ANTIHYPERTENSIVE AND VASOPROTECTIVE EFFECTS OF EPIGALLOCATECHIN-3-GALLATE (EGCG) IN SPONTANEOUSLY HYPERTENSIVE RATS

By

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ABSTRACT

ANTIHYPERTENSIVE AND VASOPROTECTIVE EFFECTS OF EPIGALLOCATECHIN-3-GALLATE (EGCG) IN SPONTANEOUSLY HYPERTENSIVE RATS

YUCINDA KHOR YEE YAN

Epigallocatechin-3-gallate (EGCG), a catechin found in green tea is demonstrated to exert blood pressure-lowering effect in hypertensive subjects with elevated oxidative stress. However, the exact underlying mechanism remains unknown. The current study investigated whether the decrease in ROS production via the modulation of angiotensin type I receptor (AT_1) contributes to the vasoprotective action of EGCG observed in an animal model with primary hypertension. Wistar-Kyoto (WKY) rats and Spontaneously Hypertensive Rats (SHR) were grouped into WKY Control, SHR Control, SHR treated with EGCG (50mg/kg/day) and SHR treated with losartan (10mg/kg/day) respectively. The treatment was given daily for 4 weeks by oral gavage and the blood pressure was monitored by tail-cuff method every 3 days. Acetylcholine-induced vascular relaxation was assessed in isolated aortic rings contracted with phenylephrine at the end of treatment. The vascular levels of nitric oxide (NO), reactive oxygen species (ROS), tetrahydrobiopterin (BH_4) and cyclic guanosine monophosphate (cGMP) were also measured. Lastly, expression of AT_1 receptor protein was analysed. After 4 weeks of treatment, the was a significant decrease in systolic

blood pressure of the SHR treated with EGCG and losartan. In line with this, there was significant improvement in endothelium-dependent relaxation in aortic ring isolated from EGCG and losartan-treated SHR groups. The ROS level was also decreased in these groups. Besides that, the levels of NO, BH_4 and cGMP were also significantly increased in SHR treated with EGCG or losartan. It was also observed that there was a decreasing trend in the protein expression of AT_1 receptor in SHR treated with EGCG and losartan. In conclusion, this study shows that EGCG improves endothelial function in SHR by attenuating oxidative stress and elevating the bioavailability of vascular NO, which may be modulated partly by downregulation of vascular AT_1 receptors. The increase in endothelium-dependent relaxation in part may have contributed to the blood pressure lowering effect of EGCG in the hypertensive animals.

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APPROVAL SHEET

This dissertation entitled **"ANTIHYPERTENSIVE AND VASOPROTECTIVE EFFECTS OF EPIGALLOCATECHIN-3-GALLATE (EGCG) IN SPONTANEOUSLY HYPERTENSIVE RATS"** was prepared by YUCINDA KHOR YEE YAN and submitted as partial fulfillment of the requirements for the degree of Master of Medical Science in M. Kandiah Faculty of Medicine and Health Sciences at Universiti Tunku Abdul Rahman.

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LIST OF ABBREVATIONS

ACE	Angiotensin-converting enzyme
ACh	Acetylcholine
AEU	Animal Experimental Unit
Ang I	Angiotensin I
AR	Aldose reductase
ARBs	Angiotensin II receptor blockers
AT_1	Angiotensin II type 1
AT_2	Angiotensin II type II
ATP	Adenosine triphosphate
AUC	Area under the curve
BH_2	Dihydrobiopterin
BH_4	Tetrahydrobiopterin
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
cGMP	Cyclic guanosine monophosphate
CR	Carbonyl reductase
CVDs	Cardiovascular diseases
DAF	Diacetate 4-Amino-5-Methylamino-2',7' Difluorofluorescein
DBP	Diastolic blood pressure

DHFR	Dihydrofolate reductase
DiOHF	3′,4′-Dihydroxyflavonol
EC	Epicatechin
ECG	Epicatechin-3-gallate
EDCFs	Endothelium-derived contracting factors
EDHF	Endothelium-derived hyperpolarizing factor
EDRFs	Endothelium-derived relaxing factors
EGC	Epigallocatechin
EGCG	Epigallocatechin-3-gallate
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
GPCRs	G protein-coupled receptors
GTP	Guanosine 5'-triphosphate
GTPCH	GTP cyclohydrolase
H_2-NTP	Dihydroneopterin triphosphate
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase
HUVECs	Human umbilical vein endothelial cells
iNOS	Inducible nitric oxide synthase
mTOR	Mammalian target of rapamycin
NFκB	Nuclear factor kappa B
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide

NOS	Nitric oxide synthase
NOx	Nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase
NPs	Natriuretic peptides
O_2^-	Superoxide anions
OCT	Optimal cutting temperature
$ONOO^-$	Peroxynitrite
PBS	Phosphate-buffer saline
PDEs	Phosphodiesterases
PE	Phenylephrine
PGI_2	Prostacyclin
PKGs	Protein kinases
RAAS	Renin-angiotensin-aldosterone system
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
sGC	Soluble guanlyl cyclase
SHR	Spontaneously Hypertensive Rats
SNP	Sodium nitroprusside
TBST	Tris-buffered saline with 0.1% Tween® 20 detergent
tMCAO	Transit middle cerebral artery occlusion
TXA_2	Thromboxane
VPR	Volume pressure recording

VSMCs	Vascular smooth muscle cells
WKY	Wistar-Kyoto

CHAPTER 1

INTRODUCTION

1.1 Research background

Cardiovascular diseases (CVDs) are one of the primary causes of death globally, taking the life of approximately 17.9 million people each year (WHO, 2019). Hypertension, a condition whereby a person's blood pressure is elevated, is a well-established risk factor for CVDs such as coronary heart disease, myocardial infarction, heart failure and stroke. If untreated, this condition would quickly become a public health burden due to increasing number of fatalities and disabilities as the prevalence of hypertension rises (Miazgowski *et al.*, 2021). In the past decades, hypertension has become more prevalent especially in developing countries with low- and middle-income due to multiple factors such as an aging population and a development in economy (Zaki *et al.*, 2021). A common phenomenon observed among hypertensive patients is an increase in total peripheral resistance with a normal cardiac output (Delong and Sharma, 2019). One of the factors that attributes to the increase in peripheral resistance is the abnormalities in the structure and function of the blood vessels leading to the occurrence of endothelial dysfunction (Gallo, Volpe and Savoia, 2022).

Endothelial dysfunction can be defined as the imbalance in production of vasodilator molecules such as endothelium-derived hyperpolarizing factor (EDHF), nitric oxide (NO) as well as prostacyclin (PGI_2) and vasoconstrictor

molecules such as endothelin-1 (ET-1), superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) as well as angiotensin II (Ang II) (Laurindo *et al.*, 2018), resulting the loss of its normal function (Medina-Leyte *et al.*, 2021). Endothelial dysfunction can be characterized by reduced release and/or the depletion of the bioavailability of NO, the key regulator in the vasculature, leading to a compromised NO-cyclic GMP (cGMP) signalling pathway which mediates vasodilation (Ataei Ataabadi *et al.*, 2020).

NO has been associated with the physiological and pharmacological function of various blood vessels either as an antioxidant or to maintain homeostasis (Cyr *et al.*, 2020). NO acts as an endothelium-derived relaxing factor (EDRF) which leads to vascular relaxation (Schmidt and de Wit, 2020). NO in the blood vessels is mainly produced by endothelial nitric oxide synthase (eNOS) (Nauli, 2022). Thus, eNOS plays a crucial part in maintaining contraction and relaxation of the vascular tissues (Nappi *et al.*, 2022). Tetrahydrobiopterin (BH_4) is one of the important cofactors for the proper functioning of eNOS to generate NO from its precursor L-arginine (Janaszak-jasiecka *et al.*, 2021). The produced NO is then released into the vascular smooth muscle cells (VSMCs) leading to activation of soluble guanlyl cyclase (sGC) which later converts guanosine 5'-triphosphate (GTP*)* to cyclic guanosine monophosphate (cGMP*)*. The increased production of cGMP will induce smooth muscle relaxation thus results in vasodilation (Migliato Martinelli *et al.*, 2018).

Oxidative stress or a decrease in BH_4 levels will disrupt the coupled state of eNOS (Shaito *et al.*, 2022). Studies done using experimental animals such as spontaneously hypertensive rats (SHR) (Long *et al.*, 2018), atherosclerosis mice (Ponnuswamy *et al.*, 2012), transit middle cerebral artery occlusion (tMCAO) mice (Mahmood *et al.*, 2017) have shown that the fundamental cause of endothelial dysfunction is eNOS uncoupling. The uncoupling of eNOS is linked to the enhanced oxidation of BH_4 by reactive oxygen species (ROS) including superoxide (O_2^-) derived from vasculature NADPH oxidases (NOx) (Förstermann, Xia and Li, 2017). Under this condition, O_2^- scavenges NO to produce the toxic radical which is peroxynitrite ($ONOO^-$) which further reduce the bioavailability of NO and worsen endothelial dysfunction (Tejero, Shiva and Gladwin, 2019).

Angiotensin II type 1 (AT_1) receptor is a component of the renin-angiotensin-aldosterone system (RAAS) (Ziaja *et al.*, 2021). Activation of AT_1 receptors by Ang II promotes vasoconstriction (Eguchi *et al.*, 2018), oxidative stress (Birk et al., 2021), inflammation (Sun *et al.*, 2020) as well as salt and water reabsorption (Leite *et al.*, 2022). Overactivation of AT_1 receptors frequently leads to cardiovascular dysfunction. The expression of AT_1 receptor is elevated in the vasculatures during cardiovascular pathogenesis (Verma *et al.*, 2021). It has been demonstrated that the binding of angiotensin II to its receptor that is AT_1 receptor, activates it. The activation of AT_1 receptor then led to the activation of NOx which subsequently led to formation of ROS thus increasing oxidative stress (Vermot *et al.*, 2021). A study had demonstrated that endothelial dysfunction D-galactose-induced aging rats is associated with

the inhibition of oxidative stress accompanied by the downregulation of the angiotensin II/AT_1 receptor pathway (Dai *et al.*, 2018). Since elevation of O_2^- is strongly associated with a decrease in BH_4 level, an increase in AT_1 receptors will in part lead to endothelial dysfunction due to eNOS uncoupling. Indeed, an increase in Ang II has been shown to cause endothelial dysfunction through NADPH-derived ROS production in several previous studies. Ding and colleagues had demonstrated that endothelial dysfunction occurred when $PP2_A$ is activated by Ang II/ AT_1 receptor pathway through the downregulation of catalytic subunit Tyr307 phosphorylation (Ding *et al.*, 2020). An animal study also demonstrated Ang II induced endothelial dysfunction in mouse ophthalmic arteries through activation of AT_1 receptor and NOx2-dependent ROS formation (Birk et al., 2021).

Tea obtained from *Camellia sinensis* plant is usually taken to improve health. Teas are categorised into three main groups which are oolong tea, black tea and green tea (Malabadi *et al.*, 2022). Among these, green tea has gained increased attention due to the beneficial effects of its various composition on health (Prasanth *et al.*, 2019). These compositions including vitamins, protein, sterols, minerals, some trace elements especially polyphenols called catechins (Prasanth *et al.*, 2019). The most abundant tea polyphenol in green tea is epigallocatechin-3-gallate (EGCG) (Nain *et al.*, 2022). It has been demonstrated that EGCG is able to improve vascular function, has antioxidative and anti-inflammatory properties which is able to lower the risk of cardiovascular diseases (Mokra, Joskova and Mokry, 2023). An *in vivo* study has demonstrated that green tea administration in SHR delayed the

development of hypertension due to the antioxidative properties of catechins (Qian *et al.*, 2018). Further on, a study on human umbilical vein endothelial cells (HUVECs) by Alvarez-Cilleros and colleagues has demonstrated that EGCG improved endothelial dysfunction by decreasing the generation of ROS and the activation of stress-related pathway (Álvarez-Cilleros *et al.*, 2018). On the same track, Aggio's team have shown that EGCG leads to endothelium-dependent vasorelaxation in rat thoracic aorta (Aggio *et al.*, 2013). Mohd Sabri' team had also reported that EGCG demonstrated improvement in vascular function by lowering oxidative stress and eNOS uncoupling, thus increasing NO production in Ang II-hypertensive mice (Mohd Sabri *et al.*, 2022).

One of the main mechanisms that induce oxidative stress is the activation of NOx due to stimulation of AT_1 receptor. Although studies have shown that treatment with EGCG decreases oxidative stress and eNOS uncoupling thus attenuates endothelial dysfunction, the modulation of EGCG on AT_1 receptors is yet to be elucidated. Hence, the aim of this study is to investigate the effect of EGCG on the modulation of AT_1 receptors in improving endothelial dysfunction in a genetically hypertensive animal model.

1.2 Problem statement

According to previous study, EGCG has been demonstrated to exhibit antihypertensive property (Potenza *et al.*, 2007). A recent study has also shown that the antihypertensive effect of EGCG is attributed to its ability to increase eNOS coupling and decrease oxidative stress (Mohd Sabri *et al.*, 2022). The stimulation of the AT_1 receptor leads to the activation of NOx, which is one of the primary mechanisms causing oxidative stress. Although research has shown that treatment with EGCG reduces oxidative stress and eNOS uncoupling, the effect of EGCG on AT_1 receptors is yet to be elucidated. Therefore, the study aims to investigate the effect of EGCG on the modulation of AT_1 receptor in improving endothelial dysfunction in a genetically hypertensive animal model.

1.3 Research hypothesis

EGCG modulates AT_1 receptor leading to a decrease in oxidative stress and an increase in NO levels resulting in an improvement of endothelial function in SHR.

1.4 Research objectives

This study aims to investigate the modulation of AT_1 receptor on vasoprotective effect of *in vivo* treatment with EGCG in SHR

i. To investigate if treatment with EGCG decrease blood pressure in SHR

ii. To investigate if treatment with EGCG improve endothelial function via the decrease in oxidative stress

iii. To investigate if treatment with EGCG modulate the expression of AT_1 receptor

CHAPTER 2

LITERATURE REVIEW

2.1 Cardiovascular diseases (CVDs)

Cardiovascular diseases (CVDs), the leading cause of death worldwide, have resulted in a decrease in the quality of life for those who are suffering from these conditions (Abbafati *et al.*, 2020; Vaduganathan *et al.*, 2022). Globally, there is an increase in the burdens of CVD especially disability-adjusted life years and fatalities (Roth *et al.*, 2020). CVDs are a group of diseases or conditions that impacted the heart and blood vessels, such as coronary heart disease, peripheral arterial disease, stroke and aortic disease (Wilson, 2019). There are numerous risk factors of CVDs, the common ones including hypertension, smoking, diabetes/glucose intolerance, consumption of alcohol, obesity and unhealthy diet (Bays *et al.*, 2022).

2.2 Hypertension

Hypertension or elevated blood pressure is known to be one of the highest risk factors of CVDs. The outcome of the 2019 National Health and Morbidity Survey has shown that 30% out of 15000 Malaysian adults aged 18 and above have an elevated blood pressure (Ariffin *et al.*, 2022). Another study conducted on Malaysian adults aged 30 and above showed that 42% out of

11,000 participants have hypertension (Ab Majid *et al.*, 2018). Hypertension is thus often referred as the 'silent killer'. There are two categories of hypertension, namely primary hypertension and secondary hypertension (Litwin and Kułaga, 2021). Primary hypertension refers to a condition whereby there is an elevated blood pressure without any medical causes whereas secondary hypertension refers to a condition whereby there is an elevated blood pressure due to a medical cause (Oparil *et al.*, 2018). Hypertension can be diagnosed through the measurement of blood pressure, where patients with persistently raised pressure (140/90 mmHg or higher) is considered as hypertensive (Flack and Adekola, 2020). The classification of hypertension in Malaysia according to the Ministry of Health clinical practice guideline is shown in Table 1 (Malaysia, 2018).

Table 1: Classification of blood pressure in Malaysia (Kamal, 2023)

Classification	**Systolic blood pressure (SBP; mmHg)**	**Diastolic blood pressure (DBP; mmHg)**
Optimal	<120	<80
Normal	<130	<85
High normal	130-139	85-89
Hypertension	≥140	≥90

2.2.1 Pathophysiology of hypertension

The increased blood pressure in hypertensive individuals is caused by a large number of interrelated factors, many of which may have relative importance depending on the individual. Salt intake (Grillo *et al.*, 2019), obesity (Leggio *et al.*, 2017), the renin-angiotensin aldosterone system (RAAS) (Fountain and Lappin, 2018), insulin resistance (Sinha and Haque, 2022) and the sympathetic nervous system (DeLalio, Sved and Stocker, 2020) are some of the aspects that have been thoroughly investigated. Other variables including genetics (Maaliki, Itani and Itani, 2022), endothelial dysfunction (seen by alterations in endothelin and nitric oxide) (Gallo, Volpe and Savoia, 2022a), low birth weight and nutrition throughout pregnancy (Jebasingh and Thomas, 2022), and neurovascular abnormalities (Iadecola and Gottesman, 2019) have all been examined in the recent years. Vascular endothelial cells are essential in the regulation of cardiovascular by secreting a variety of potent local vasoactive chemicals, such as the vasodilator molecule NO and the vasoconstrictor peptide endothelin (Kostov, 2021). Endothelial dysfunction has been linked to human essential hypertension. An appealing therapeutic strategy for reducing some of the significant consequences of hypertension is through the modulation of endothelial function.

