SAFETY AND EFFICACY OF ORAL EPIGALLOCATECHIN GALLATE IN ATTENUATING HYPERTENSION IN SPONTANEOUSLY HYPERTENSIVE RATS VIA MODULATION OF THE INTRARENAL RENIN-ANGIOTENSIN SYSTEM

By

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ABSTRACT

SAFETY AND EFFICACY OF ORAL EPIGALLOCATECHIN GALLATE IN ATTENUATING HYPERTENSION IN SPONTANEOUSLY HYPERTENSIVE RATS VIA MODULATION OF THE INTRARENAL RENIN-ANGIOTENSIN SYSTEM

Parn Kim Wai

Epigallocatechin gallate (EGCG) has been frequently recommended as a potential antihypertensive compound. Nevertheless, multiple cases of hepatotoxicity are associated with consumption of high dose EGCG-containing health supplements. To date, there is lacking scientific evidence on the safety profile of EGCG and the underlying mechanism of its antihypertensive effects in any cardiovascular disease model. This study aimed to identify the no-observed-adverse-effect level (NOAEL) of EGCG and its possible antihypertensive effects through modulation of the intrarenal renin-angiotensin system (RAS) gene expression in spontaneously hypertensive rats (SHR). EGCG (50, 250, 500 or 1000 mg/kg *b.w. i.g.*, once daily) was administered to SHR for 28 days. All the SHR survived with no signs of systemic toxicity. Increased alanine aminotransferase and aspartate aminotransferase levels were evident in SHR administrated with 1000 mg/kg *b.w.* while increased thiobarbituric acid reactive substances level were present in SHR receiving EGCG equal or higher than 500 mg/kg *b.w.*. However, these effects were not found in those administrated with lower doses of EGCG. Subsequently, the NOAEL of EGCG was established at 250 mg/kg *b.w.*, and the same protocol was replicated to assess its effects on blood pressure and renal RAS-related

genes in SHR. The systolic blood pressure (SBP) of the EGCG group was consistently lower than the control group. The mRNA levels of cortical *Agtr2* and *Ace2* and medullary *Agtr2*, *Ace* and *Mas1* were upregulated while medullary *Ren* was downregulated in EGCG group. The Pearson correlation analysis showed that SBP reduction was associated with the changes in medullary *Agtr2*, *Ace*, and *Ren*. EGCG treatment exhibits antihypertensive effects through activation of intrarenal *Ace* and *Agtr2* and suppression of *Ren* mediators, while a high dose of EGCG induced liver damage in SHR. In future clinical studies, liver damage biomarkers should be closely monitored to further establish the safety of the long-term use of EGCG.

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APPROVAL SHEET

This dissertation entitled "**SAFETY AND EFFICACY OF ORAL EPIGALLOCATECHIN GALLATE IN ATTENUATING HYPERTENSION IN SPONTANEOUSLY HYPERTENSIVE RATS VIA MODULATION OF THE INTRARENAL RENIN-ANGIOTENSIN SYSTEM"** was prepared by PARN KIM WAI and submitted as partial fulfillment of the requirements for the degree of Master of Medical Science at Universiti Tunku Abdul Rahman.

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DECLARATION

I PARN KIM WAI hereby declare that the dissertation and published article (Nutrients 2022, 14(21), p.4605) are based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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LIST OF ABBREVATIONS

(P)RR	(pro)renin receptor
DCFDA	2,7-Dichlorofluoroscin Diacetate
ACEi	ACE inhibitors
AGT	Angiotensinogen
ALT	Alanine transaminase
Ang I	Angiotensin I
Ang II	Angiotensin II
AT1R	Angiotensin II type 1 receptor
AT2R	Angiotensin II type 2 receptor
ARB	Angiotensin receptor blockers
Ang-(1-7)	Angiotensin-(1-7)
ACE	Angiotensin-converting enzyme
ACE2	Angiotensin-converting enzyme 2
AST	Aspartate transaminase
b.w.	Body weight
BUN	Blood urea nitrogen
BSA	Bovine serum albumin
C	Catechin
CG	Catechin gallate
C21	Compound 21
cAng (1-7)	Cyclic Ang (1-7)
CCB	Calcium channel blockers
DOCA	Deoxycorticosterone acetate

