Mohd Shafie *et al.,* 2022), and pain perception in rats (Mohd Shafie *et al.,* 2022) and humans (Iacovides *et al.,* 2017) were also affected by REMsd. Previous studies have also demonstrated that REMsd was associated with mania-like behaviour in mice (Siddique *et al.,* 2018) and anxiety-depression-like behaviour in rats (Turan *et al.,* 2021). Based on the literature, most of the studies on REMsd were associated with memory and behaviour; however, recently, the effects of REMsd on the cardiovascular system were also reported. For example, REMsd results in impairment of the vascular endothelium (Jiang *et al.,* 2017; Tengku Adnan *et al.,* 2017) and brain capillaries or pericytes (Medina-Flores *et al.*, 2020).

2.3 Animal models of REM sleep deprivation

Animal-based research is crucial in sleep medicine research, especially in studying sleep physiology and identifying the underlying mechanisms of sleep disorders (Toth & Bhargava, 2013). There are substantial drawbacks to using humans in research, especially when testing specific drugs or treatments. Thus, an appropriate model that can provide a convincing evidence for developing pathophysiology of disease is essential. Animal models, including small and large animal models in sleep deprivation studies, have been established to elucidate the adverse effects of sleep deprivation (Zaragoza *et al.* 2011). The basic constructs on which animal models are built include a similar phenotype of illness to humans, and their response to pharmacological and non-pharmacological interventions is as effective as in humans (Nestler & Hyman, 2010).

Different models were utilised in the study of REMsd in rodents, which include the flowerpot technique (classic single platform, double platforms, and multiple small platforms), disk-over water, electrical stimulation, pendulum technique, and cold ambient environment. The flowerpot approach technique is widely used since it is simple, easy, cost-effective (Mahmoudi *et al.*, 2017; Tengku Adnan *et al.,* 2017), and does not require continuous monitoring and surgical intervention (Mehta *et al*., 2018; Pandey *et al.,* 2018). Jouvet et al. (1964) first applied this technique on cats (Jouvet *et al.,* 1964), and it has been extensively used on rats (Siran *et al.,* 2014; Jeddi *et al.,* 2016; Mahmoudi *et al.,* 2017; Tengku Adnan *et al.,* 2017) and mice (Yin *et al.,* 2017; Nasehi *et al.,* 2019). This technique did not increase the levels of serum corticosterone, a marker of stress, in REM sleep-deprived rats (Khan *et al.,* 2021). In addition, the technique has been demonstrated to deprive rats of 90-99% of their REM sleep (May

et al., 2005), while the amount of NREM sleep is reduced by approximately 1-10% (May *et al*., 2005; Elizabeth *et al*., 2017).

In the flowerpot technique, animals are maintained on small, raised platforms individually, typically inverted flowerpots about 4-10 cm in diameter in individual containers filled with water up to 1 cm below the platform surface, as shown in Figure 2.3 (Mohmed Nor *et al.,* 2021). The animals can sit on this small platform, crouch, and move around freely; however, they do not have enough space to relax completely. Atonia, a characteristic feature of REMsd is utilised in this technique (Khan *et al.,* 2021). When the animals reach REM sleep, they cannot maintain their extended, relaxed body position owing to atonia, and they fall into the water and wake up immediately (Pandey *et al.,* 2018). The longer duration of deprivation will cause the animal to fall into the water more frequently. Interestingly, in this technique, the animals quickly learn to wake up at the onset of REM sleep whereby they try to avoid REM sleep, and as a result, they do not frequently fall into the water (Mehta *et al.*, 2018; Khan *et al.,* 2021). The flowerpot technique was utilised in many REMsd studies, including systemic lipid peroxidation (Thamaraiselvi *et al.*, 2012), appetitive behaviour (Hanlo *et al.*, 2005), and memory (Alzoubi *et al.*, 2012). Other studies include pain-related gene expression (Siran *et al.*, 2014), and vascular endothelial function (Tengku Adnan *et al.,* 2017). Previous studies used varying durations of REMsd, such as 24-h and 48-h (Thamaraiselvi *et al.,* 2012), 72-h (Siran *et al.,* 2014; Tengu Adnan *et al.,* 2017), 96-h (Koban *et al.,* 2005), 5 days (Hanlon *et al.,* 2005), and 10 days (Koban *et al.,* 2008).