2.3 Vascular endothelium

The cross section of an artery (Figure 2.1) shows that the walls of arteries are thicker compared to other blood vessels as they are constantly subjected to high pressure. The arterial walls are made up of several layers namely tunica intima, tunica media and tunica adventitia. Tunica intima, the innermost layer, is composed of endothelial cells that are attached to the basal lamina (connective tissue). Tunica medica, the middle layer, is composed of vascular smooth muscle cells that are responsible in controlling the vascular tone. The outermost layer, called tunica adventitia, is composed of connective tissue, perivascular adipose tissue, and nerve endings (Medina-Leyte *et al.*, 2021). The endothelium comprised of a monolayer of endothelial cells (0.2– 4 μm-thick) that lines the inner wall of the blood vessels. Although the amount of smooth muscle as well as connective tissue in the vessel wall varies depending on the vessel's diameter and function, the endothelium is always present (Mironov *et al.*, 2023). It is distinguished by scant intercellular gaps, thus forming a dynamic barrier between the blood and the underlying tissues (Neubauer and Zieger, 2022). A number of factors regulate the endothelial integrity and permeability. These include the composition of the basement membrane, endothelial membrane electrostatic charges, and intercellular junctions (Mironov *et al.*, 2023). The endothelium has antiplatelet, anticoagulant, and fibrinolytic characteristics under normal physiological conditions (Neubauer and Zieger, 2022).

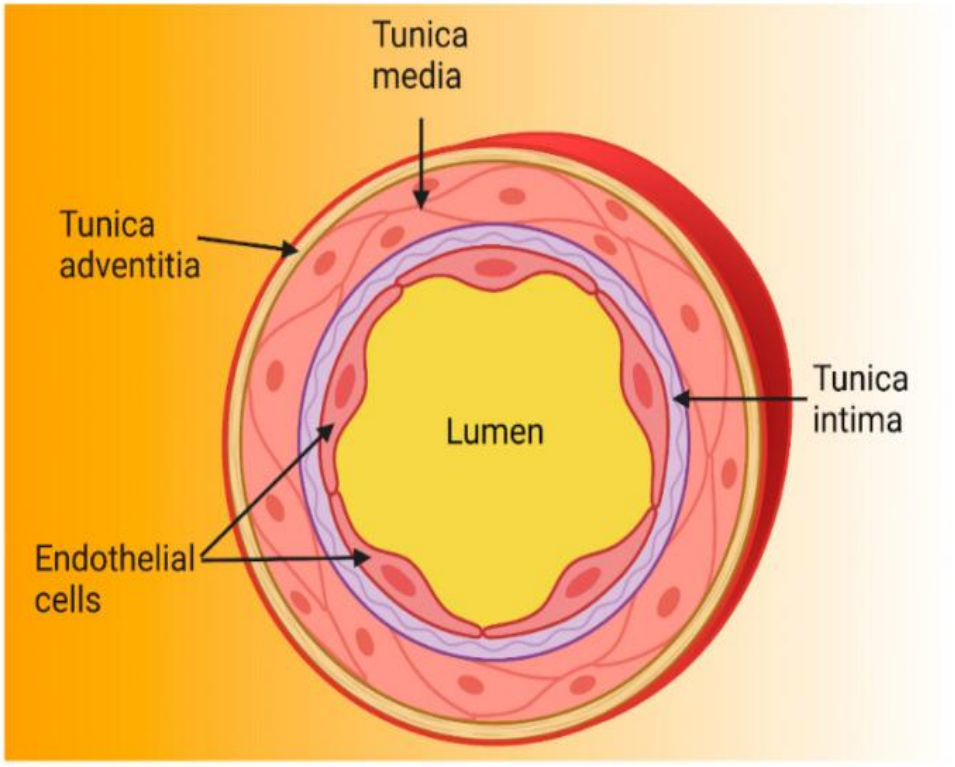

Figure 2.1 Diagram of the cross section of an artery (Medina-Leyte *et al.*, 2021)

2.3.1 Endothelium-derived vasoactive substances

Multiple vasoactive substances are released by the endothelium. These includes vasodilatory substances such as NO, prostacyclin (PGI_2), and EDHF as well as vasoconstrictive substances such as thromboxane A_2 (TXA_2) and endothelin-1 (ET-1) (Mangana, Lorigo and Cairrao, 2021). Early on in the development of vascular disease, alterations in vascular reactivity are caused by changes in the release and action of the vasoactive mediators produced from the endothelium (Mangana, Lorigo and Cairrao, 2021).

2.3.1.1 Endothelium-derived relaxing factors (EDRFs)

NO, PGI_2 and EDHF are examples of a various of EDRFs that are released by the endothelium (Laurindo *et al.*, 2018). NO acts as a common endothelium-dependent vasodilator and is crucial for the maintenance of basal vasodilator tone (Ahmad *et al.*, 2018). The nitric oxide synthase (NOS) enzyme forms NO by converting the L-arginine to NO (Król and Kepinska, 2021). Endothelial cells respond to shear stress, hypoxia, and a variety of other stimuli that also release PGI_2, in addition to NO (Krüger-Genge *et al.*, 2019). PGI_2 that is released into the endothelial cells can cross endothelial cell membranes and operate as a local vasorelaxing agent since it is lipid soluble (Kang, 2014). As an essential vasodilator in the microcirculation, EDHF works by opening potassium channels to promote potassium ion efflux which leads to hyperpolarization and relaxation of vascular smooth muscle (Jackson, 2022). The current discussion will centre on NO because it is largely responsible for a blood vessel's capacity to dilate. There are three different types of NOS: neuronal (nNOS), which produces NO to act as a neuronal messenger that regulates the release of synaptic neurotransmitters (Picón-Pagès, Garcia-Buendia and Muñoz, 2019), inducible NOS (iNOS), which is only expressed in cells that have been exposed to inflammatory mediators or other harmful stimuli that activate the macrophages (Ning *et al.*, 2019), and eNOS, which produces NO in the vasculature (Li *et al.*, 2016).

2.3.1.2 Endothelium-derived contracting factors (EDCFs)

A variety of EDCFs mediate endothelium-dependent vasoconstriction such as ET-1, Ang II, TXA_2, prostaglandin H_2, and ROS. Inflammatory cells such as interleukins and TNFα increase the regulation of ET-1 synthesis. However, this regulation is inhibited by the release of NO and PGI_2. (Houde, Desbiens and D'Orléans-Juste, 2016). ET-1 receptors are expressed in endothelial cells (ET_{-B1}) and smooth muscle cells (ET_A and ET_{-B2}). When ET-1 binds to ET_A and ET_{-B2} receptors, extracellular Ca^{2+} molecules enter the vascular smooth muscle cell through the Ca^{2+} channels and causing vasoconstriction (Mangana, Lorigo and Cairrao, 2021). It has been shown that TXA_2 promotes smooth muscle contraction and hypertrophy as well as platelet shape change and aggregation. It is generated by endothelial cells, neutrophils, platelets, and macrophages (Rucker and Dhamoon, 2019). The RAAS predominates in the homeostatic equilibrium of the cardiovascular system and body fluids. The most significant effector in the renin-angiotensin system is Ang II, a linear polypeptide of 8 amino acids (Zhu *et al.*, 2022). Angiotensin I (Ang I) is transformed to Ang II by ACE upon activation of the ACE-Ang II-AT_1 receptor axis. The AT_1 receptor and the AT_2 receptor are the two receptors that Ang II binds to (Fountain and Lappin, 2018). Vasoconstriction, inflammation, and oxidative stress are all made worse when Ang II binds to the angiotensin type 1 receptor.

2.3.2 Normal endothelial function

eNOS in the endothelial cells produces NO through the interaction of oxygen and L-arginine (Cahill and Redmond, 2016) with Ca^{2+} and calmodulin as co-factors in this oxidation process. After that, NO and L-citrulline are generated in stoichiometric amounts (Jamwal and Sharma, 2018). In the presence of fluid shear stress and vasoactive substances such as acetylcholine, bradykinin and adenosine triphosphate (ATP), eNOS is activated via the phosphorylation of proteins, protein-protein interactions, Ca^{2+} signaling, and subcellular protein translocations (Pi, Xie and Patterson, 2018). NO is released when eNOS is activated, and it diffuses easily through the membrane of endothelial cells. The NO that diffused into the vascular smooth muscle cells activates sGC which subsequently converts GTP to cGMP and lead to a rise in intracellular cGMP. By activating a protein kinase, this second messenger reduces the activation of calcium-calmodulin of the myosin light chain kinase and inhibits calcium influx into the cell. Following that, there is a reduction in the phosphorylation of the myosin light chain which prevents the development of tension in the surrounding smooth muscle cells and causes vasodilation (Figure 2.2) (Cahill and Redmond, 2016).

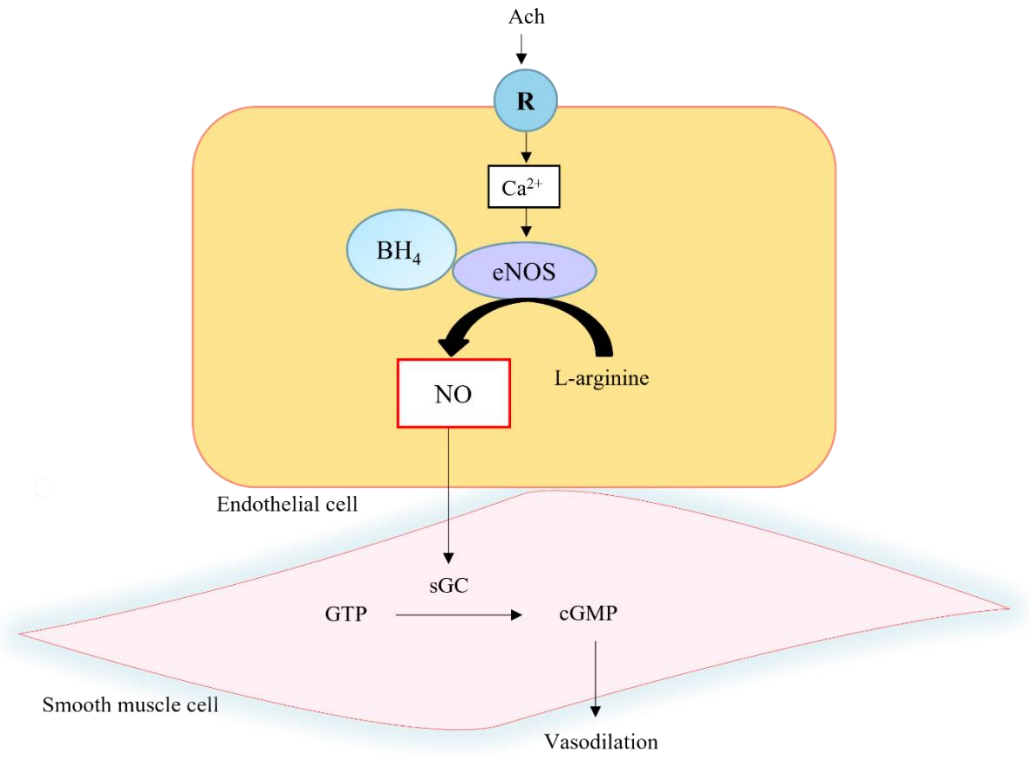

Figure 2.2: Mechanism of nitric oxide (NO) production involving the eNOS pathway in the endothelium (modified from da Silva et al., 2021; Lee and Im, 2021). Ach: acetylcholine; R: acetylcholine receptor; Ca^{2+}: calcium ion; BH_4: tertrahydrobiopterin; eNOS: endothelial nitric oxide synthase; NO: nitric oxide; sGC: soluble guanlyl cyclase; GTP: guanosine 5'-triphosphate; cGMP: cyclic guanosine monophosphate

2.3.2.1 Tetrahydrobiopterin (BH_4)

BH_4 is an animal intracellular antioxidant and a multifunctional cofactor of aromatic amino acid hydroxylases and NOS (Eichwald *et al.*, 2023). Through controlling NOS activity, BH_4 is essential for a number of normal cellular processes as well as the pathophysiology of cardiovascular illnesses, which arise from oxidative stress. It seems that cellular destiny depends on a balanced interaction between BH_4 and NOS. L-arginine is converted to L-citrulline and NO by functional eNOS in the presence of the powerful natural reducing agent BH_4 (Janaszak-Jasiecka *et al.*, 2023). Xie and colleague have demonstrated that exogenous injection of 1mg liposomal BH_4 in male Sprague-Dawley rats can sustain eNOS coupling, function, and post-translational activation (Xie *et al.*, 2015). It was also suggested that to make up the oxidative loss by increasing BH_4 levels to restore the cellular redox equilibrium in the eNOS-GFP mouse model would reduce hyperoxia-induced vascular regression (Edgar *et al.*, 2017). Adding on, Guan and colleagues have demonstrated that BH_4 and nebivolol protect against SHR model damage by increasing eNOS recoupling and protecting diastolic function (Guan *et al.*, 2020).

2.3.2.2 Cyclic guanosine 3',5' monophosphate (cGMP)

As a ubiquitous intracellular second messenger, cGMP mediates a few human physiological activities such as ion channel conductance, cell development and death, as well as cellular mobility and contractility. cGMP signalling is essential for the normal functioning of endothelial cells, cardiac

myocyte and vascular smooth muscle cells (Fajmut, 2021). The activities of cGMP are mediated by cGMP-regulated phosphodiesterases (PDEs), cGMP-dependent protein kinases (PKGs), and cGMP-gated cation channels, which in turn hydrolyse cyclic nucleotides. In NO/cGMP pathway, eNOS works together with cofactor BH_4 to synthesised NO (Golshiri *et al.*, 2020). The NO released in the endothelial cell will rapidly diffuses inside the vascular smooth muscle cells and bind to sGC. This subsequently led to the conversion of guanosine triphosphate (GTP) to cGMP. cGMP then mediates the relaxation of vascular smooth muscle cells (Premont *et al.*, 2020).

2.4 Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are compounds derived from molecular oxygen, by partial chemical reduction (Collin, 2019). ROS is also known as free radical or oxygen radical because it has "extra" electrons. There are a variety of ROS, the common ones such as superoxide, hydroxyl radical and hydrogen peroxide are involved in many biological oxidative cascades (Checa and Aran, 2020). When ROS is produced in uncontrolled amount, it can lead to abnormal metabolism thus damaging protein, DNA and lipids resulting in development of vascular diseases (Chen *et al.*, 2018).

2.4.1 Oxidative stress

A significant underlying cause of various diseases including cardiovascular diseases is the occurrence of oxidative stress. It is brought on by a change in the ratio of antioxidant defense mechanisms to ROS generation (Pizzino *et al.*, 2017). An increased in oxidative stress resulted in vascular remodelling, constriction of blood vessels, inflammation and fibrosis (Sena *et al.*, 2018). The majority of ROS in biological systems are produced by mitochondria. The pathogenesis and progression of many diseases are influenced by mitochondrial dysfunction, which is brought on by energy excess and oxidative stress, which in turn promotes ROS generation and oxidative stress (Sharifi-Rad *et al.*, 2020; Andrés Juan *et al.*, 2021).

2.4.1.1 NADPH oxidase (NOx)

The oxidative stress in vascular cells is mainly produced by activation of NOx. VSMCs, fibroblasts, endothelial cells and perivascular adipocytes expresses many NOx isoforms in a constitutive manner (Martínez-martínez *et al.*, 2021). Endothelial cells express NOx1, NOx2, NOx4 and NOx5 whereas VSMCs express NOx1, NOx4 and NOx5 (Poznyak *et al.*, 2020). NOx1 expression in blood vessels is minimal at rest and significantly increases in response to stimulation (Parascandolo and Laukkanen, 2019). NOx2 directly affects the bioavailability of NO and the contractile patterns of the vasculature by producing both O_2^- and H_2O_2 (Martínez-martínez *et al.*, 2021). NOx4 produces ROS, which alters the redox state of several proteins (Poznyak *et al.*,

2020). Pro-contractile NOx isoform NOx5 is crucial for redox-sensitive contraction (Martínez-martínez *et al.*, 2021).

2.4.1.2 Uncoupled eNOS

Instead of NO, eNOS generated O_2^- through eNOS uncoupling when the concentrations of either its cofactor BH_4 or the substrate L-arginine is low (Lee *et al.*, 2020). This superoxide will then interact with NO to create peroxynitrite which further promotes eNOS uncoupling (Gonçalves, Jasiulionis and de Melo, 2021). Continuous production of superoxide anions will result in a vicious cycle whereby BH_4 is further oxidized to BH_2 and leads to aggravation of eNOS uncoupling.