DNA	Deoxyribonucleic acid
DBP	Diastolic blood pressure
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
gDNA	genomic deoxyribonucleic acid
GTE	Green tea extract
H_2O_2	Hydrogen peroxide
i.g.	Intragastric
i.p.	Intraperitoneal
JGA	Juxtaglomerular apparatus
LDH	Lactate dehydrogenase
MasR	Mas receptor
MBP	Mean blood pressure
NHE3	Na^+/H^+ exchanger 3
NKA	Na^+/K^+-ATPase
NADH	Nicotinamide adenine dinucleotide
NO	Nitric oxide
NOAEL	No-observed-adverse-effect level
OECD	Organisation for Economic Co-operation and Development
PVN	Paraventricular nucleus
PBS	Phosphate-buffered saline
PRA	Plasma renin activity
ROS	Reactive oxygen species

RAS	Renin-angiotensin system
RO	Reverse osmosis
RNA	Ribonucleic acid
SP1	Specificity protein 1
SHR	Spontaneously hypertensive rats
SD	Sprague-Dawley
SD	Standard deviation
O_2^-	Superoxide anion
SBP	Systolic blood pressure
TBARS	Thiobarbituric acid reactive substances
UTAR	Universiti Tunku Abdul Rahman
VPR	Volume pressure recording

CHAPTER 1

INTRODUCTION

1.1 Research Background

Dysregulation of the renin-angiotensin system (RAS), the main blood pressure regulator, is associated with several pathological conditions which include hypertension, atherosclerosis, heart and kidney diseases. Over the past decades, research has been focusing on overactivity of the classical RAS in the development of hypertension while the roles of the counter-regulatory RAS in promoting cardiovascular health are gaining popularity in recent decades (Nehme et al., 2019).

The angiotensin-converting enzyme/angiotensin II/angiotensin II type 1 receptor [ACE/Ang II/AT1R] and prorenin/(pro)renin receptor [(P)RR] are the two axes in classical RAS in which activation of these axes leads to vasoconstriction and subsequently elevated systemic blood pressure. Activation of the counter-regulatory arm via ACE/Ang II/angiotensin II type 2 receptor (AT2R) or angiotensin-converting enzyme 2/angiotensin (1-7)/Mas receptor [ACE2/Ang (1-7)/MasR] axes counteracts the action of the classical arm, leading to vasodilation and reduction in systemic blood pressure (Nehme et al., 2019).

The RAS can operate as an endocrine (commonly knowns as circulating or systemic RAS) and autocrine/paracrine (commonly known as local, or tissue-based RAS) in the vasculature, brain, heart, kidney, etc. The regulatory significance of local RAS components like renin, angiotensinogen (AGT), ACE, angiotensin I (Ang I) and II is well recognized (Nehme et al., 2015). It has been demonstrated that systemic RAS is involved in short-term hemodynamic effects regulation while local RAS is involved in structural rebuilding of the vascular wall, brain, heart or kidney (Simko et al., 2021). Studies on transgenic animal models have uncovered that intrarenal RAS is implicated in the pathogenesis of hypertension and renal diseases, and manipulation of intrarenal RAS mediators is associated with blood pressure reduction (Kobori et al., 2007).

Overactivation of the classical RAS is frequently found in hypertensive individuals, usually evidenced by increased Ang II and AT1R levels (Szczepanska-Sadowska et al., 2018). Uses of RAS-blockade agents *i.e.* ACE inhibitors (ACEi) and angiotensin receptor blockers (ARB) which aim to suppress Ang II production are the therapeutic strategies in the management of hypertension. However, given limitations in using the current synthetic RAS inhibitors that cause various undesirable effects and intolerance in some patient populations (Marcum and Fried, 2011, Mishima et al., 2019), the need on exploration of novel RAS modulators is indeed in demand. Recent studies have shown that targeting counter-regulatory RAS mediators *e.g.* AT2R and ACE2 could be a novel approach in treating hypertension (Dai et al., 2016, Sartorio et al., 2020).