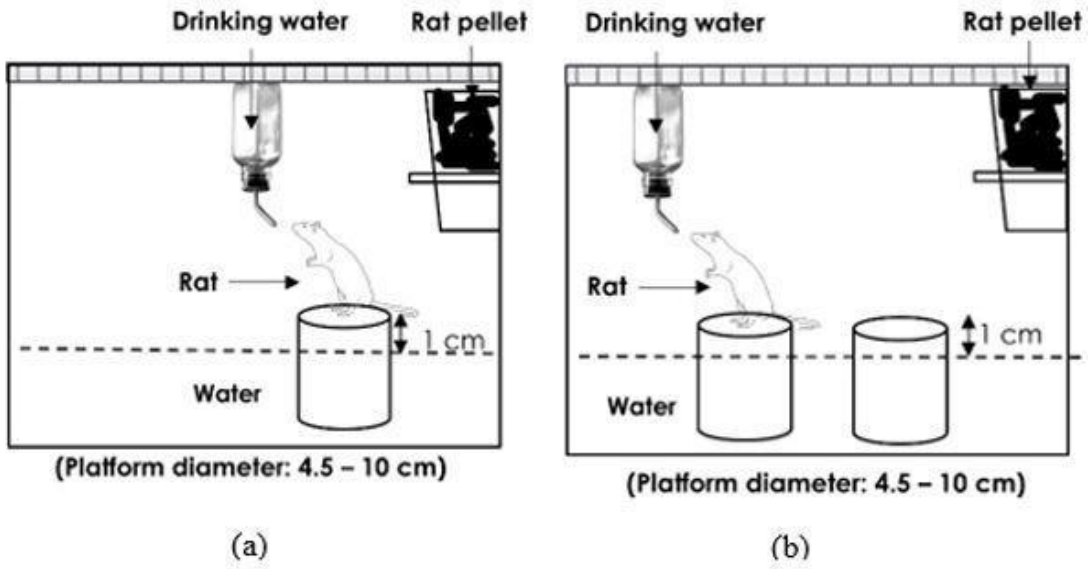

Figure 2.3: Schematic diagram of REM sleep deprivation animal models, (a) Classic single platform (b) Double platforms. The height of the platform is 1 cm above a pool of water (Mohmed Nor *et al.,* 2021).

2.4 Hypertension and its pathogenesis

According to the Malaysia Guideline on the Management of Hypertension, hypertension is defined as a persistent elevation of systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥90 mmHg (Ministry of Health, 2018). In Malaysia, hypertension is prevalent among adults, especially those with older age, higher body mass index (BMI), and diabetes (Zaki *et al.,* 2021). Lifestyle modification, including a healthy dietary pattern, weight loss, low sodium intake, and physical activity are key in treating hypertension (Carey *et al.,* 2022). Angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, calcium channel blockers, and diuretics are the first-line agents for hypertension (Brouwers *et al.,* 2021). Hypertension can be associated with target organ damage and organ function impairment, especially in the heart, kidneys, and brain (Konukoglu & Uzun, 2017).

In rats, hypertension is diagnosed when the average SBP/DBP exceeds 124/82 mmHg for male rats and 121/80 mmHg for female rats (Luo *et al.,* 2008). Rats have been a popular animal model for hypertensive research because their genomes have 99% sequence homology with human genomes. The pathogenesis of hypertension in rats are largely similar to humans in terms of response to environmental factors, haemodynamic factors (vascular resistance), mechanisms regulating arteriolar and venous constriction, sympathetic nerve activity, and humoral influences by the renin-angiotensin-aldosterone system (RAAS) and NOS (Lin *et al.,* 2016). Blood pressure in rats can be accurately measured using non-invasive tail-cuff plethysmography (Jama *et al.,* 2022). This method enables the determination of SBP that is close to genuine intra-arterial BP and is reproducible.