2.5 Renin-angiotensin-aldosterone system (RAAS)

RAAS is an endocrine system that is critically involved in the regulation of blood pressure and fluid balance (Menikdiwela *et al.*, 2020). Activation of the classical RAAS originates with the synthesis of renin at the renal afferent arteriole (Sequeira-Lopez and Gomez, 2021). Prorenin, converted from preprorenin, activates renin in the juxtaglomerular cells before it is released into the blood stream (Bakris and Sorrentino, 2017). The release of renal renin is stimulated by high salt content in the distal tubules, renal sympathetic nerve activity and reduced renal perfusion (Díaz-Morales *et al.*, 2023). Ang II is a major effector peptide. The effects of Ang II are manifested via activation of two G protein-coupled receptors (GPCRs), AT_1 receptor and AT_2 receptor, which in

general act in opposite directions (Philippe *et al.*, 2022). AT_1 receptor is presented in adipose tissue, endothelium, vascular smooth muscle, liver, kidney, heart, lung, adrenal cortex, and pituitary (Forrester *et al.*, 2018). The binding of Ang II to AT_1 receptor results in stimulation of many intracellular signalling pathways, resulting in hypertension, endothelial dysfunction, vascular remodelling and end organ damage (Figure 2.3) (de Mello-Aires *et al.*, 2017; Forrester *et al.*, 2018).

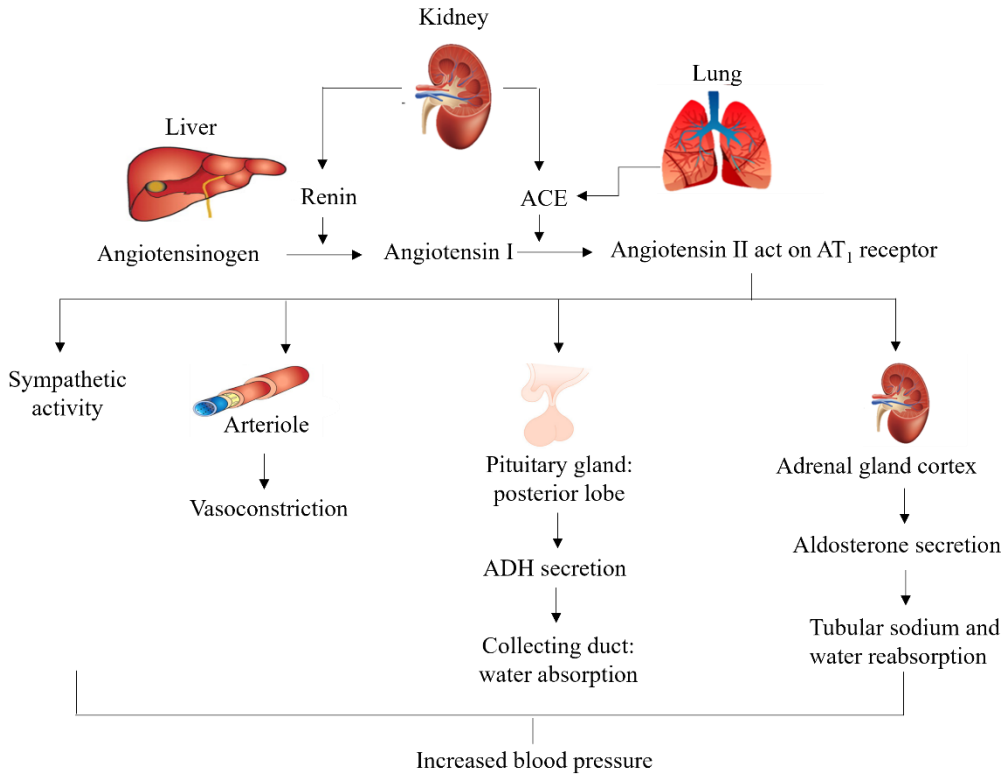

Figure 2.3: Renin-angiotensin aldosterone system and its effects on various organs to increase blood pressure (modified from Fountain and Lappin, 2018; Ames, Atkins and Pitt, 2019). ACE: angiotensin converting enzyme; AT_1: angiotensin II type I

2.5.1 Upregulation of AT_1 receptor and oxidative stress

Ang II is a potent mediator of ROS which later accumulates and resulted in oxidative stress (Forrester *et al.*, 2018). It manifests this effect via the activation of AT_1 receptor. A series of evidence has shown that Ang II-induced ROS production plays a crucial role in the initiation and development of cardiovascular dysfunction related to disease such as hypertension, obesity, angina, chronic heart failure and diabetes mellitus (He and Zuo, 2015; Senoner and Dichtl, 2019). Collectively, Ang II activates NOx in vascular smooth muscle cells to produce ROS (Takaishi *et al.*, 2021). One of the well-known consequences of AT_1 receptor-induced ROS production is the inactivation of endothelial-derived relaxing factor (eg: NO) (Yasiukaits and Pavlova, 2021). The activation of AT_1 receptor-induced ROS production not only reduce the bioavailability of NO but also lead to a depletion of BH_4 which act as an important cofactor for eNOS to produce sufficient NO (Verma *et al.*, 2021). This causes the impairment of endothelium to release NO for vasodilation. One experimental study showed that the elevation of vascular AT_1 receptor expression in human aortic smooth muscle cells as well as SHR aortic smooth muscle cells is a result of oxidative stress through mechanisms involving nuclear factor kappa B (NFκB) hence contributed to the development of hypertension (Bhatt, Lokhandwala and Banday, 2014). Another finding suggests that upregulation of the ACE-AngII-AT_1 receptor axis on the left ventricle is a crucial in increasing the oxidative/pro-inflammatory profile and reducing antioxidative mechanism in hypertension-induced cardiac remodelling (Silva *et al.*, 2017). In line with that, Dai and colleagues showed that increase in expression of AT_1

receptor protein in the vasculature of D-gal-induced aging rats lead to increase in ROS level thus resulted in the occurrence of endothelial dysfunction in the animals (Dai *et al.*, 2018). An investigation by Birk's teams has demonstrated that Ang II causes oxidative stress and endothelial dysfunction through activation of AT_1 receptor and subsequent stimulation of NOx2 in mouse ocular arteries of C57BL/6J mice (Birk *et al.*, 2021b) .

2.5.2 Angiotensin II receptor blockers (ARBs)

The pathophysiology of many diseases is closely entwined with the RAAS (Banerjee *et al.*, 2022). These include hypertension, congestive heart failure, and chronic kidney disease of various kinds, including diabetic nephropathy. In each of these disorders, pharmaceutical RAAS blockage is a well-established and effective treatment option. Angiotensin receptor blockers, often known as ARBs, are commonly prescribed to treat hypertension, diabetic nephropathy and congestive heart failure (Hill and Vaidya, 2020). They bind to and inhibit the AT_1 receptors. The common ARBs used in the commercial market are losartan, telmisartan, candesartan, valsartan and etc. A clinical study done on randomised controlled trials has demonstrated that ARBs improve peripheral endothelial function and lower the blood pressure (Li *et al.*, 2014). According to another clinical study, ARBs can decrease systolic and diastolic blood pressure in people with primary hypertension while also reducing arterial stiffness (Jatic *et al.*, 2019). Besides that, animal study has also demonstrated that losartan reduced blood pressure by restoring anti-inflammatory defence and reducing tubular injury by improving blood pressure and kidney NOx2

expression to that of SHR control (Karanovic *et al.*, 2016). In Halabi's findings, they reported that therapy with losartan and nicardipine reduced blood pressure and pulse pressure in elastin-deficient mice (Halabi *et al.*, 2015). However, there are certain adverse side effects that is associated with prolonged use of ARBs such as headache, nasal congestion, diarrhoea, fainting and back pain (Ferrario and Mullick, 2017).

2.6 Endothelial dysfunction

Endothelial dysfunction is a condition in which a multitude of destructive stimuli such as pathophysiological stress, sheer stress and inflammation cause the endothelium to phenotypically change to a non-adaptive state (Meza *et al.*, 2019). The important homeostatic processes in healthy endothelium cells are lost or dysregulated in endothelial dysfunction (Li, Sun and Carmeliet, 2019). This dysfunctional is characterised by an imbalance of endothelium-derived relaxing and contracting factors, increased oxidative stress, and abnormal modulation of vasoactive pathways, which may lead to different functional manifestations, including, but not limited to, impaired endothelium dependent vasodilation (Ray *et al.*, 2023). In many instances, the 'fault' seems to be in the pathway that generates NO (Mónica, Bian and Murad, 2016). Reduction of NO has been recognised in a variety of conditions related with a predisposition to cardiovascular events, including hypertension, diabetes, hypercholesterolaemia and heart failure (Cyr *et al.*, 2020).

2.6.1 eNOS uncoupling

eNOS uncoupling is one of the main factors contributing to endothelial dysfunction (Janaszak-Jasiecka *et al.*, 2023). Figure 2.4 illustrates the mechanism of eNOS uncoupling in endothelial dysfunction. When eNOS produces superoxide instead of NO, eNOS uncoupling happened and this turned eNOS into a source of harmful free radicals. Peroxynitrite, a by-product of superoxide and NO results in a decrease in eNOS activity and eNOS dimer disintegration (Janaszak-Jasiecka *et al.*, 2023). BH_4 is a vital component in the generation of NO. Each eNOS dimer is bound by two molecules of BH_4, which promotes electron transport for L-arginine oxidation. When BH_4 production or oxidation are restricted, superoxide is produced by the uncoupled (Gonçalves, Jasiulionis and de Melo, 2021). This will lead to restriction of vasodilation in smooth muscles cells (Figure 2.4).

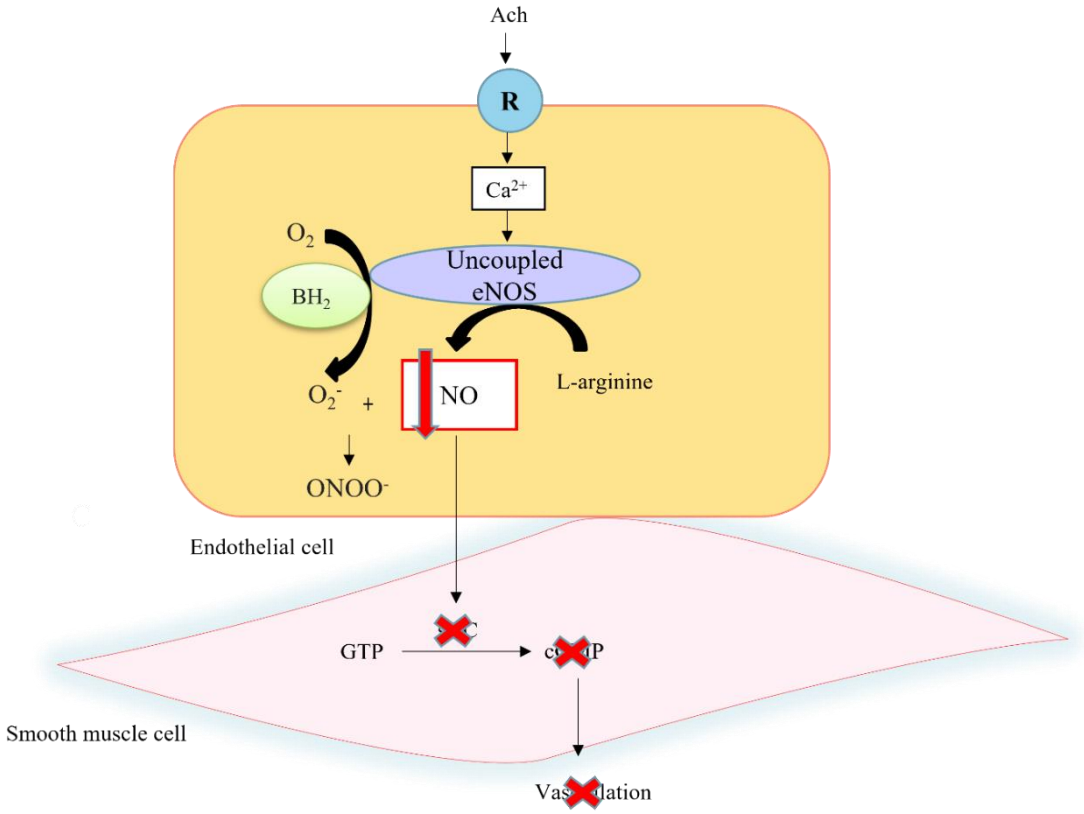

Figure 2.4: Mechanism of eNOS uncoupling in endothelial dysfunction-modified from (da Silva *et al.*, 2021; Lee and Im, 2021). Ach: acetylcholine; R: receptor; Ca^{2+}: calcium ion; BH_2: dihydrobiopterin; O_2^-: superoxide anion; O_2: oxygen; eNOS: endothelial nitric oxide synthase; NO: nitric oxide; sGC: soluble guanlyl cyclase; GTP: guanosine 5'-triphosphate; cGMP: cyclic guanosine monophosphate

2.6.2 Endothelial dysfunction and hypertension

There is always a casual role of endothelial dysfunction leading to hypertension. It is worth noting that manifestation of endothelial dysfunction may occur prior to the onset of hypertension (Gallo, Volpe and Savoia, 2022b). Resistance arteries exhibit anatomical and functional changes in essential hypertension, causing the peripheral vascular resistance to rise (Delong and Sharma, 2019). Endothelial dysfunction may lead to the development and progression of hypertension by a number of mechanisms including increased vasoconstriction and vascular remodeling of resistance arteries (Ambrosino *et al.*, 2022). The activation of RAAS contributes to endothelial dysfunction, which causes resistance to blood flow, an increase in myogenic tone in resistance arteries, and finally a rise in peripheral blood pressure (Ameer, 2022). Several evidences have demonstrated that endothelial dysfunction is linked to hypertension. Pan and colleagues have demonstrated that treatment with salusin-b is able to reduce the ROS level in SHR thus resulted in improvement of endothelial function which subsequently led to a decrease in blood pressure of the treated animal (Pan *et al.*, 2021). Another finding shows that hypertension causes local Ca^{2+} signaling circuits at myoendothelial projections to become disorganised, leading to an increase in contractile responses in SHR model (Wilson *et al.*, 2019). Researchers also reported that hypertension causes lymphatic endothelial dysfunction, which is driven by an increased in oxidative stress through the p38 MAPK/NADPH oxidase 2 pathways. Eventually, hydrochlorothiazide and reserpine or hydrochlorothiazide and hydralazine

treatment for six weeks fully restored lymphatic endothelial function in SHR (Mukohda, Mizuno and Ozaki, 2020).

2.6.3 Oxidative stress and endothelial dysfunction

Cardiovascular risk factors, such as hypertension, obesity diabetes, hypercholesterolemia, and smoking, are characterised by oxidative stress that also serves as a significant contributor to endothelial dysfunction (Chaudhary *et al.*, 2020). Oxidative stress and endothelial dysfunction are closely related. ROS substantially reduce NO generation (Gradinaru *et al.*, 2015). In addition, endothelial dysfunction, inflammation, and oxidative stress all work together to speed up the damaging process (Higashi, 2022). The outnumbering of antioxidant defence by prooxidant stimulants including ROS, Ang II, ET-1 and inflammatory cells can cause oxidative stress (Griendling *et al.*, 2021). Inflammation brought on by oxidative stress decreases the control of vascular tone, increases the risk of foam cell production, and induces unfavorable vascular remodeling, all of which contribute to endothelial dysfunction (El Hadri *et al.*, 2022). When stimuli that cause vasorelaxation in the presence of intact vascular endothelium (like acetylcholine) act directly on the underlying smooth muscle cells in vascular regions with injured endothelium, vasoconstriction happened (Drożdż, Drożdż and Wójcik, 2022). Study has reported that icarlin is able to reduce the expression of NADPH oxidase thus reduce oxidative stress and increase eNOS coupling whereby improve endothelial function in SHR (Long *et al.*, 2018). Zhang's results demonstrated that maternal exercise reduces oxidative stress and reverse impaired endothelium-dependent vasodilation via

sirtuin 1-regulated deacetylation of NOx4, which may help enhance vascular function in SHR (Zhang *et al.*, 2023). Another study showed acacetin prevented endothelial dysfunction in hypertension via triggering the AKT/eNOS pathway and modifying mitochondrial function by concentrating on mitochondrial permeability transition pore and dynamin-related protein 1 optic atrophy1-dependent dynamics (Li *et al.*, 2022).

2.7 Tea

Tea is a beverage that is brewed by infusing the dried leaves of the evergreen shrub Camellia sinensis in boiling water (Sanlier, Atik and Atik, 2018). Tea is the second most consumed drinks in Asian countries, such as Japan and China (Valavanidis, 2019). In the past, tea was prescribed to treat gastroenteritis (Sadeghian *et al.*, 2022), oedema (Choo, 2023), common colds (Furushima, Ide and Yamada, 2018) and allergies (Maeda-Yamamoto, Ema and Shibuichi, 2007). It was also known to promote a good balance of intestinal microbiota and oral hygiene (Bond and Derbyshire, 2019). Studies have demonstrated that drinking tea reduced the risk of several main diseases that cause premature death, many of which are linked to opulent lives and polluted environments. These include cancer, cardiovascular diseases, diabetes, neurological issues, liver and kidney illnesses (van den Brandt, 2018). The mechanism of action of tea and its constituents against these diseases as well as their metabolism in human tissues and plasma have been studied in clinical and laboratory settings using animal models, human volunteers, and cell studies, as well as other *in vitro* techniques (Tang *et al.*, 2019).