Natural products play important role in the development of antihypertensive drugs as they serve as a reservoir of novel compounds with significant biological activities. Many natural products including alkaloids, coumarins, diterpenes, flavonoids and peptides exhibit antihypertensive effects (Bai et al., 2015). Reserpine, the first natural antihypertensive drug isolated from the roots of the tropical plants, *Rauwolfia serpentina*, had been used clinically as earlier in 1955 (Cheung and Parmar, 2022). The current synthetic antihypertensive drugs targeting the RAS are often accompanied by angioedema, headache and hyperkalemia (Verma et al., 2010). These adverse effects often become intolerable after an extended period of using synthetic drugs. Thus, natural products gained the interest of people as they have better safety profiles and cause lesser side effects (Zhu et al., 2022).

Epigallocatechin gallate (EGCG), a polyphenol predominantly found in green tea is a potent antioxidative and anti-inflammatory molecule and has been recommended as a cardioprotective compound (Cao et al., 2019). It has been shown that EGCG modulates deoxyribonucleic acid (DNA) transcription and protein synthesis, functionality of receptors and subsequent cellular responses and biological activities (Krupkova et al., 2016). Uses of green tea or green tea extract in relation to its blood pressure-lowering effect have been demonstrated (Xu et al., 2020). In *in vivo* studies, it has been demonstrated that the blood pressure-lowering effect of EGCG is in a time and dose-dependent manner (Zhang et al., 2018, Tan et al., 2021). Exploratory research has showed that EGCG is a potential blockage agent in inhibiting ACE and renin activities in *in silico* and *in vitro* studies (Li et al., 2013, Ke et al., 2017). Nevertheless, there

is lacking scientific evidence in correlating the modulatory effects of EGCG on RAS with its blood pressure-lowering effect.

EGCG is in general considered to be safe and well-tolerated in humans and it is widely available in the market as a health supplement (Younes et al., 2018). A recent comprehensive investigation by the European Food Safety Authority concluded that (i) catechins from green tea infusion prepared traditionally are in general considered to be safe (ii) liver injury reported after consumption of green tea infusions/EGCG supplements are rare and most probably due to an idiosyncratic reaction (iii) consumption of EGCG at doses equal or above 800 mg/day as a food supplement resulted in increased serum transaminases in EGCG treated subjects compared to control (Younes et al., 2018). Nevertheless, these reports did not elucidate the mechanism underlying the hepatoxicity effect of EGCG, and there is still lacking scientific evidence in addressing the association of EGCG ingestion with organ damage.

1.2 Problem Statement and Rationale of the Study

The antihypertensive effects of EGCG had been demonstrated in previous studies (Potenza et al., 2007). However, the blood pressure of EGCG-treated hypertensive animals was not reduced to desired levels. On the other hand, high doses EGCG was found to be associated with hepatotoxicity and nephrotoxicity (Lambert et al., 2010, Rasheed et al., 2017). Despite the efficacy of EGCG in treating hypertension, the safety profile of EGCG in the cardiovascular disease model is not well established. There is also a need to

explore the role of EGCG on modulating the intrarenal RAS which is the main blood pressure regulatory system in human body.

This present study postulates that supplementation with high doses of EGCG over an extended period may cause a greater blood pressure reduction in spontaneously hypertensive rats (SHR) which is a genetically hypertensive rodent model. With the consideration of possible toxic effect of EGCG, this study aimed to establish the no-observed-adverse-effect level (NOAEL) of EGCG before proceeding to examine the antihypertensive effect of EGCG.

The second phase of the study aimed to investigate the efficacy of EGCG in attenuating hypertension via its modulatory activities on the transcriptional levels of intrarenal RAS in SHR. SHR is selected as the study model as it is a model of primary hypertension, and it has been established that the intrarenal RAS is inappropriately activated and attributed to the development of hypertension and renal damage (Williamson et al., 2017).