Hypertension is an important risk factor for endothelial dysfunction and atherosclerosis (Konukoglu & Uzun, 2017). Various factors have been attributed to the pathogenesis of hypertension, including endothelial dysfunction, genetics, activation of the sympathetic nervous system, and inflammatory mediators (Konukoglu & Uzun, 2017). When exposed to mechanical stimuli, endothelial cells can convert the stimuli into intracellular or biochemical signals, including apoptosis, proliferation, migration, remodelling, and gene expression (Konukoglu & Uzun, 2017) that can lead to hypertension (Rajendran *et al.,* 2013).

Oxidative stress-induced endothelial dysfunction is a well-known factor for the development of hypertension. Although sleep deprivation is linked to hypertension, the mechanisms behind the pathogenesis of increased blood pressure are still poorly understood. The pathogenesis involved in endothelial dysfunction that leads to hypertension includes oxidative stress (Brandes, 2014; Ye *et al.,* 2023), decreased $NO^{\cdot}$

bioavailability (Jiang *et al.,* 2017), and impaired fibrinolysis (Tabak *et al.,* 2009). Inflammation (Alexander *et al.,* 2021; Theofilis *et al.,* 2021), and parasympathetic dysfunction (Dimova *et al.,* 2020) have also been attributed to its pathogenesis. Oxidative stress and vascular inflammation increase vascular resistance by enhancing vascular smooth muscle cell proliferation and disrupting vasoconstriction and relaxation activities (Alexander *et al.,* 2021). There were limited studies on the effects of REMsd on blood pressure. Previous research has shown higher SBP in rats that were sleep-deprived for 72-h (Joukar *et al.,* 2013), 96-h (Jeddi *et al.,* 2016), 114-h (De Mesquita & Hale, 1992), and 120-h or five days (Jiang *et al.,* 2017) when compared to control rats.

In the *in vitro* functional study (myograph), impaired endothelial-dependent vasorelaxation was demonstrated in REMsd rats which indicated the occurrence of endothelial dysfunction following REMsd (Tengku Adnan *et al.,* 2017). Alterations in the structures of microcirculatory beds, including remodelling and rarefaction have been linked to hypertension, which leads to increased systemic vascular resistance (Konukoglu & Uzun, 2017).

2.5 Basic structure of blood vessel

The human body's vascular system comprises a huge number of vessels that play an important part in circulating oxygen, nutrients, and metabolic wastes (Pan *et al.,* 2022). The blood vessels are involved in the regulation of blood pressure, body temperature, metabolites, and immune cells (Weigel *et al.,* 2023). Three main types of blood vessels are arteries, veins, and capillaries that exert different functions (Pan *et al.,* 2022; Weigel *et al.,* 2023). The arteries are classified into two types: muscular arteries and elastic arteries. Muscular arteries include anatomically termed arteries

such as the brachial, radial, and femoral arteries, whereas elastic arteries include aorta and pulmonary arteries. The elastic arteries have more elastic tissue, but less smooth muscle than muscular arteries (Tucker *et al.,* 2023).

Histologically, the wall of the blood vessel is divided into three layers, or ‘tunics’; from innermost to outermost layers are tunica intima (internal), tunica media, and tunica adventitia (externa), as shown in Figure 2.4. Meanwhile, the structure of blood vessels in rats is similar to that of humans (Figure 2.4(d)). Tunica intima, the innermost and thinnest layer, comprises a monolayer of endothelial cells or endothelium that line the whole vasculature, basal lamina, and subendothelial connective tissue (Pushpalatha & Bhat, 2020). Tunica media, the middle layer, predominantly consists of extracellular matrix, including elastin, collagen, and proteoglycans, and multiple layers of smooth muscle cells (Jana *et al.,* 2019; Pan *et al.,* 2022).

The extracellular matrix is essential for maintaining the vessel's structural integrity because it protects the wall of big elastic arteries from severe wall stresses. Tight and gap junctions connect smooth muscle cells, facilitating the intercellular transfer of signalling molecules and, as a result, coordinated control of vessel diameter. Smooth muscle cells have a variety of functions based on their phenotype, which is typically characterised as contractile or synthetic (Dave *et al.,* 2022). Contractile smooth muscle cells have a long spindle structure with a single, elongated nucleus, whereas synthetic smooth muscle cells have a "cobblestone" morphology. Primary role of contractile smooth muscle cells is to modulate vessel diameter, thus, regulate blood pressure and tissue perfusion. Synthetic smooth muscle cells secrete extracellular matrix and matrix metalloproteinases, which help to remodel the extracellular matrix.