2.7.1 Green tea

Tea has been categorised into three groups which are oolong tea, black tea and green tea (Malabadi *et al.*, 2022). Among these, green tea is the most common tea and has gained popularity in many other parts of the world due to the recognition of its health benefits (Reygaert, 2017). Since green tea is not fermented, the essential ingredients in the fresh leaves are preserved (Zhao *et al.*, 2022). A significant number of studies have demonstrated that green tea contains chemical components that are beneficial to human health (Musial, Kuban-Jankowska and Gorska-Ponikowska, 2020). Tea polyphenols, caffeine, theanine, polysaccharides, and other substances have pharmacological properties that include anti-cancer, anti-oxidation, nervous system protection, and blood sugar reducing. Patients with hypertension, hyperlipidaemia, coronary heart disease, atherosclerosis, and diabetes have been shown to benefit from green tea consumption (Zhao *et al.*, 2022). A study has demonstrated that drinking 3 cups green tea per day could lower the level of total cholesterol and diastolic blood pressure in male Chinese patients with coronary heart diseases (Pang *et al.*, 2015). Another study reported that long term green tea consumption around 2.1-4.0 gram per day has systolic blood pressure lowering effect in patients with high blood pressure (Zhao *et al.*, 2023).

2.7.2 Epigallocatechin3-gallate (EGCG)

An abundance of polyphenols such as flavanols, flavandiols, flavonoids, and phenolic acids are found in green tea (Musial, Kuban-Jankowska and Gorska-Ponikowska, 2020). The primary constituents of green tea polyphenols are catechins. Of these, (-)-epigallocatechin-3-gallate (EGCG), (-)-epicatechin (EC), (-)-epicatechin-3-gallate (ECG), and (-)-epigallocatechin (EGC) are the most abundant catechins (Hoang *et al.*, 2019). EGCG is the primary polyphenol found in tea, making up 50% of all the polyphenols in green tea (Mokra, Joskova and Mokry, 2023). EGCG, with its wide range of beneficial properties such as anti-inflammatory, antioxidant, antifibrotic and anti-remodelling, may be helpful in the treatment of various diseases. These includes the treatment of cancer, in particular, as well as neurological, cardiovascular, respiratory, and metabolic disorders. The beneficial effects observed from the treatment with EGCG is due to its multiple interactions with cell surface receptors, intracellular signalling pathways, and nuclear transcription factors (Mokra, Joskova and Mokry, 2023).

2.7.3 Effect of EGCG on hypertension

EGCG exerts various beneficials effect in cardiovascular disorders. One of the major effects is that EGCG has the ability to reduce blood pressure. One of the studies in Malaysia has showed that administration of 250mg EGCG daily by oral gavage for 28 days exhibits antihypertensive effects in SHR (Parn *et al.*, 2022). Mohd Sabri and her team have shown that treatment with EGCG for two weeks significantly reduced the SBP in angiotensin II-infused hypertensive mice

by 40% compared to the non-treated mice (Mohd Sabri *et al.*, 2022). Adding on, the SBP of Dahl salt-sensitive rats was significantly reduced following oral administration with EGCG for six weeks (Luo *et al.*, 2020). Lastly, 12 weeks daily oral treatment of 200mg EGCG given to SHR group has significantly reduced their systolic, diastolic and mean blood pressure in Hsieh's findings (Hsieh *et al.*, 2021).

2.7.4 Effect of EGCG on endothelial function

EGCG has been demonstrated to exert beneficial effects on endothelial function through several experimental studies. Zhang and colleagues have demonstrated that 10-50 μM of EGCG treatment on high glucose-treated human umbilical vein endothelial cells elevated the expression of BH_4 and eNOS (dimer) as well as increased NO production and attenuated ROS formation (Zhang and Zhang, 2020). Another ex-vivo findings show that functioning NO synthase in endothelial cells and subsequent stimulation of NO production in arteries are essential for low doses of EGCG (5–15 μM) induced vasodilation in eNOS−/− mice (Lorenz *et al.*, 2015). Apart from that, Mohd Sabri and her team has reported that *in vivo* EGCG (50 mg/kg/day) treatment for 14 days was able to increase the p-eNOS expression and NO production whereby improving endothelial function in angiotensin II-infused hypertensive mice (Mohd Sabri *et al.*, 2022).

2.7.5 Effect of EGCG on oxidative stress

There is also a crosstalk between effect of EGCG on oxidative stress. A few findings have proven that EGCG is involved in the modulation of oxidative stress. Two weeks of treatment with EGCG in angiotensin II-infused mice is shown to successfully reduce DHE fluorescence intensity indicating a decrease in ROS formation and oxidative stress (Mohd Sabri *et al.*, 2022). Othman and colleagues have demonstrated that administration of EGCG (2mg/kg) on alternate days for one month decreased oxidative stress via the increase of antioxidant enzymes in an induced diabetes rat model (Othman *et al.*, 2017). Bulboaca study has showed that 2.5 mg/100 g liposomal-EGCG pretreatment reduced the indicators of oxidative stress and matrix-metalloproteinase plasma levels 48 h after diabetes mellitus induction in rat model (Bulboaca *et al.*, 2020). Additionally, data had shown that 30∼50 μM EGCG is able to inhibit the angiotensin II-induced upregulation of NADPH oxidase subunits in human umbilical vein endothelial cells (Ahn, Kim and Ha, 2010a).

2.7.6 Effect of EGCG on RAAS

EGCG also had demonstrated several effects in RAAS. It is demonstrated that administration of EGCG (50mg/kg/day) for 28 days in SHR exhibited antihypertensive and nutrigenomics benefits through the activation of intrarenal ACE and Agtr2 and reduction of *Ren* mediators in RAAS (Parn *et al.*, 2022). A recent finding by Wu et al. shows that 10 mg/kg of EGCG-derived

polymeric oxidation products for 3 weeks treatment inhibits the harmful axis of RAS, renal PEPCK/G6Pase-α, SELENOP, and TXNIP, and activates the beneficial axis of RAS in *db/db* mice (Wu *et al.*, 2022). Adding on, a study done by Aiza and colleagues has also shown that 50 mg daily oral gavage of EGCG for 14 days reduced the oxidative stress and improved endothelial dysfunction of angiotensin II-infused hypertensive mice (Mohd Sabri *et al.*, 2022).

CHAPTER 3

MATERIALS AND METHODS

3.1 Research design

Wistar-Kyoto (WKY) rats and Spontaneously Hypertensive Rats (SHR) were grouped into WKY Control, SHR Control, SHR treated with EGCG (50mg/kg/day) and SHR treated with losartan (10mg/kg/day) respectively. The treatment was given daily for 4 weeks by oral gavage and the blood pressure was monitored by tail-cuff method every 3 days. At the end of the treatment, the aortic tissues of the animals were isolated for vascular functional study and the measurement of vascular levels of nitric oxide (NO), reactive oxygen species (ROS), tetrahydrobiopterin (BH_4) and cyclic guanosine monophosphate (cGMP) as well as the expression of angiotensin type I (AT_1) receptor protein. The simplified diagram of experiment research design is shown in Figure 3.1.

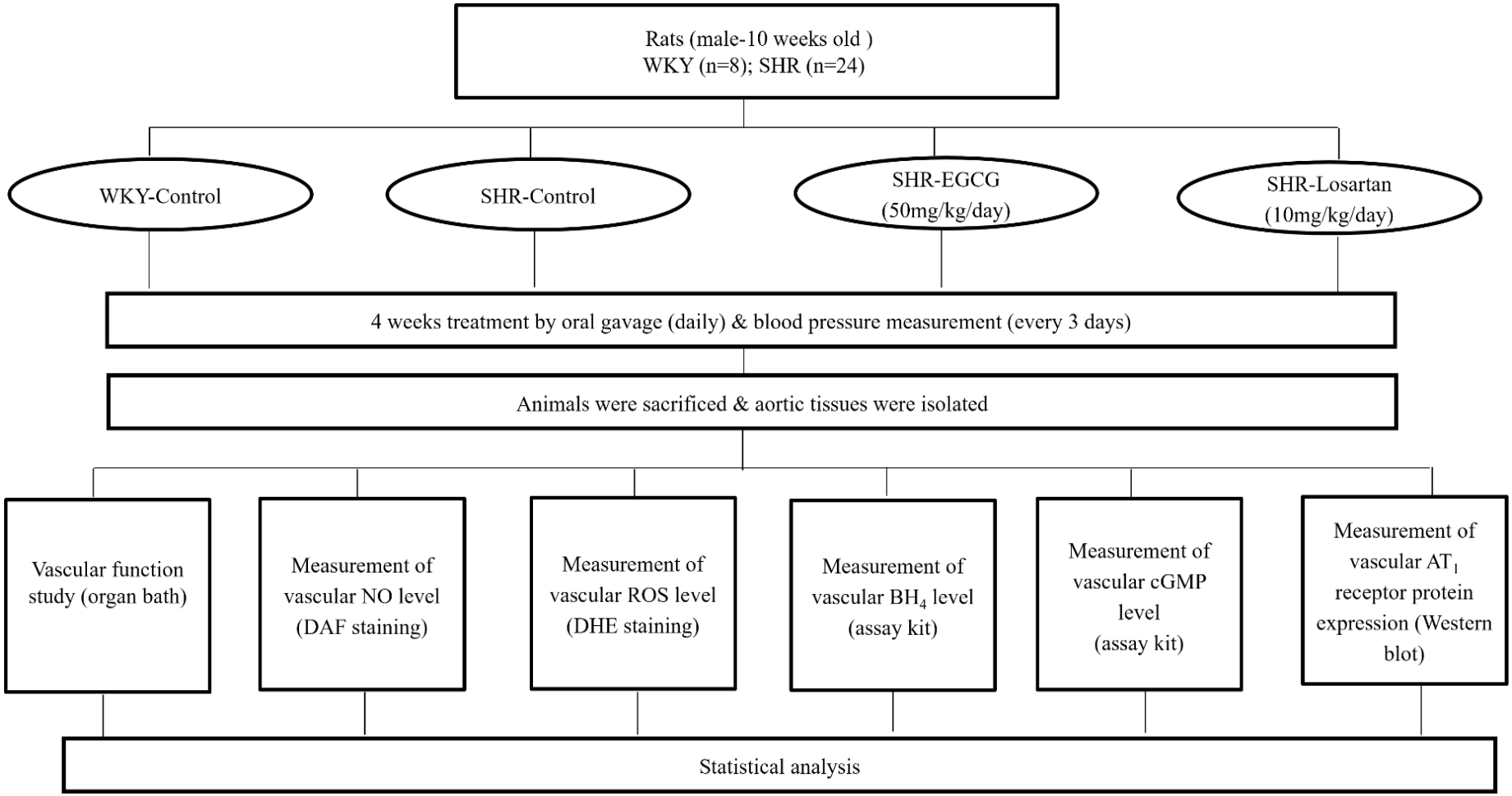

Figure 3.1 is the research design of experiment. WKY: Wistar-Kyoto rats; SHR: Spontaneously Hypertensive Rats; NO: nitric oxide; DAF: 4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate; ROS: reactive oxygen species; DHE: dihydroethidium; cGMP: cyclic guanosine monophosphate; BH_4: tetrahydrobiopterin; AT_1**:** angiotensin II type 1

3.2 Drugs and chemicals

PE, Ach, SNP and Tween-20 were purchased from Sigma Chemicals (St Louis, MO, USA). EGCG with a purity of ≥ 96% was purchased from Cayman chemicals. NaCl was purchased from Calbiochem® Merck (Darmstadt, Germany). $MgSO_4$, KCl, KH_2PO_2, glucose and $CaCl_2$ were purchased from BDH Laboratory Supplies (Poole, UK). BSA was purchased from Santa Cruz (Dallas, Texas, USA). All chemicals were dissolved in deionised water.

3.3 Experimental animals

Wistar-Kyoto rats (WKY; male; 10-weeks-old) and Spontaneously Hypertensive Rats (SHR; male; 10-weeks-old) were used for the *in vivo* study. The experimental rats were purchased from the Animal Experimental Unit (AEU), Universiti Malaya, Malaysia. All animals were housed at the Animal Holding Facility, Universiti Tunku Abdul Rahman (Sungai Long campus). They were maintained at a temperature of 24±1ºC, 12:12 light/dark cycle and unrestricted access to normal food and drink. The Ethics Committee of Universiti Tunku Abdul Rahman (U/SERC/38/2020) accepted and approved that all animal studies adhered to the UK Animals (Scientific Procedures) Act of 1986.

3.4 Experimental treatment

All WKY and SHR were divided randomly into four groups, namely WKY Control, SHR Control, SHR treated with EGCG (50mg/kg/day; Cayman Chemical Company, Ann Arbor, MI, USA) (Mohd Sabri *et al.*, 2022) and SHR treated with losartan (10mg/kg/day; Sigma Company, St Louis, MO, USA). Losartan is the inhibitor for AT_1 receptor and serve as the positive control for EGCG treatment group. All treatment were given via oral gavage daily for 4 weeks to investigate the sub-acute effect of EGCG. The following is the calculation to prepare the treatment given to the animals:

EGCG (50mg/kg) or losartan (10mg/kg)

$$\frac{\text{Weight of animal}}{1000} \times \text{Dosage of treatment (50 or 10)} = \text{Amount of drugs}$$

Amount of drugs + 1 ml of Milli Q water $\xrightarrow{\text{Dissolved}}$ Ready for oral gavage

3.5 Measurement of systolic blood pressure by tail-cuff method

A modified tail-cuff method utilising the CODA monitoring system (Torrington, CT, USA) was used to measure the systolic blood pressure of the rats every three days starting from day 0 of the treatment period. In order to stop the blood from flowing to the tail, an occlusion tail cuff was inflated.

Slowly deflating the cuff allowed a second tail cuff equipped with a volume pressure recording (VPR) sensor to monitor the physiological traits of the blood flow returning to the heart. The VPR sensor cuff measured the tail swelling brought on by arterial pulsations by the blood flow as it returned to the tail. All animals were trained for a week before the actual measurement of blood pressure to ensure that they were adapted to the procedure. This minimised the stress associated with blood pressure measurements thus ensuring a more reproducible result. The restrained animals were pre-heated in a chamber by warming pad for 30 minutes prior each cycle of measurement. A cycle of five to six measurements was taken, and the average values of all the readings were calculated and reported.

3.6 Samples Collection and Preparation

The animals were sacrificed at the end of the treatment period by excessive carbon dioxide inhalation. The animal's blood was promptly drawn by cardiac puncture. The thoracic aorta was isolated and placed immediately in ice-cold Krebs solution (in mM: NaCl 118.93, $NaHCO_3$ 25.00, $MgSO_4$ 1.18, KCl 4.69, KH_2PO_4 1.03, Glucose 11.10, $CaCl_2$ 2.38). After that, the adhering connective tissues and adipose tissues were removed. The aorta was sliced into segments of around 5mm for vascular function investigation. A portion of the isolated aortic tissues were embedded in an optimal cutting temperature (OCT) compound and some were snap-frozen in liquid nitrogen before being stored at -80°C for subsequent processing.

3.7 Vascular function study (Organ bath)

An arrangement of chambers known as an "organ bath" is used to study the physiology and pharmacology of *in vitro* tissue preparations. The protocol of vascular function study was followed and modified from (Ling *et al.*, 2016; Matsumoto *et al.*, 2021). In an organ chamber with a controlled temperature, the tissues can be maintained for a number of hours. Fresh cleaned aortic ring isolated from experimental animals was prepared in a petri dish filled with ice-cold Krebs solutions. The end side of the aortic ring was then attached to a mounting hook and a transducer for the measurement of isometric tension. An organ bath containing 5ml of Krebs solution kept at 37°C was used to suspend the produced aortic ring and constantly ventilated with 95% O_2 and 5% CO_2 throughout the experiment. The change in the isometric tension was recorded using a Powerlab recording system (AD Instrument, Sydney, NSW, Australia). The rings were stretched to 2.5g optimal basal tension for 1 hour to maintain the equilibrium of the rings. After that, each ring was contracted with 60mM KCl until a stable contraction is achieved to test its viability. The rings were then washed three times with Krebs solution before the endothelial function of the endothelium was investigated. The presence of endothelium was pre-investigated by one dose of acetylcholine (Ach; 10 µM) to phenylephrine (PE; 300 nM – 1 µM)-contracted rings. The rings were then washed three times with Krebs solution. After that, cumulative addition of Ach (3 nM – 10 µM) to PE (300 nM – 1 µM)-contracted rings was performed again to generate endothelium-dependent relaxation. The rings were then washed three times with Krebs solution. Cumulative concentration-response curve to endothelium

independent agonist, sodium nitroprusside (SNP; 10 nM-10 µM) was also obtained to investigate if there are any changes of the vascular smooth muscle sensitivity to nitric oxide.

3.8 Measurement of vascular nitric oxide (NO) level

DAF-FM mediated fluorescence microscopy was used to determine the intracellular NO production at the vascular tissues (Ling *et al.*, 2016; Mohd Sabri *et al.*, 2022). The thoracic aorta was sliced into 10 µm-thick cryostat slices after being embedded in OCT compound (Sakura Finetik, AJ Alphen aan den Rijn, Netherlands). The prepared slide was washed using phosphate-buffer saline (PBS) (Sigma, Aldrich, USA) three times every 5 minutes prior to incubation with 5µM 4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate (DAF-FM) diacetate fluorescence dye (Invitrogen, Carlsbad, CA, USA) for half an hour at 37°C. After incubation, the aortic segments were washed again with PBS three times every 5 minutes to remove excessive staining dye. A cover slip was placed on the slides and the sections were observed by using fluorescence microscope (ZIESS, Oberkochen, Germany). ZIESS software is used to detect the NO intensity and measure the intensity using excitation at 495 nm whereas the emission at 515 nm. The NO level was compared to that of the WKY control.