1.3 Research Questions

i. What are the effects of EGCG on body weight, food and water intake?

ii. What are the effects of EGCG on the mortality rate, behavioural changes, observable clinical signs, gross anatomy and relative organ weights of thoracic and abdominal organs?

iii. What are the effects of EGCG on the plasma alanine transaminase (ALT), aspartate transaminase (AST), creatinine and urea and hepatic reactive oxygen species (ROS), thiobarbituric acid reactive substances (TBARS) and caspase-3 levels?

iv. What are the effects of EGCG on the systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean blood pressure (MBP) in SHR?

v. What are the effects of EGCG on the mRNA expression of RAS-related genes included angiotensin II receptor type 1a (*Agtr1a*), angiotensin II receptor type 2 (*Agtr2*), angiotensin-converting enzyme (*Ace*), angiotensin-converting enzyme 2 (*Ace2*), angiotensinogen (*Agt*), Mas receptor (*Mas1*), renin (*Ren*), and (pro)renin receptor [*Atp6ap2*] in SHR?

vi. Do the antihypertensive effects of EGCG correlate with the transcriptional level of RAS-related genes in research question (v)?

1.4 Research Aims

This study aims:

1. To establish the NOAEL of EGCG treatment in SHR.

2. To examine the antihypertensive effects of EGCG in modulating intrarenal RAS transcriptional activities in SHR.

1.5 Research Objectives

Phase I: To determine the NOAEL of oral EGCG in SHR via the following specific objectives

i. To determine effects of EGCG on body weight, food and water intake.

ii. To determine effects of EGCG on mortality rate, behavioural changes, observable clinical signs, gross anatomy and relative organ weights of thoracic and abdominal organs.

iii. To determine effects of EGCG on plasma ALT, AST, creatinine and urea and hepatic ROS, TBARS and caspase-3 levels.

Phase II: To determine the roles of EGCG as an intrarenal renin-angiotensin system modulator in amelioration of raised blood pressure in SHR

iv. To determine effects of EGCG on SBP, DBP and MBP in SHR.

v. To determine effects of EGCG on mRNA expression of RAS-related genes included *Agtr1a*, *Agtr2*, *Ace*, *Ace2*, *Agt*, *Mas1*, *Ren*, and *Atp6ap2* in SHR.

vi. To correlate antihypertensive effects of EGCG with the transcriptional level of RAS-related genes in objective (v).

1.6 Research Hypotheses

i. EGCG will not affect the body weight, food and water intake.

ii. EGCG will not affect the mortality rate, behavioural changes, observable clinical signs, gross anatomy and relative organ weights of thoracic and abdominal organs.

iii. High dose EGCG will increase plasma ALT, AST, creatinine and urea and hepatic ROS, TBARS and caspase-3 levels while these effects will not observed with SHR treated with low dose EGCG.

iv. EGCG will reduce the SBP, DBP and MBP in SHR.

v. EGCG will downregulate the *Agtr1a*, *Ace*, *Agt*, *Ren* and *Atp6ap2* gene expression while upregulate the *Agtr2*, *Ace2* and *Mas1* gene expression in SHR.

vi. The antihypertensive effect of EGCG will significantly correlate with the transcriptional levels of RAS-related gene expression in hypotheses (v).

CHAPTER 2

LITERATURE REVIEW

2.1 Physiology of Blood Pressure Regulation

Blood pressure is determined by the cardiac output and peripheral resistance. Cardiac output represents the amount of blood pumped by the left ventricle of the heart while peripheral resistance is determined by the small peripheral arteries and arterioles (Touyz, 2014). Cardiac output is stable most of the time, thus, increased in peripheral resistance is often the cause of hypertension. The heart, nervous system and kidneys are the main organs that involve in blood pressure regulation. The blood pressure is tightly controlled to maintain sufficient perfusion to various tissues and organs to supply oxygen and nutrients to meet the metabolic demands (Touyz, 2014).

The regulation of blood pressure can be classified into short-term and long-term regulations depending on the onset of the effect and how long it lasts. The short-term blood pressure regulation is mediated through autonomic nervous systems. The blood pressure is closely monitored by the high-pressure arterial baroreceptors present on the aortic arch and carotid sinuses, and the low-pressure volume receptors present on the atria, ventricles and pulmonary vasculature (Armstrong et al., 2022). An elevated arterial pressure will lead to stretching of the artery wall, and triggers stimulation of the baroreceptors and

subsequently increases the parasympathetic activity to reduce heart rate and increase arterial diameter which ultimately results in blood pressure reduction.