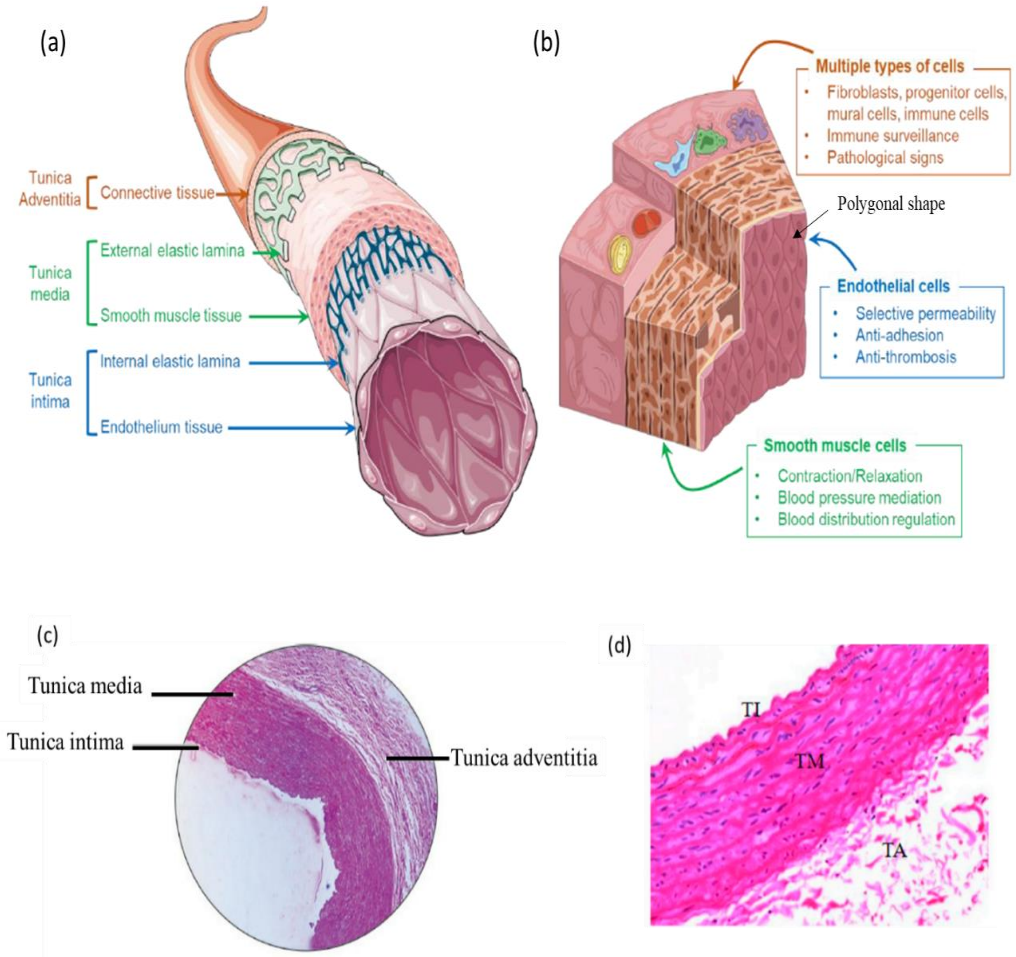

Figure 2.4: Structure of blood vessel. Schematic illustration of the artery (a) and (b). Photomicrographs of normal arteries from a human at 200x magnification (c) and a rat at 400x magnification (d). The wall consists of three layers: tunica intima (TI), tunica media (TM), and tunica adventitia (TA). (Pushpalatha & Bhat, 2020; Hamzah *et al.,* 2021; Pan *et al.,* 2022). The external elastic laminae and internal elastic laminae separate the tunica media from the intima and externa, respectively. The outermost layer of the vessels, the tunica adventitia or externa, is fibroelastic with numerous capillaries and arterioles. There are various cells in this layer that include active fibroblasts, phagocytic, and endothelial cells embedded in the fibrous stroma.