3.9 Measurement of vascular reactive oxygen species (ROS) level

DHE mediated fluorescence microscopy was used to determine the intracellular ROS production at the vascular tissues (Ling *et al.*, 2016; Mohd Sabri *et al.*, 2022). A segment of the thoracic aorta embedded in OCT compound (Sakura Finetik, AJ Alphen aan den Rijn, Netherlands) was cut into cryostat sections of 10μm thick. The prepared glass slide was washed using PBS three times every 5 minutes prior to incubation with 5μM dihydroethidium (DHE) fluorescence dye (Invitrogen, Carlsbad, CA, USA). The slides were incubated for half an hour at 37°C. After incubation, the aortic segments were washed again with PBS three times every 5 minutes to remove excessive staining dye. A cover slip was placed on the slides and the sections were observed by using fluorescence microscope (ZIESS, Oberkochen, Germany). ZIESS software is used to detect the ROS intensity and measure the intensity using excitation at 516 nm whereas the emission at 606 nm. The ROS level was compared to that of the WKY control.

3.10 Measurement of vascular tetrahydrobiopterin (BH_4) level

The frozen samples of isolated aortic tissues were homogenized in PBS and centrifuged at 15,000g for 15 minutes at 4°C. A commercially available BH_4 ELISA assay kit (Elabscience, USA) was used to determine the total vascular BH_4 level in the collected supernatant. Each well received 50 µl of the standard or sample, followed immediately by 50 µl of the biotinylated detection antibody, which was incubated at 37 °C for 45 minutes. The wells

were aspirated and washed three times after incubation. Following that, 100µl of horseradish peroxidase (HRP) conjugated solution was added into each well and incubated for 30 minutes at 37°C. Following five rounds of washing and aspiration, 90 µl of substrate reagent was added to the wells, and they were then incubated at 37°C for 15 minutes. After that, at 50µl stop solution was added. The plate was read at a wavelength of 450nm (Hidex, Turku Finland, Sweden) immediately. The BH_4 level was compared to that of the WKY control.

3.11 Measurement of vascular cyclic guanosine monophosphate (cGMP) level

The frozen samples of isolated aortic tissues were homogenized in PBS and centrifuged at 15,000g for 15 minutes at 4°C. A commercially available ELISA test kit (Cayman Chemical Company, Ann Arbour, MI, USA) was used to measure the total cGMP in the collected supernatant. In non-specific binding wells, 100 µl of ELISA buffer was added, at the same time maximum binding wells received an additional 50 µl of ELISA buffer. 50µl of prepared standard solution was aliquoted and placed in the standard wells. Then, 50 µl of sample and 50 µl of the cGMP AChE tracer were added to each well, with the exception of the total activity and blank wells. Each well except total activity, non-specific binding, and blank wells received 50 µl of cGMP ELISA antiserum. A plastic film was placed over the plate, and it was let to incubate at room temperature for 18 hours. The wells were emptied and washed with wash buffer for five times. Each well received 200 µl of Ellman's reagent, and

the total activity wells received 5 µl of tracer. The plate was placed on a shaker for 60-90 minutes and covered with plastic film for optimum development. After incubation, a clean tissue was used to wipe away any debris or fingerprints from the plate's bottom. To prevent the Ellman's reagent from spilling, the plate cover was carefully removed. The plate was read at a wavelength of 405 nm using a plate reader (Hidex, Turku Finland, Sweden) immediately. The cGMP level was compared to that of the WKY control.

3.12 Measurement of vascular angiotensin II type 1 (AT_1) receptor protein expression via Western blot

Treated aortic tissues were homogenised in ice-cold radio immunoprecipitation (RIPA) lysis buffer (1 µg/ml leupeptin, 5 µg/ml aprotonin, 100 µg/ml phenylmethanesulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM egtazic acid, 1 mM ethelynenediamine tetraacetic acid, 1 mM NAF, 2 mg/ml glycerol phosphate). The supernatant from the centrifuged lysates was used for a Western blot. The protein concentration was determined by a modified Lowry assay (Bio-Rad Laboratories, Hercules, CA, USA). Each sample was loaded with 20 µg of protein concentration, separated in a 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel, and then transferred to an immobilon-P polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) at 110 v for one and a half hours. The blots were gently shaken at room temperature for an hour while being blocked with 5% bovine serum albumin (BSA). The blots were then incubated at 4°C for an overnight period with primary antibodies against the angiotensin type 1 (AT_1) receptor and β-actin

(1:10000, Santa Cruz Biotechnology). The blots were then incubated at room temperature with the appropriate HRP-conjugated secondary antibodies for two hours after being three times washed in tris-buffered saline with 0.1% Tween® 20 detergent (TBST). The membranes were created using an enhanced chemiluminescence detection system (Millipore, Billerica, Massachusetts, USA), and the c600 Ultimate Western System from Azure Biosystems (Dublin, California, USA) was used to record protein expression images. Quantity one software (Bio-Rad Laboratories, Hercules, CA, USA) was used to analyse the protein expression. The protein expression of AT_1 was normalised to β-actin and then compared with WKY control.

3.13 Statistical analysis

The number of animals (n) in each group was used to calculate the means and standard error of the means (SEM) for all results. Non-linear regression fitting was used to examine the concentration-response curves in GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). Using the same statistical software, a one-way ANOVA was followed by Bonferroni's multiple comparison tests (for more than two groups). The result is considered statistically significant when there is a P value of less than 0.05.

CHAPTER 4

RESULTS

4.1 Treatment with epigallocatechin-3-gallate (EGCG) decreased systolic blood pressure (SBP) of SHR

There is a significantly decreased in SBP of SHR treated with EGCG. The basal SBP of SHR from different treatment groups was similar before treatment and was significantly higher than WKY rats (Figure 4.1). There was a sustained decrease in SBP of SHR treated with EGCG and losartan from day 3 onwards. At the end of the treatment period, the SBP of SHR treated with EGCG and losartan was 8% and 17% lower compared to SHR control, respectively. The SBP of WKY and SHR control was not significantly altered before and after treatment.

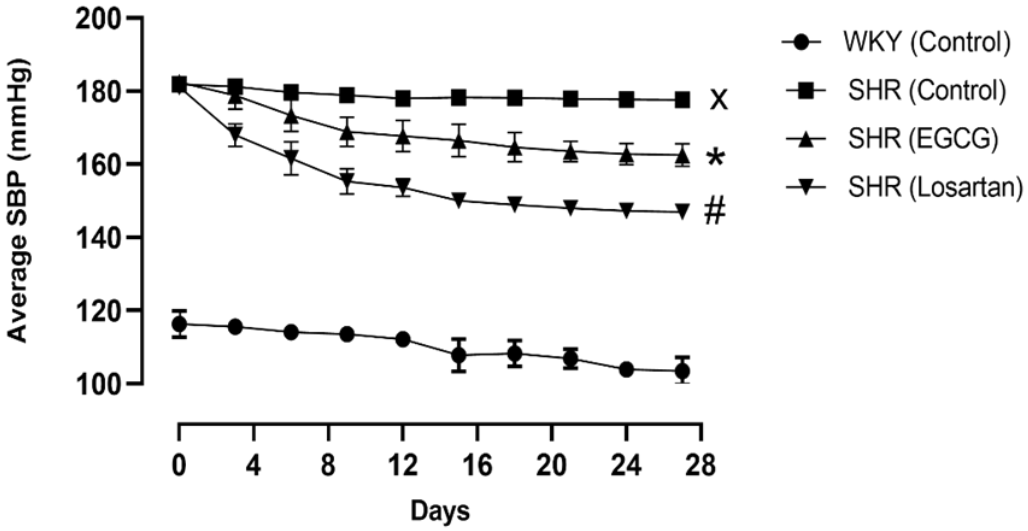

Figure 4.1: Average systolic blood pressure (SBP) in Wistar Kyoto (WKY) rats and Spontaneously Hypertensive Rats (SHR), with and without *in vivo* treatment with either epigallocatechin-3-gallate (EGCG; 50 mg/kg/day) or losartan (10mg/kg/day) for four weeks. Data are presented as mean ± SEM (n=4-6). $^{x}p < 0.05$ SHR (Control) compared to WKY (Control), $*p < 0.05$ SHR (EGCG) compared to SHR (Control) and $^{\#}p < 0.05$ SHR (Losartan) compared to SHR (Control).

4.2 Treatment with epigallocatechin-3-gallate (EGCG) improved the vascular relaxation in SHR

There was a significant decrease in the relaxation of the aortic rings from SHR control compared to that of WKY control groups. Treatment with EGCG and losartan improved the acetylcholine-induced relaxation in aortic rings of SHR significantly compared to that of SHR Control as shown in representative tracing (Figure 4.2A) and relaxation curve (Figure 4.2B). The total relaxation of treatment with EGCG and losartan in aortic rings of SHR compared to WKY control groups was 11% and 23% respectively as shown in area under the curve (AUC) (Figure 4.2C). The relaxation to sodium nitroprusside (SNP), an exogenous nitric oxide donor was similar in the aorta of all experimental groups (Figure 4.2D and 4.2E).

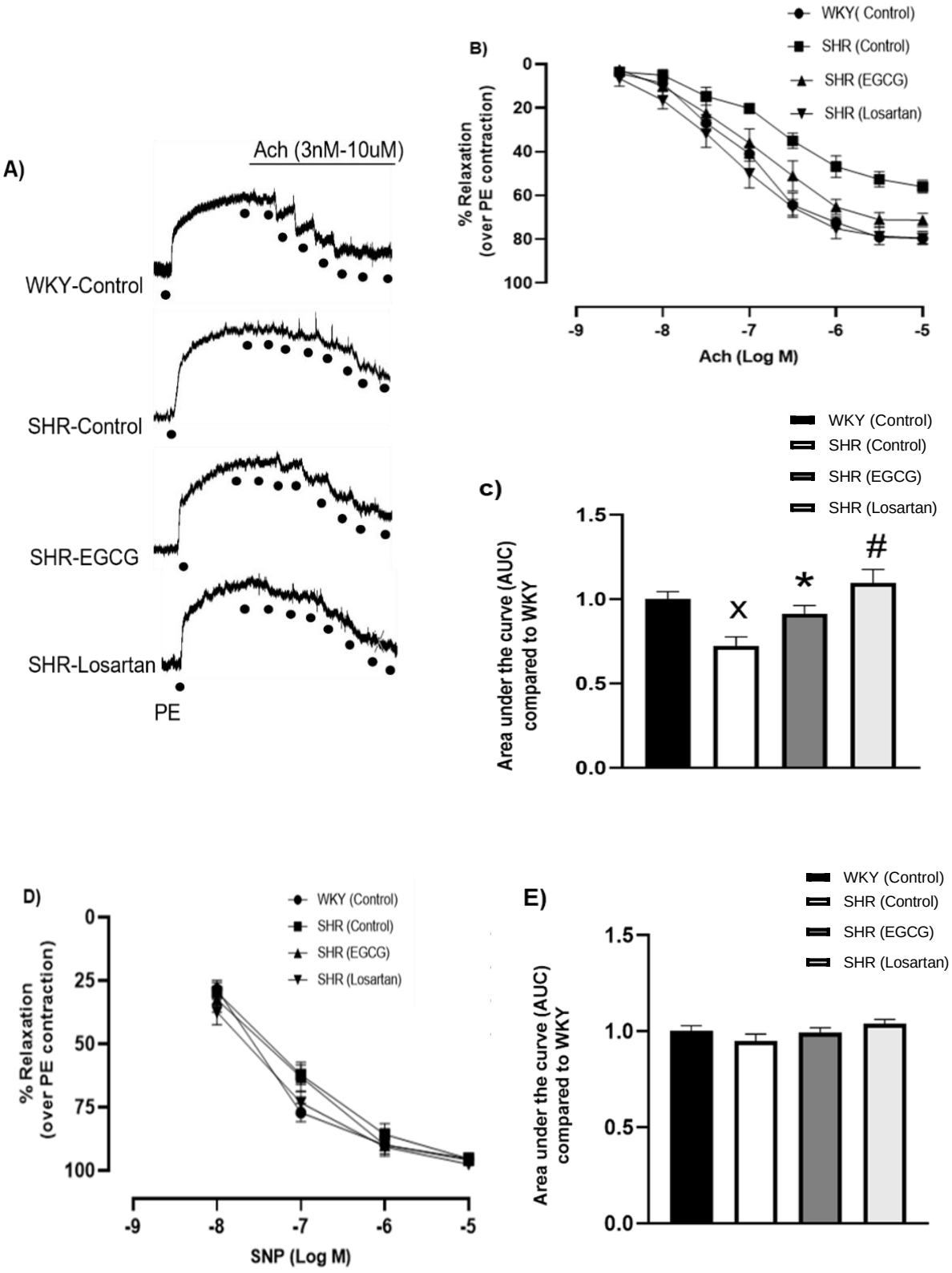
A)
Ach (3nM-10uM)
WKY-Control
SHR-Control
SHR-EGCG
SHR-Losartan
PE
B)
WKY(Control)
SHR (Control)
SHR (EGCG)
SHR (Losartan)
% Relaxation (over PE contraction)
0
20
40
60
80
100
-9
-8
-7
-6
-5
Ach (Log M)
c)
WKY (Control)
SHR (Control)
SHR (EGCG)
SHR (Losartan)
Area under the curve (AUC) compared to WKY
1.5
1.0
0.5
0.0
X
*
#
D)
WKY (Control)
SHR (Control)
SHR (EGCG)
SHR (Losartan)
% Relaxation (over PE contraction)
0
25
50
75
100
-9
-8
-7
-6
-5
SNP (Log M)
E)
WKY (Control)
SHR (Control)
SHR (EGCG)
SHR (Losartan)
Area under the curve (AUC) compared to WKY
1.5
1.0
0.5
0.0

Figure 4.2: Relaxation curve to increasing concentration of acetylcholine (Ach) and sodium nitroprusside (SNP) of aortic rings from Wistar Kyoto (WKY) rats and Spontaneously Hypertensive rats (SHR) with and without *in vivo* treatment with epigallocatechin-3-gallate (EGCG) and losartan. (**A**) Representative tracings of acetylcholine-induced relaxation in phenylephrine (PE)-contracted aortic rings of all experimental groups. (**B**) Relaxation curve and (**C**) area under the curve graph for percentage of relaxation to Ach of all experimental groups. (**D**) Relaxation curves and area under the curve graph for percentage of relaxation to SNP of all experimental groups. Data are presented as mean ± SEM (n=4-6). $^{x}p < 0.05$ SHR (Control) compared to WKY (Control), $*p < 0.05$ SHR (EGCG) compared to SHR (Control) and $^{\#}p < 0.05$ SHR (Losartan) compared to SHR (Control).

4.3 Treatment with epigallocatechin-3-gallate (EGCG) increased the level of vascular nitric oxide (NO) in SHR

The measurement of DAF-FM fluorescence indicates that SHR displayed a significantly lower level of vascular NO compared to WKY control group (Figure 4.3). The intensity of DAF-FM fluorescence was significantly higher 27.7% and 30% respectively in aortic tissues of SHR treated with EGCG and losartan compared to that of SHR control, implying that four weeks of treatment with EGCG and losartan significantly increased the level of vascular NO in SHR.

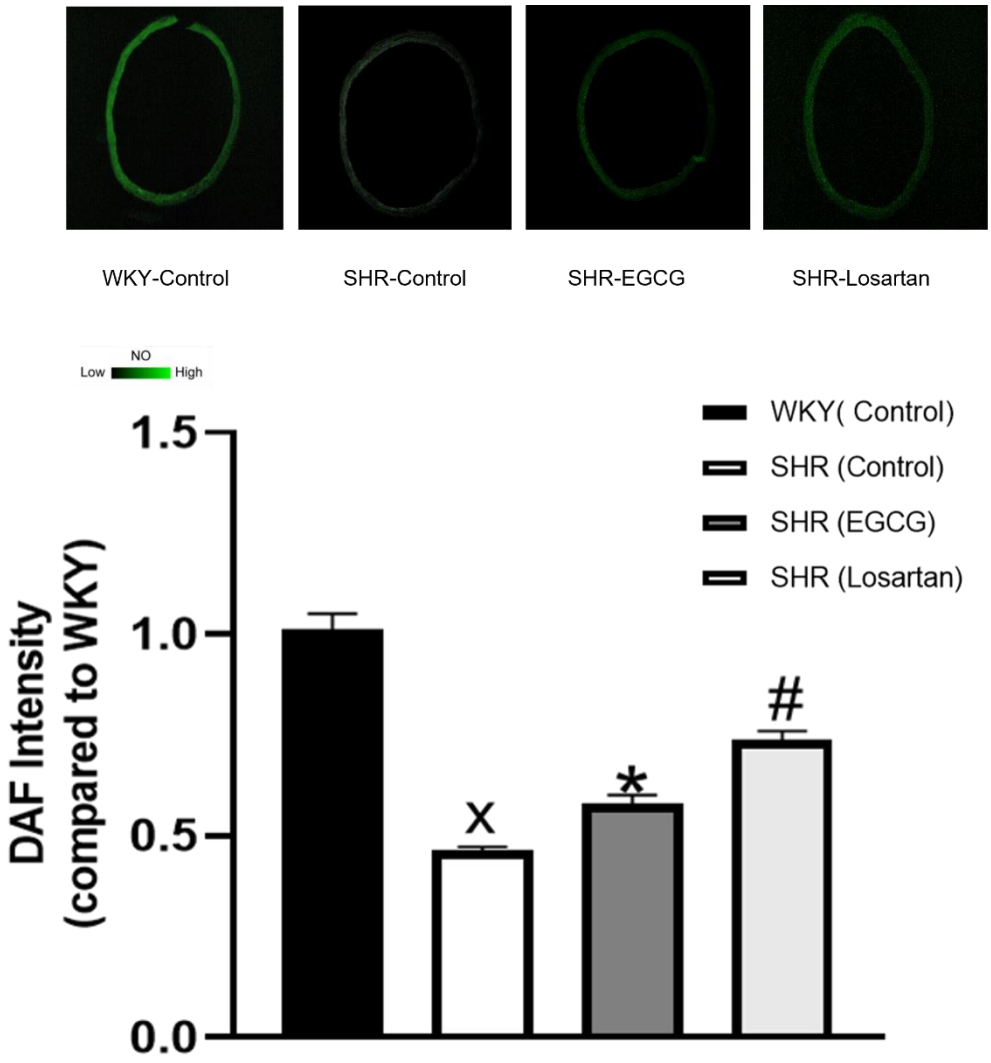

Figure 4.3: Measurement of the level of 4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate (DAF-FM) fluorescence intensity in aortic rings of Wistar Kyoto (WKY) rats and Spontaneously Hypertensive rats (SHR), with and without *in vivo* treatment with either epigallocatechin-3-gallate (EGCG; 50 mg/kg/day) or losartan (10mg/kg/day). The upper panel showed the representative image of DAF-stained aortic rings whereas the lower panel showed the quantified fluorescence intensity for all treatment group. Data are presented as mean ± SEM (n=4-6). $^{x}p < 0.05$ SHR (Control) compared to WKY (Control), *$p < 0.05$ SHR (EGCG) compared to SHR (Control) and $^{\#}p < 0.05$ SHR (Losartan) compared to SHR (Control).

4.4 Treatment with epigallocatechin-3-gallate (EGCG) decreased the level of vascular reactive oxygen species (ROS) in SHR

The measurement of DHE fluorescence intensity showed that SHR displayed a significantly higher level of vascular ROS compared to WKY control group (Figure 4.4). Treatment with EGCG and losartan for four weeks decreased the intensity of DHE fluorescence 43.2% and 51.3% significantly, indicating a significant decrease in the level of ROS in SHR aorta.

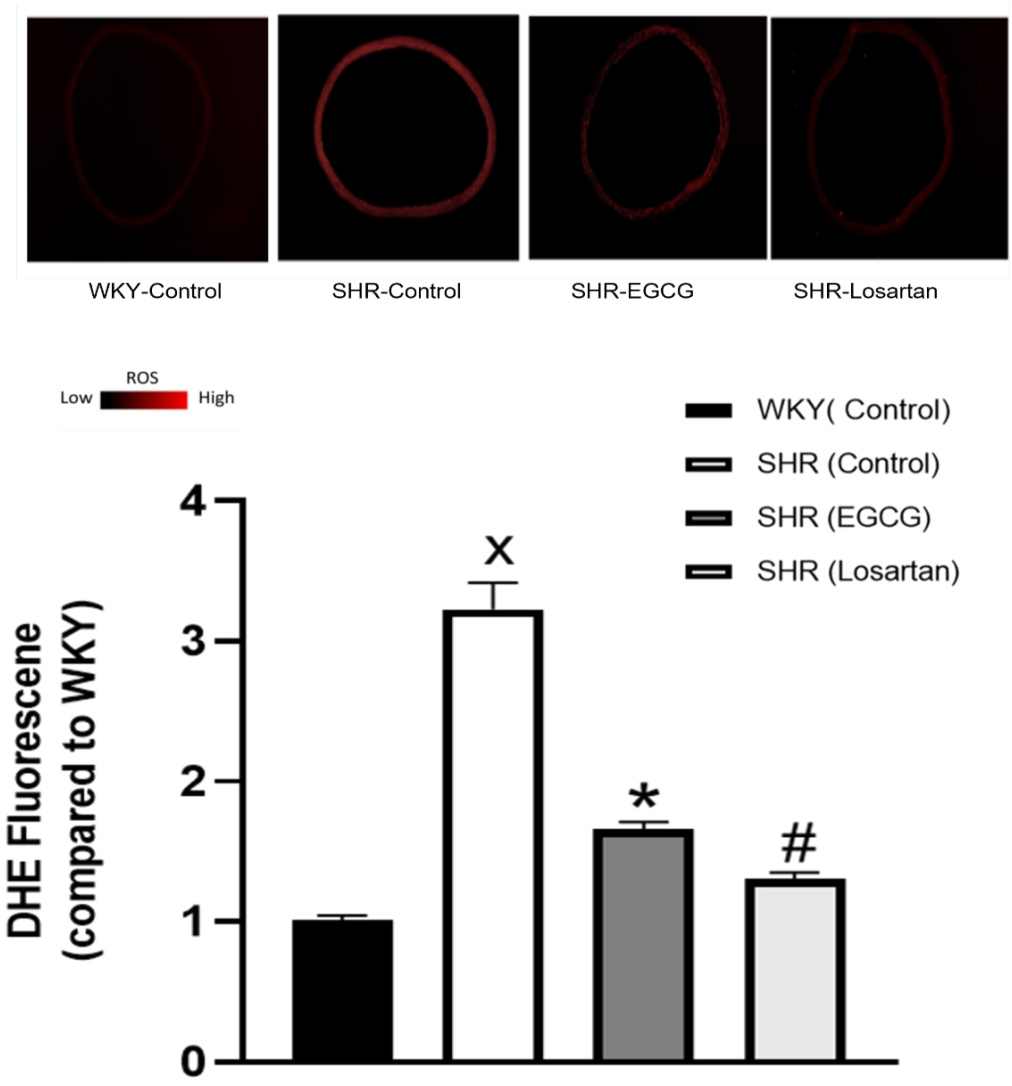

Figure 4.4: Measurement of the level of reactive oxygen species (ROS) in aortic rings of Wistar Kyoto (WKY) rats and Spontaneously Hypertensive rats (SHR), with and without *in vivo* treatment with either epigallocatechin-3-gallate (EGCG; 50 mg/kg/day) or losartan (10mg/kg/day) after four weeks by DHE fluorescence. The upper panel showed the representative image of DHE-stained aortic rings whereas the lower panel showed the quantified fluorescence intensity. Data are presented as mean ± SEM (n=4-6). $^{x}p < 0.05$ SHR (Control) compared to WKY (Control), $*p < 0.05$ SHR (EGCG) compared to SHR (Control) and $^{\#}p < 0.05$ SHR (Losartan) compared to SHR (Control).

4.5 Treatment with epigallocatechin-3-gallate (EGCG) increased the level of vascular tetrahydrobiopterin (BH_4) in SHR

The measurement of BH_4 in SHR control groups showed that the animals displayed a significant lower level of vascular BH_4 compared to WKY control groups (Figure 4.5). *In vivo* treatment with EGCG and losartan for four weeks in SHR increased 41.1% and 52.8% of the BH_4 level significantly compared to non-treated SHR.

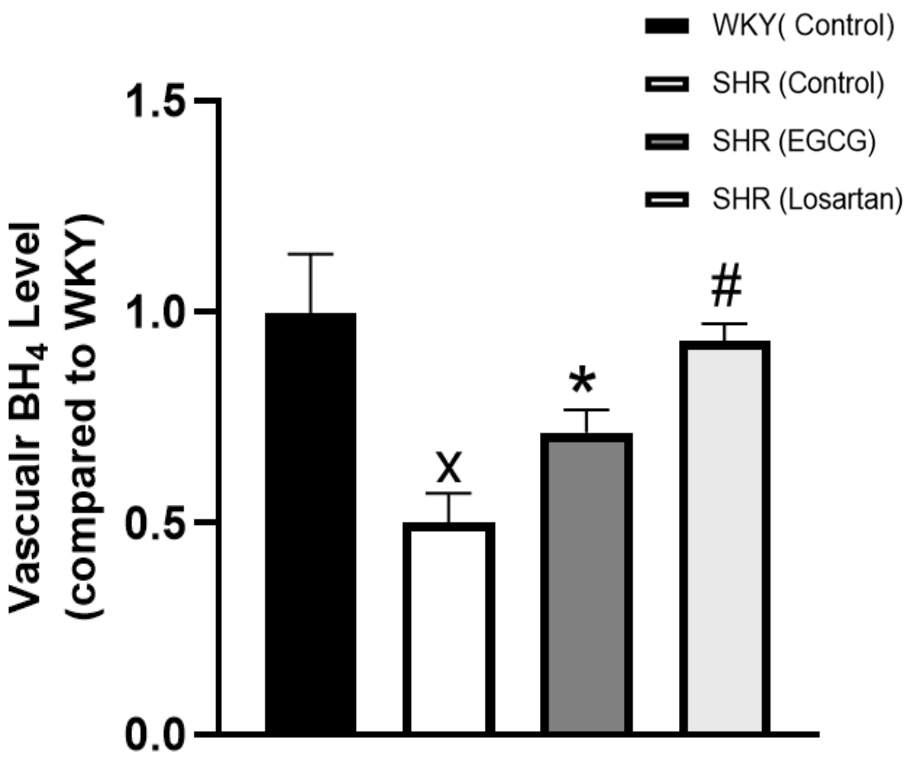

Figure 4.5: Measurement of the level of vascular tetrahydrobiopterin (BH_4) in aortic rings of Wistar Kyoto (WKY) rats and Spontaneously Hypertensive rats (SHR), with and without *in vivo* treatment with either epigallocatechin-3-gallate (EGCG; 50 mg/kg/day) or losartan (10mg/kg/day) after four weeks. Data are presented as mean ± SEM (n=4-6). $^{x}p < 0.05$ SHR (Control) compared to WKY (Control), *$p < 0.05$ SHR (EGCG) compared to SHR (Control) and $^{\#}p < 0.05$ SHR (Losartan) compared to SHR (Control).

4.6 Treatment with epigallocatechin-3-gallate (EGCG) increased the level of vascular cyclic guanosine monophosphate (cGMP) in SHR

The measurement of the level of cGMP showed that there is a significant lower level of cGMP in aorta of SHR control groups compared to that of WKY control groups (Figure 4.6). Four weeks of *in vivo* treatment with EGCG and losartan significantly increase 30.9% and 39.5% of cGMP level in the aorta of SHR.

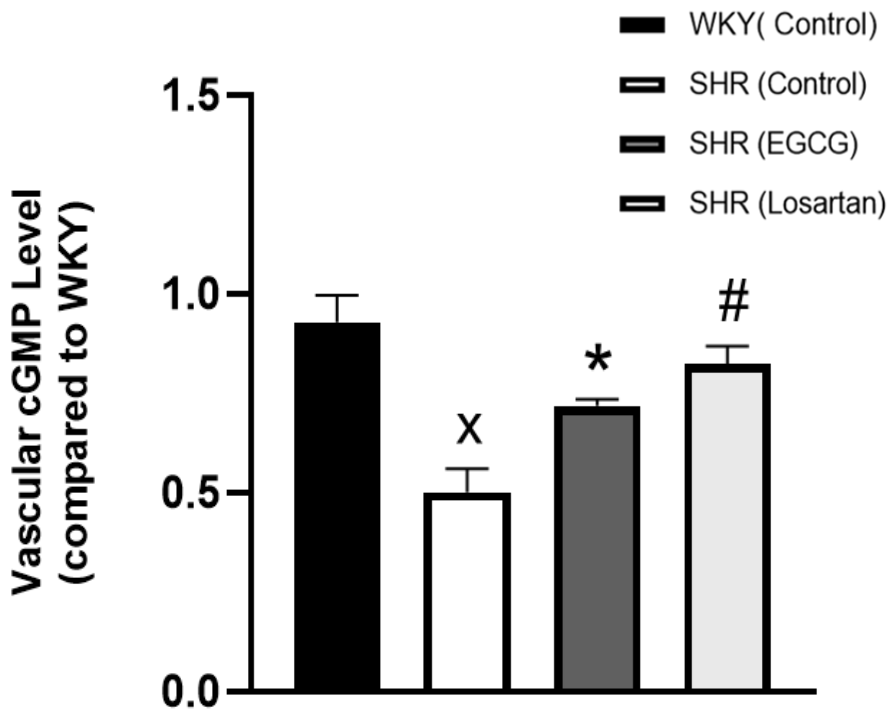

Figure 4.6: Measurement of the level of vascular cyclic guanosine monophosphate (cGMP) in aortic rings of Wistar Kyoto (WKY) rats and Spontaneously Hypertensive rats (SHR), with and without *in vivo* treatment with either epigallocatechin-3-gallate (EGCG; 50 mg/kg/day) or losartan (10mg/kg/day) after four weeks. Data are presented as mean ± SEM (n=4-6). $^{x}p < 0.05$ SHR (Control) compared to WKY (Control), *$p < 0.05$ SHR (EGCG) compared to SHR (Control) and $^{\#}p < 0.05$ SHR (Losartan) compared to SHR (Control).

4.7 Treatment with epigallocatechin-3-gallate (EGCG) decreased angiotensin II type I (AT_1) receptor protein expression in SHR

The measurement of the protein expression of AT_1 receptors in aorta of SHR demonstrated that SHR has a slightly higher level of AT_1 protein compared to WKY (Figure 4.7). Treatment with EGCG and losartan reduced 11.1% and 16.2% of AT_1 protein expression in SHR compared to SHR control group. Four weeks of treatment with EGCG and losartan resulted in a decreasing trend of the AT_1 receptor in the aortic tissues of SHR.

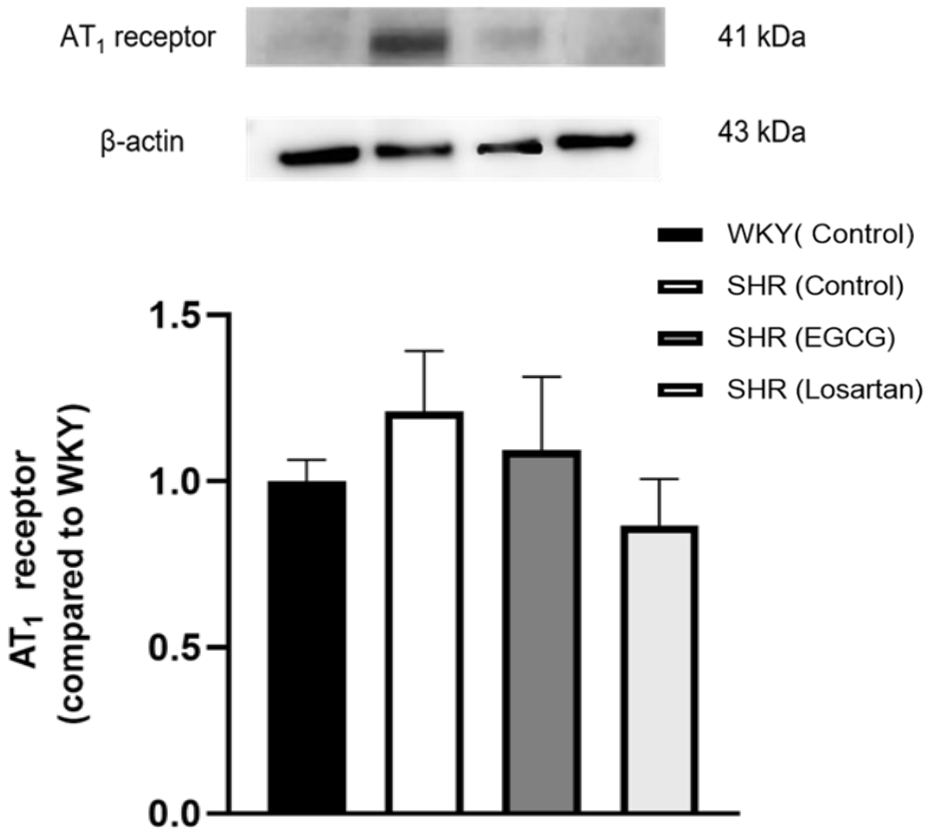

Figure 4.7: The expression of angiotensin II type 1 (AT_1) receptor protein in aortic rings of Wistar Kyoto (WKY) rats and Spontaneously Hypertensive rats (SHR), with and without *in vivo* treatment with either epigallocatechin-3-gallate (EGCG; 50 mg/kg/day) or losartan (10mg/kg/day) for four weeks. The upper panel shows representative Western blots and the bottom panel shows the ratio of protein to β-actin. Data are presented as mean ± SEM (n=4-6).