2.6 Endothelium

The endothelium is a monolayer of flattened polygonal cells, approximately 10 to 60 × 10^{12} cells that rest on the basement membrane (Pushpalatha & Bhat, 2020). It lines the vessel walls of the entire circulatory system and plays an important function in maintaining vascular homeostasis (Wang *et al.,* 2022). The endothelium acts as a haemocompatible lining, assisting in maintaining and regulating systemic blood flow, the blood coagulation system, and tissue perfusion (Yau *et al.,* 2015). The endothelial cell surface is covered by a layer of surface glycoprotein (glycocalyx) comprising negatively charged proteoglycans, glycosaminoglycans, and adsorbed plasma proteins. Glycocalyx protects the endothelium against leukocyte attachment and modulates haemostatic and inflammatory reactions (Yilmaz *et al.,* 2019).

The endothelium regulates cellular adhesion, smooth muscle cell proliferation, vascular tone, leukocyte trafficking, the coagulation pathway, all of which may contribute to the development of CVD (Godo & Shimokawa, 2017; Fang *et al.,* 2019). Vascular tone refers to the degree of constriction experienced by a blood artery relative to its maximally dilated state (Suganya *et al.,* 2016). The importance of endothelium is initially discovered by its effect on vascular tone. This is achieved by producing and releasing a variety of vasoactive molecules that either constrict or relax the vessel, as well as responding to and modifying circulating vasoactive mediators such as bradykinin and thrombin.

The endothelium regulates vascular tone through interactions with peripheral nervous system components and is engaged in thrombolysis and coagulation processes (Pan *et al.,* 2022). It releases multiple vasoactive factors by smooth muscle cells that modulate vasorelaxation (endothelium-derived relaxing factors - EDRFs) and

vasoconstriction (endothelium-derived contraction factors - EDCFs) (Mangana *et al.,* 2021). EDRFs include nitric oxide ($NO^{\cdot}$), prostacyclin, bradykinin, and endothelium-derived hyperpolarising factors (EDHFs), while the EDCFs include endothelin-1 (ET-1), angiotensin II (Ang II), and thromboxane A_2 (TXA_2) (Mangana *et al.,* 2021). Damage to the endothelium causes an imbalance between vasodilation and vasoconstriction, leading to leucocyte adhesion, increased endothelial permeability, cytokine production, and platelet aggregation (Rajendran *et al.*, 2013; Suganya *et al.*, 2016).

The endothelium is also a barrier between blood coagulation factors and subendothelial prothrombotic extracellular matrix components. It releases vasoactive substances, which influence platelet reactivity, coagulation, and fibrinolysis that contribute to thrombotic formation (Wang *et al.,* 2018). In healthy endothelium, the thrombosis is suppressed by antithrombotic factors such as tissue plasminogen activator (tPA) and thrombomodulin (TM), which inhibit platelet aggregation (Suganya *et al.*, 2016; Wang *et al.,* 2018). Meanwhile, in damaged endothelium, the prothrombotic factors such as tissue factors (TF), plasminogen activator inhibitor-1 (PAI-1), and von Willebrand factor (vWF) are released and responsible for promoting platelet aggregation and adhesion (Suganya *et al.*, 2016; Wang *et al.,* 2018).

Endothelial cells secrete vascular endothelial growth factor (VEGF), the major growth factor in the endothelium that regulates endothelial cell development. In addition, growth factors such as insulin-like growth factor (IGF), platelet-derived growth factor-β (PDGF), transforming growth factor (TGF), and basic fibroblast growth factor (bFGF) are involved in vascular development and promote angiogenesis (Suganya *et al.,* 2016). Moreover, endothelium is also involved in regulating cell

adhesion and vascular permeability due to inflammation through factors such as intracellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM). These molecules are crucial for maintaining endothelial barrier integrity, and mediating paracellular and transcellular migration (Suganya *et al*., 2016; Wautier & Wautier, 2022). Hence, the vasoprotective effects of a healthy endothelium include vasodilation, suppression of smooth muscle cell growth, and inhibition of inflammatory responses, in which the vasodilators counteract the effects of EDCF (Suganya *et al.,* 2016). The factors that are secreted by a normal endothelium and a damaged endothelium are shown in Figure 2.5.