CHAPTER 5

DISCUSSION

The current investigation was to investigate the effect of *in vivo* treatment with EGCG on the endothelial function of hypertensive animal models. WKY and SHR were divided into WKY Control, SHR Control, SHR treated with EGCG (50mg/kg/day) and SHR treated with losartan (10mg/kg/day). The treatment was administered via oral gavage to the animals daily and their blood pressure was monitored by tail-cuff method every three days. After four weeks of treatment, the animals were sacrificed and the aortic tissues from WKY and SHR were isolated for vascular functional study. Experimental works such as the measurement of the level of vascular NO, ROS, BH_4 and cGMP level of the animals were carried out. In addition, the protein expression of AT_1 receptor of the animals were measured.

EGCG is the most prevalent polyphenols found in green tea and has been demonstrated to exhibit several beneficial effects for our cardiovascular system. Studies have shown that EGCG exhibits cardio protective as well as antioxidant effects in addition to its positive effect on vascular remodelling (Mokra, Joskova and Mokry, 2023). A study has demonstrated that drinking three cups of green tea per day could lower the level of total cholesterol and diastolic blood pressure in male Chinese patients with coronary heart diseases (Pang *et al.*, 2015). Another study reported that consumption of green tea (around 2.1-4.0 gram per day) in a long period of time has systolic blood pressure lowering effect in

patients with high blood pressure (Zhao *et al.*, 2023). Since EGCG is a major polyphenol in green tea, the antihypertensive effect of green tea may be attributed to the effect of EGCG. Indeed, this study has demonstrated that four weeks of treatment with EGCG has lowered the SBP of the hypertensive animals. This implies that treatment/supplementation with EGCG is able to exert blood pressure-lowering effect in hypertensive subjects. The current finding is in line with findings from other research groups. According to research by Mohd Sabri and her team, two weeks of treatment with EGCG significantly lowered the SBP in angiotensin II-infused hypertensive mice by 40% when compared to untreated mice (Mohd Sabri *et al.*, 2022). Besides, Hsieh and team have demonstrated that there was a reduction in the SBP of SHR treated with EGCG via oral gavage for 12 weeks (Hsieh *et al.*, 2021). In Parn's study, it was proven that SHR supplemented with 250mg/kg EGCG showed a significant 23mmHg reduction in their SBP compared to control animals (Parn *et al.*, 2022). Adding on, the SBP of Dahl salt-sensitive rats was significantly reduced after oral administration of EGCG for six weeks (Luo *et al.*, 2020) whereas the mean arterial pressure (MAP) of SHR was significantly reduced after 4 weeks of paraventricular nucleus of hypothalamus infusion with EGCG (Yi *et al.*, 2016). Present finding showed that SHR treated with losartan in four weeks treatment period in hypertensive animals has blood pressure-lowering effect. This finding also in line with others research findings. Chiu and team have demonstrated 20 mg/kg/day of losartan treated in SHR by daily oral gavage reduced SBP by 22.2.% compared to non-treated SHR group following eight weeks treatment (Chiu *et al.*, 2020). Another study with combination of antidiabetic agent with 50 mg/kg of losartan for 8 weeks oral treatment able to reduced SBP in hypertensive diabetic rats (Moke *et*

al., 2023). Although EGCG has been demonstrated to possess antihypertensive effect, its protective mechanism on vascular endothelial layer is yet to be fully explored.

It has been demonstrated that hypertension and endothelial dysfunction is strongly associated (Gallo, Volpe and Savoia, 2022c). Hence, treatment that targets an improvement in endothelial function may produce antihypertensive effect. Lau's team has shown that treatment with 3′,4′-dihydroxyflavonol (DiOHF) normalised the SBP of C57BL/6 J in tunicamycin-treated mice and improved the endothelial-dependent relaxation of the animals (Lau *et al.*, 2018). Besides, treatment with (−)-epicatechin has been shown to exert antihypertensive effect in aged Wistar rats and induced vascular endothelium-dependent relaxation in the animal (Ramirez-Sanchez *et al.*, 2018). On the other hand, (−)-epicatechin prevented the onset of hypertension in SHR and increased the endothelium-dependent relaxation of the treated young rats with developing hypertension (Kluknavsky *et al.*, 2016). The finding from the recent study is in line with previous studies done whereby an improvement in the endothelium-dependent relaxations in the aortic tissues was followed by a decrease in SBP of EGCG-treated SHR. The beneficial effect of EGCG to improve endothelium-dependent relaxation is also demonstrated in a study done by Potenza and colleagues whereby treatment with EGCG in SHR for three weeks improved vasodilation and insulin resistance by stimulating endothelial production of NO (Potenza *et al.*, 2007). An experiment done by Mohd Sabri and colleagues also demonstrated that 14 days of treatment with EGCG significantly improved endothelium-dependent relaxation and lower the blood pressure in angiotensin

II-infused mice (Mohd Sabri *et al.*, 2022). Besides, this finding also had shown losartan significantly improved vascular relaxation in the treated SHR. This is aligned with the previous studies as one of the studies had demonstrated that orally administrated of losartan 20mg/kg/day for 5 weeks in SHR has improved endothelium-dependent relaxation and reduced elevated blood pressure (Robles-Vera *et al.*, 2020). Another experiment done by Chiu and colleagues proven that showed 8 weeks of treatment with 20 mg/kg/day of losartan significantly improved endothelium-dependent relaxation and lower blood pressure in SHR (Chiu *et al.*, 2020). These studies imply that the improvement in vascular function may partly contribute to the decrease in SBP of the treated hypertensive animals.

NO is a highly reactive molecule that plays a crucial part in vascular homeostasis and blood pressure regulation (Cyr *et al.*, 2020). The release of NO leads to the activation of sCG to produce cGMP in vascular smooth muscle to induce vasodilation (Ahmad *et al.*, 2018). The DAF fluorescence staining on the vascular tissues of the animals in the current study has indicated that there is a raised in the level of vascular NO in EGCG-treated SHR. In addition to this, the current study has shown that there is an elevated level of BH_4 in SHR treated with EGCG. BH_4 is a cofactor of eNOS that is essential in the production of endogenous NO to maintain the normal endothelial function (Feng *et al.*, 2021; Gonçalves, Jasiulionis and de Melo, 2021). Depletion of BH_4 resulted in the uncoupled state of eNOS thus reducing NO bioavailability (Sen *et al.*, 2022). The elevated level of NO that is accompanied by an increase in the level of BH_4 in SHR treated with EGCG is aligned with the work done by Zhang and

colleagues. The team has demonstrated that treatment with 10-50 μM of EGCG in human umbilical vein endothelial cells exposed to high glucose elevated the expression of BH_4 and eNOS (dimer) followed by increased NO production and attenuated ROS formation (Zhang and Zhang, 2020). In Mohd Sabri and colleague's studies also had shown 50 mg *in vivo* treatment of EGCG in angiotensin II-infused hypertensive mice increase the expression of BH_4, eNOS and p-eNOS. Besides, the current study also had shown an increase in BH_4 and NO level in SHR treated with losartan. This is supported by previous experimental findings in Satoh and colleague's studies. They showedad that 6 weeks orally administration of 30 mg/kg/day losartan treatment has significantly increased the NO production, expression of BH_4, eNOS and p-eNOS in streptozotocin-infused diabetes rats (Satoh *et al.*, 2008). Our current data implies that treatment with EGCG is able to increase the level of BH_4 in the aortic tissues of hypertensive animals leading to increased eNOS coupling thus increase production of the NO level.

The NO produced in the endothelium diffuses into the underlying vascular smooth muscle cells and subsequently bind to the heme moiety of sGC. This results in the production of cGMP, which in turn activates the cGMP-dependent protein kinases and causes relaxation in vascular system (Durgin *et al.*, 2019; Golshiri *et al.*, 2020). In line with the increase in the level of vascular NO and BH_4, it was observed that there is an elevated level of cGMP in SHR treated with EGCG. Previous study has shown that after 2 hours of orally administered EGCG (100 mg/kg), there was an increase in the plasma cGMP level in both male and female C57BL/6J mice (Tanaka *et al.*, 2021). Chen et al.

has reported that EGCG supplement significantly increased cGMP level in aged rats which led to improvement of impaired erectile function via the NO/cGMP pathway (Chen *et al.*, 2016). Another study done by Alvarez's team reported that EGCG elevated cGMP levels through cell surface receptor 67LR that stimulated eNOS pathway hence leads to vasorelaxation and improved vascular function (Álvarez *et al.*, 2006). Similarly, SHR treated with losartan also demonstrated and increased vascular NO, BH_4 and cGMP. Li et al. has reported that 6 weeks of losartan orally treatment increased cGMP level in diabetes rats which led to improvement of impaired erectile function (W. J. Li *et al.*, 2017). Hence, our current finding implies that treatment with EGCG improves endothelial dysfunction partly via stimulation of the NO-sGC-cGMP pathway.

Excessive production of ROS leads to oxidative stress and subsequently causes vascular cell damage (Chen *et al.*, 2018). The current findings have revealed that there is a lower DHE fluorescence intensity in aortic ring of EGCG-treated SHR. This indicates that EGCG is able to scavenge the free radical and reduce the formation of ROS. This result is in line with the result obtained from DAF staining whereby there is an increase in NO level following treatment of EGCG in the animals. An increase in total vascular NO level accompanied by a decrease in the production of ROS cumulatively leads to decreased oxidative stress, thus contributing to the increase in vasodilation which partly reduces the blood pressure of SHR. The current finding is in agreement with previous finding whereby two weeks of treatment with EGCG in angiotensin II-infused mice successfully reduced the DHE fluorescence intensity indicating a decrease in ROS formation and oxidative stress (Mohd Sabri *et al.*, 2022). To add on, present

study also had proven that losartan decreased the ROS production in SHR-treated aortic rings. This is aligned with the previous finding reported by Dios's team, demonstrated that losartan is able to reduce the ROS production in human cortical neuron cell line (De Dios, Collazo and Inostroza-Nieves, 2022).

AT_1 receptor is a major bioactive peptide in the RAAS and its activation by angiotensin II leads to the increased activation of NOx (Murakami, 2015). The stimulation of the AT_1 receptor activates NOx, which produces O_2^-, increasing the amount of ROS and oxidative stress in the vascular system (Salazar, 2018). In a study, angiotensin II-induced expression of NADPH oxidase subunits in human umbilical vein endothelial cells was demonstrated to be inhibited by EGCG (Ahn, Kim and Ha, 2010b). Another study conducted by Han had also demonstrated that EGCG-induced protective effect in vascular endothelial cells via a decrease in NOx expression (Han, 2014). A study has reported that pretreatment with losartan, particularly at doses of 15 and 30 mg/kg, reduced lung injury by blocking the interaction between Ang-II and AT_1 receptors, which in turn lowered p-NF-κB expression and JAK2/STATs pathway activation (C. Li *et al.*, 2017). However, there has been no studies on the effect of EGCG on the upstream of NOx activation. The current study showed treatment with EGCG and losartan reduced the expression of AT_1 receptor, albeit statistically insignificant, in aortic tissues of SHR compared to the untreated hypertensive animals. Hence, it is possible that EGCG may also partly improve the endothelial function of SHR by attenuating AT_1 receptor dependent signaling pathway.

CHAPTER 6

CONCLUSION

6.1 Conclusion

The present findings demonstrated that *in vivo* treatment with EGCG and losartan for four weeks significantly reduced the SBP of hypertensive animals. This is followed by an improvement in endothelium-dependent relaxation in SHR aorta. Besides that, treatment with EGCG and losartan resulted in an increase in the level of total vascular NO and a decrease in the level of vascular ROS in SHR. In addition, an increase in the levels of vascular BH_4 and cGMP was observed in the aortic tissues of SHR treated with EGCG and losartan. In line with these results, it is also observed that there is a decreasing trend in the protein expression of AT_1 receptor in animals treated with EGCG and losartan.

In summary, the current findings suggests that treatment with EGCG has a potential to decrease the expression of AT_1 receptors, leading to a decrease in ROS levels thus reduce eNOS uncoupling by increasing the level of BH_4 which in turn increase the level of NO. This in part improves the vascular relaxation of SHR treated with EGCG hence contributes to a decrease in the blood pressure of the hypertensive animals.

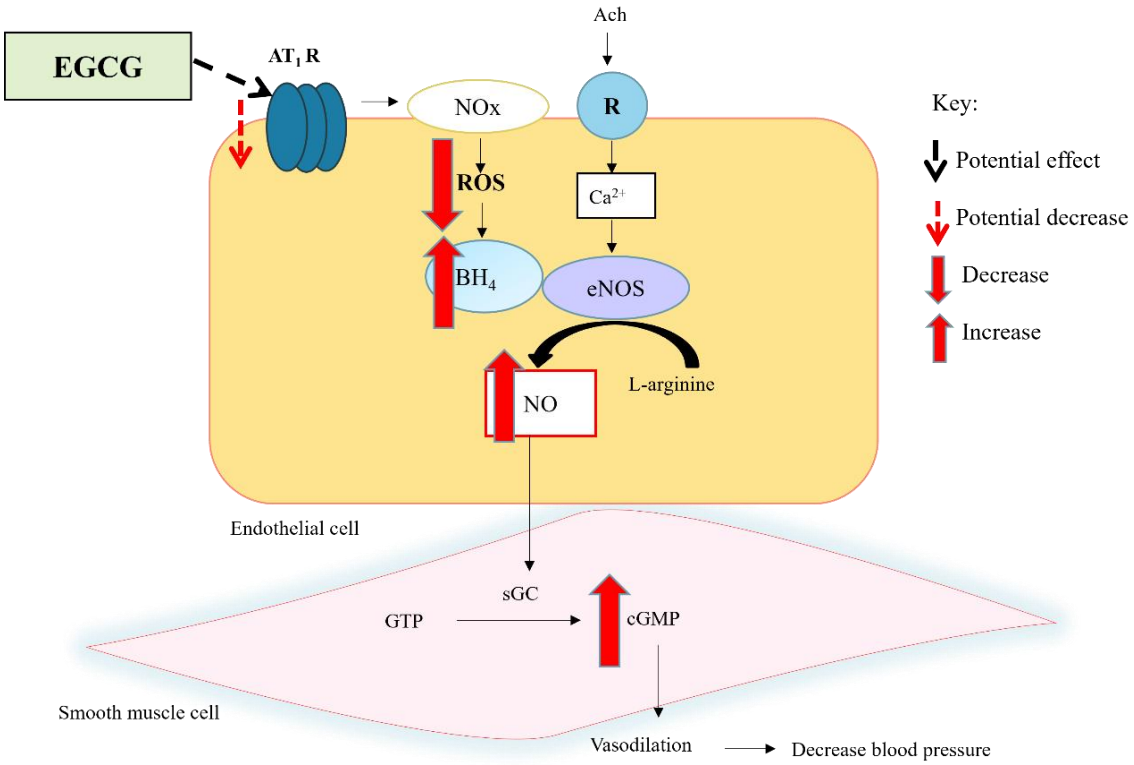

Figure 6.1: Summary diagram of the study. Ach: acetylcholine; R: acetylcholine receptor; Ca^{2+}: calcium ion; NOx: nicotinamide adenine dinucleotide phosphate oxidase; ROS: reactive oxygen species; BH_4: tertrahydrobiopterin; eNOS: endothelial nitric oxide synthase; NO: nitric oxide; sGC: soluble guanlyl cyclase; GTP: guanosine 5'-triphosphate; cGMP: cyclic guanosine monophosphate

6.2 Limitation of study

The detection of NOx expression level using Western blotting will serve as a confirmation that the enzyme is activated and hence causing the increase in the ROS level observed. Furthermore, there is insufficient evidence of AT_1 receptor mRNA expression. Since the mRNA has a wider range of expression levels than the western blotting approach (Reimegård *et al.*, 2021), the detection of AT_1 receptor mRNA expression is more sensitive. This is likely due to parallel variations in protein level. A thorough examination of cellular conditions will be possible with the combination of mRNA and protein level measurement.

6.3 Future study

Although there are mixed results from clinical trials using green tea and EGCG as regimen for antihypertensive, studies conducted to establish good safety profile of EGCG and its protective actions on the vascular endothelial tissues may provide new impetus to pursue preliminary investigations on the efficacy of the polyphenol as a treatment for hypertension or the effectiveness of it as a prophylaxis in hypertensive patients. Additionally, extended EGCG treatment period should be researched to determine its long-term impact. Finally, investigations using EGCG should also look into other possible mechanisms of the polyphenol, such as potential anti-inflammatory and the central actions of the EGCG.

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APPENDICES

APPENDIX A

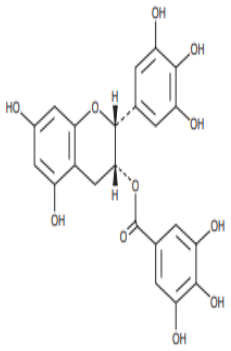
PRODUCT INFORMATION

Cayman CHEMICAL

(−)-Epigallocatechin Gallate

Item No. 70935

CAS Registry No.:	989-51-5
Formal Name:	3,4-dihydro-5,7-dihydroxy-2R-(3,4,5-trihydroxyphenyl)-2H-1-benzopyran-3R-yl-3,4,5-trihydroxy-benzoate
Synonym:	EGCG
MF:	$C_{22}H_{18}O_{11}$
FW:	458.4
Purity:	≥96%
UV/Vis.:	λ_{max}: 276 nm
Supplied as:	A crystalline solid
Storage:	-20°C
Stability:	≥4 years
Item Origin:	Plant/*Folium camelliae*



Information represents the product specifications. Batch specific analytical results are provided on each certificate of analysis.