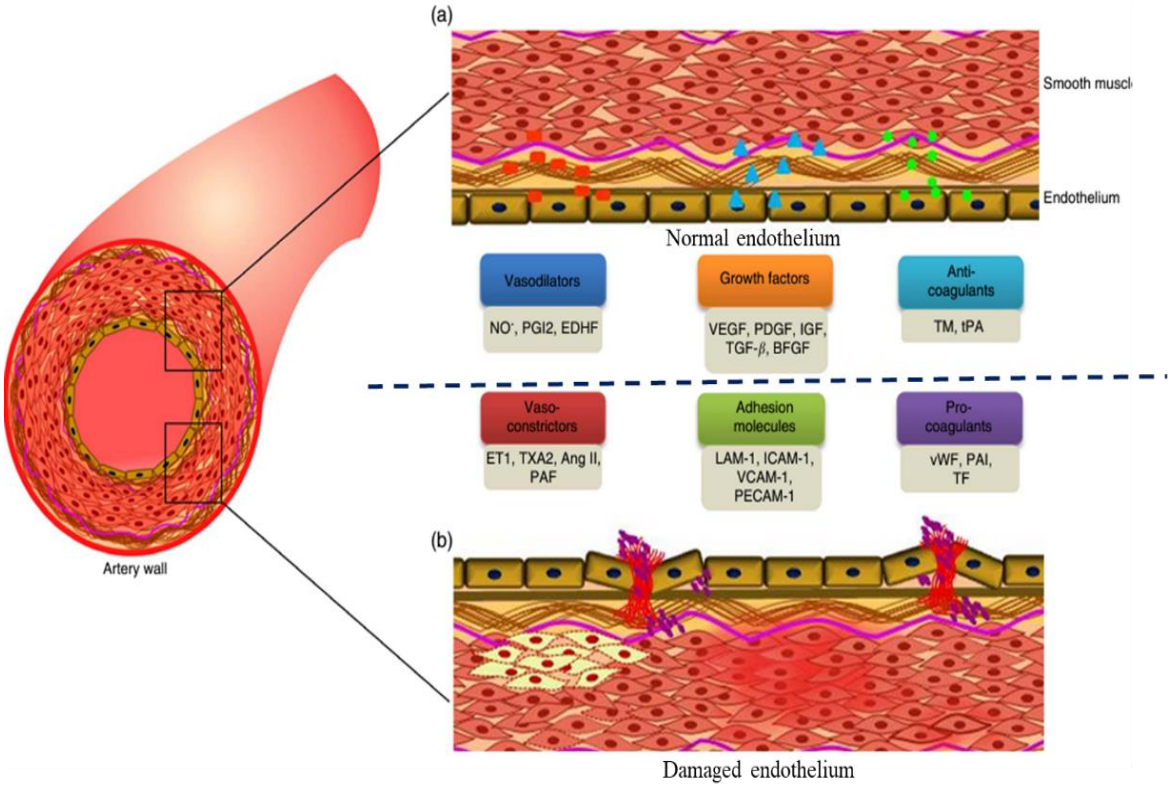

Figure 2.5: Factors secreted by the endothelium. (a) A normal or healthy endothelium secretes vasodilators, growth factors, and anti-coagulants. (b) A damaged endothelium secretes vasoconstrictors, adhesion molecules, and pro-coagulants (Suganya *et al.*, 2016).

Abbreviations: NO˙; nitric oxide, PGI2; prostacyclin, EDHF; endothelium-derived hyperpolarising factor, 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-1; endothelin-1, TXA_2; thromboxane A_2, Ang II; angiotensin II, PAF; platelet-activating factor, LAM-1; leucocyte adhesion molecule-1, ICAM-1; intercellular adhesion molecule 1, VCAM-1; vascular cell adhesion molecule 1, PECAM-1; platelet cell adhesion molecule-1, vWF; vonWillebrand factor, PAI-1; plasminogen activator inhibitor type-1, TF; tissue factor.