APPENDIX B

LIST OF PUBLICATION

1. Khor, Y. *et al.* (2023) 'Epigallocatechin-3-gallate exerts antihypertensive effects and improves endothelial function in spontaneously hypertensive rats', *Asian Pacific Journal of Tropical Biomedicine*, 13(7), p. 287. doi:10.4103/2221-1691.380560.

APPENDIX C

LIST OF CONFERENCE PARTICIPATION

Name of Conference	Title of Abstract
34th Malaysian Society of Pharmacology and Physiology (MSPP) • Poster presentation	Investigation of the vasoprotective mechanism of epigallocatechin-3-gallate (EGCG) in Spontaneously Hypertensive Rats
Third Biennial International Medical and Health Sciences Conference (2021) • Poster submission	The beneficial effect of epigallocatechin-3-gallate (EGCG) on the vascular function of Spontaneously Hypertensive Rats
Global Young Scientists Summit 2023 • Poster submission	The vasoprotective effect of epigallocatechin-3-gallate (EGCG) on the Spontaneously Hypertensive Rats

APPENDIX D

Investigation of the role of Epigallocatechin-3-gallate (EGCG) in reducing oxidative stress level in Spontaneously hypertensive rats

Yucinda Khor Yee Yan[1], Lee Siew-Keah[1], Dharmani Devi Murugan[2], Ling Wei Chih[1]

[1]Faculty of Medicine and Health Sciences, Universiti Tunku Abdul Rahman, 43000 Kajang, Selangor, Malaysia
[2]Department of Pharmacology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

BACKGROUND OF STUDY

Epigallocatechin-3-gallate (EGCG), the major catechin found in green tea has been demonstrated to potentially reduce the risk factors for cardiovascular disease (Chu *et al.*, 2017). EGCG has been demonstrated to lower the blood pressure of hypertensive animals (Luo *et al.*, 2020) but whether the decrease in blood pressure is contributed by its vascular protective mechanism is yet to be elucidated.

OBJECTIVE

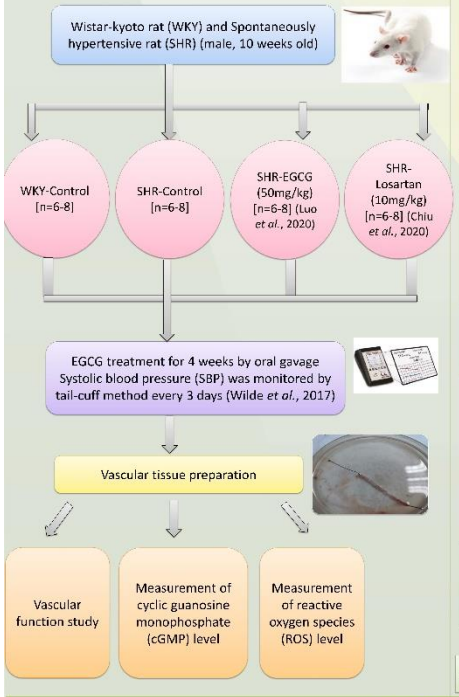
This current study aims to investigate if treatment with EGCG reduces the vascular oxidative stress level in Spontaneously hypertensive rats (SHR).

METHODOLOGY

Animals

The protocol described below was approved by the Animal Care and Ethics Committee of the Universiti Tunku Abdul Rahman and University of Malaya.

Experiment design



RESULTS

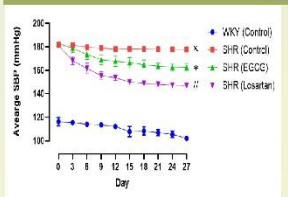
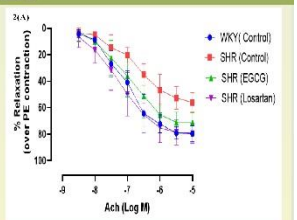
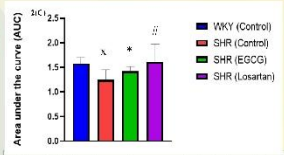


Figure 1: Average systolic blood pressure (SBP) in WKY control and SHR, with and without in vivo treatment with either epigallocatechin gallate (EGCG; 50 mg/kg/day) or losartan (10mg/kg/day) for four weeks. Data are expressed as mean ± SEM. * p ≤ 0.05 WKY (Control) compared to SHR (Control), * p ≤ 0.05 SHR (Control) compared to SHR (EGCG) and # p ≤ 0.05 SHR (Control) compared to SHR (Losartan)





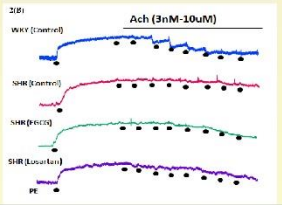
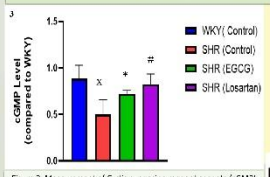


Figure 2: (A) Relaxation curve for percentage of relaxation in aortic rings in WKY (Control) and Spontaneously hypertensive rats with and without *in vivo* EGCG treatment and losartan treatment. (B) Representative tracings of acetylcholine induced relaxation in phenylephrine (PE)-contracted aortic rings. (C) Area under the curve for acetylcholine induce relaxation in phenylephrine (PE)-contracted aortic rings in WKY (Control) and Spontaneously hypertensive rats with and without *in vivo* EGCG treatment, and losartan treatment. Data are expressed as mean ± SEM. * p < 0.05 WKY (Control) compared to SHR (Control), * p < 0.05 SHR (Control) compared to SHR (EGCG) and # p < 0.05 SHR (Control) compared to SHR (Losartan)



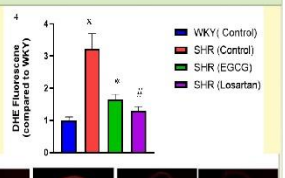


Figure 3: Measurement of Cyclic guanosine monophosphate (cGMP) level in aortic rings of WKY control and SHR, with and without *in vivo* treatment with either epigallocatechin gallate (EGCG; 50 mg/kg/day) or losartan (10mg/kg/day) for four weeks. Data are expressed as mean ± SEM. * p ≤ 0.05 WKY (Control) compared to SHR (Control), * p ≤ 0.05 SHR (Control) compared to SHR (EGCG) and # p ≤ 0.05 SHR (Control) compared to SHR (Losartan)

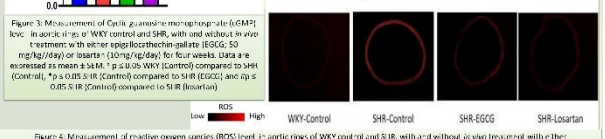


Figure 4: Measurement of reactive oxygen species (ROS) level in aortic rings of WKY control and SHR, with and without *in vivo* treatment with either epigallocatechin gallate (EGCG; 50 mg/kg/day) or losartan (10mg/kg/day) for four weeks. Data are expressed as mean ± SEM. * p ≤ 0.05 WKY (Control) compared to SHR (Control), * p ≤ 0.05 SHR (Control) compared to SHR (EGCG) and # p ≤ 0.05 SHR (Control) compared to SHR (Losartan)

DISCUSSION AND CONCLUSION

- *In vivo* treatment with EGCG and losartan attenuated the increase in SBP and improved the vascular function in SHR respectively.
- The increase of cGMP level SHR treated with EGCG implies that there is an increase in nitrite oxide (NO) level in the vascular tissue, contributing to the increase in relaxation in treated SHR.
- The decrease of ROS level in SHR treated with EGCG implies that treatment with EGCG decreased the production of ROS, thus leads to an increase in NO level.
- In conclusion, this study shows that treatment with EGCG decrease oxidative stress and improves the vascular function of SHR which in part contributes to the decrease in blood pressure of the animals

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ACKNOWLEGEMENT

This study is funded by the Universiti Tunku Abdul Rahman Research fund [IPSR/RMC/UTARRF/2019-C2/L08]

APPENDIX E

The beneficial effect of Epigallocatechin-3-gallate (EGCG) on the vascular function of Spontaneously Hypertensive Rats

Yucinda Khor Yee Yan[1], Lee Siew-Keah[1], Dharmani Devi Murugan[2], Ling Wei Chih[1]

[1]Department of Pre-clinical Sciences, Faculty of Medicine and Health Sciences, Universiti Tunku Abdul Rahman, Selangor, Malaysia
[2]Department of Pharmacology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

BACKGROUND OF STUDY

Epigallocatechin-3-gallate (EGCG), the major catechin found in green tea has been demonstrated to potentially reduce the risk factors for cardiovascular disease (Chu *et al.*, 2017) and lower the blood pressure of hypertensive animals (Luo *et al.*, 2020). However, whether the decrease in blood pressure is contributed by its vascular protective mechanism is yet to be elucidated.

OBJECTIVE

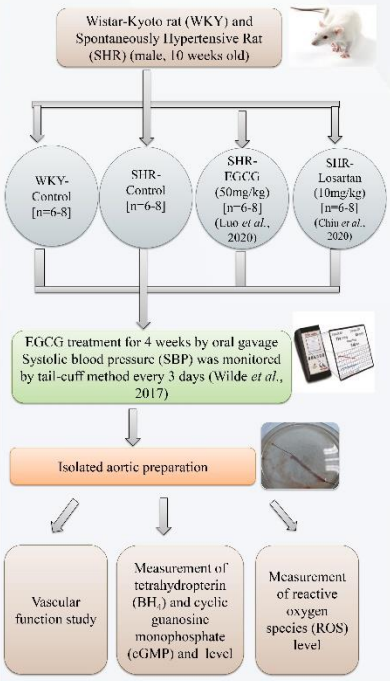
This current study aims to investigate if treatment with EGCG improves vascular function in Spontaneously Hypertensive Rats (SHR).

METHODOLOGY

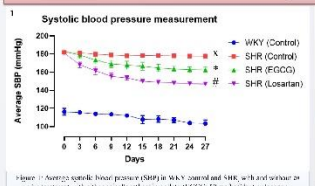
Animals

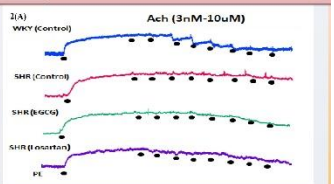
The protocol described below was approved by the Animal Care and Ethics Committee of the Universiti Tunku Abdul Rahman and University of Malaya.

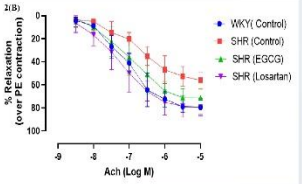
Experiment design

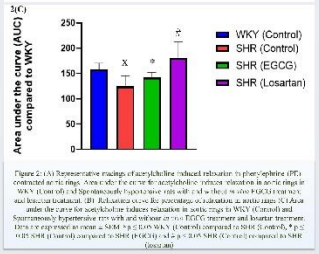


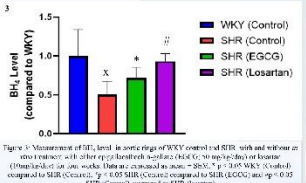
RESULTS

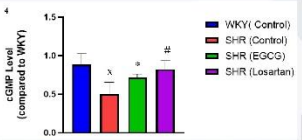


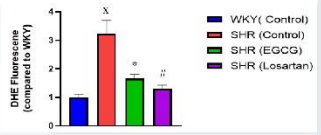












DISCUSSION AND CONCLUSION

- *In vivo* treatment with EGCG and losartan attenuated the increase in SBP and improved the vascular function in SHR, respectively.
- The increase of BH_4 level in SHR treated with EGCG is in line with the increase in cGMP level, implying there might an increase of eNOS coupling as BH4 is a cofactor for eNOS coupling. This could lead to an increase in nitric oxide (NO) level in the vascular tissue, contributing to the increase in vasorelaxation in treated SHR.
- The decrease of ROS level in SHR treated with EGCG implies that treatment with EGCG decreased the production of ROS which may contribute to a decrease in NO oxidation.
- In conclusion, this study shows that treatment with EGCG improves the vascular function of SHR via an increase in eNOS coupling and a decrease in ROS level, which in part contributes to the decrease in blood pressure of the animals.

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APPENDIX F

The vasoprotective effect of Epigallocatechin-3-gallate (EGCG) on Spontaneously Hypertensive Rats

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ABSTRACT

EGCG, a major catechin found in green tea, is claimed to reduce the risk factor for cardiovascular diseases. The current study investigated the vasoprotective action of EGCG in hypertensive animals. SHR is treated with EGCG (50mg/kg/day) or losartan (10mg/kg/day) for 4 weeks by oral gavage. The SBP was significantly decreased in SHR treated with EGCG and losartan group. Endothelium-dependent relaxation was significantly improved in aortic ring isolated from EGCG and losartan-treated SHR groups. There is also a decrease in the ROS level of these animals. The levels of BH_4 and cGMP were also significantly increased in SHR treated with EGCG and losartan respectively. In conclusion, this study shows that EGCG improves the vascular function of SHR by reducing oxidative stress which in part may have contributed to the decrease in blood pressure of the animals.

INTRODUCTION

Epigallocatechin-3-gallate (EGCG), the major catechin found in green tea has been demonstrated to potentially reduce the risk factors for cardiovascular disease (Chu *et al.*, 2017) and lower the blood pressure of hypertensive animals (Luo *et al.*, 2020). However, whether the decrease in blood pressure is contributed by its vascular protective mechanism is yet to be elucidated.

OBJECTIVE

This current study aims to investigate if treatment with EGCG improves vascular function in Spontaneously Hypertensive Rats (SHR).

JUSTIFICATION OF STUDY

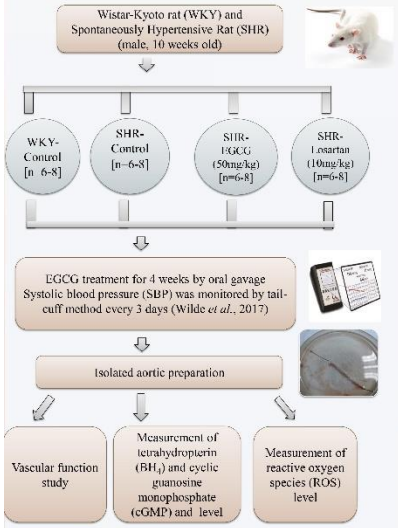
The understanding of this fundamental mechanism will help in the drug discovery and development process whereby EGCG with its vascular protective property, maybe consumed as an antihypertensive agent, either as a supplement or a drug.

METHODOLOGY

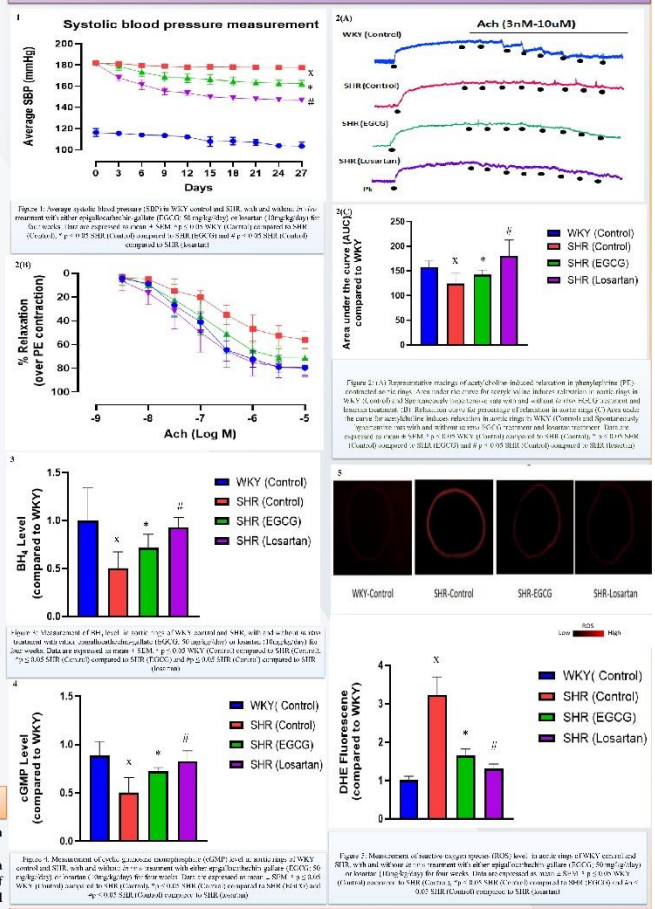
Animals

The protocol described below was approved by the Animal Care and Ethics Committee of the Universiti Tunku Abdul Rahman and University of Malaya.

Experiment design



RESULTS



DISCUSSION AND CONCLUSION

- *In vivo* treatment with EGCG and losartan attenuated the increase in SBP and improved the vascular function in SHR, respectively.
- The increase of BH_4 level in SHR treated with EGCG is in line with the increase in cGMP level, implying there might an increase of eNOS coupling as BH4 is a cofactor for eNOS coupling. This could lead to an increase in nitric oxide (NO) level in the vascular tissue, contributing to the increase in vasorelaxation in treated SHR.
- The decrease of ROS level in SHR treated with EGCG implies that treatment with EGCG decreased the production of ROS which may contribute to a decrease in NO oxidation.
- In conclusion, this study shows that treatment with EGCG improves the vascular function of SHR via an increase in eNOS coupling and a decrease in ROS level, which in part contributes to the decrease in blood pressure of the animals.

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