The long-term blood pressure regulation involves both nervous and endocrine systems. The paraventricular nucleus (PVN) is responsible for long-term neurogenic blood pressure regulation. The PVN can be divided into autonomic and neuroendocrine compartments. The autonomic PVN regulates the sympathetic outflow of the heart, blood vessels and kidneys, thus, controls the blood pressure through modulation of the renal function, vasomotor tone and cardiac function (Becker, 2018). The neurosecretory parvocellular neurons involved in the adrenal glands' hormones production through the hypothalamic-pituitary-adrenal axis while vasopressin is produced directly by the neurosecretory magnocellular neurons of the PVN (Savic et al., 2022). The vasopressin induce vasoconstriction upon binding to the vasopressin receptor 1a located on blood vessel and water reabsorption through binding to the vasopressin receptor 2 in the kidneys.

The RAS is often identified as the most important system in long term regulation of blood pressure. The renin catalyses the conversion of AGT into Ang I which is further being catalysed into Ang II by ACE. Ang II is a potent vasoconstrictor. Binding of Ang II to AT1R at different locations namely blood vessels, adrenal glands, brain and kidney induce different reactions such as vasoconstriction, secretion of aldosterone and vasopressin, increased sympathetic nervous system activity as well as retention of sodium and water. A decrease in blood pressure stimulates the production and release of renin to

produce Ang II in order to increase the blood pressure back to the normal levels. The raised blood pressure exerts negative feedback mechanism to suppress further renin biosynthesis (Fountain and Lappin, 2022).

2.2 Hypertension

Hypertension, known as persistent raised or high blood pressure, is the highest risk factor for cardiovascular diseases, which affects 1 billion people worldwide (World Health Organization, 2021). Hypertension is a manageable and treatable disease, but unfortunately, despite extensive understanding of its pathophysiology and the availability of effective treatment strategies, only 37.4% of hypertensive patients achieved satisfactory blood pressure control (<140/90 mm Hg) during their treatment (Minister of Health Malaysia, 2018)

Hypertension can be classified into primary or secondary hypertension based on its aetiologias. Around 90-95% cases are primary hypertension, and its aetiology is multifactorial. Meanwhile, the remaining 5-10% cases are classified as the secondary hypertension which is due to an identifiable cause such as chronic kidney diseases, endocrine diseases, vascular diseases and neurogenic diseases (Hegde and Aeddula, 2022).

Primary hypertension also known as essential hypertension which the etiology remains unidentified. In addition to non-modifiable risk factors (family history of hypertension, gender, age), modifiable risk factors which include excessive alcohol consumption, high salt diet, obesity, physical inactivity and

smoking are associated with pathogenesis of hypertension (Andriolo et al., 2019). Dysregulation of the renin-angiotensin system, impairment of renal sodium handling and overactivation of sympathetic nervous system are associated with the pathogenesis of the primary hypertension (Iqbal and Jamal, 2022). The following review focuses on the physiological function of RAS and its dysregulation that leads to pathogenesis of hypertension.

2.3 Classical and Counter-regulatory Renin-Angiotensin System

The classical RAS or commonly known as RAS is the main blood pressure regulator in our body. It regulates sodium balance, extracellular fluid volume and ultimately arterial blood pressure. Decreased blood sodium levels, blood pressure, blood volumes and/or increased stress/trauma trigger baroreceptor, macula densa mechanisms and sympathetic nervous system that causes renin release and a series of sequential actions that leads to Ang II production, the determinant of blood pressure control (Figure 2.1).

The RAS is well-known for its function in increasing blood pressure. Nevertheless, discovery of several RAS new components that has been collectively known as the counter-regulatory RAS has re-established the roles of RAS in blood pressure regulation. The counter-regulatory RAS plays a central role in blood pressure reduction and commonly known as “the protective arm” of the RAS (Figure 2.2).

The ACE/Ang II/AT1R and prorenin/(P)RR the two axes in classical RAS in which activation of these axes leads to vasoconstriction and subsequently elevated systemic blood pressure. Activation of the counter-regulatory arm via ACE/Ang II/ AT2R or ACE2/Ang-(1-7)/MasR axes counteracts the action of the classical arm, leading to vasodilation and reduction in systemic blood pressure (Figure 2.2) (Nehme et al., 2019).

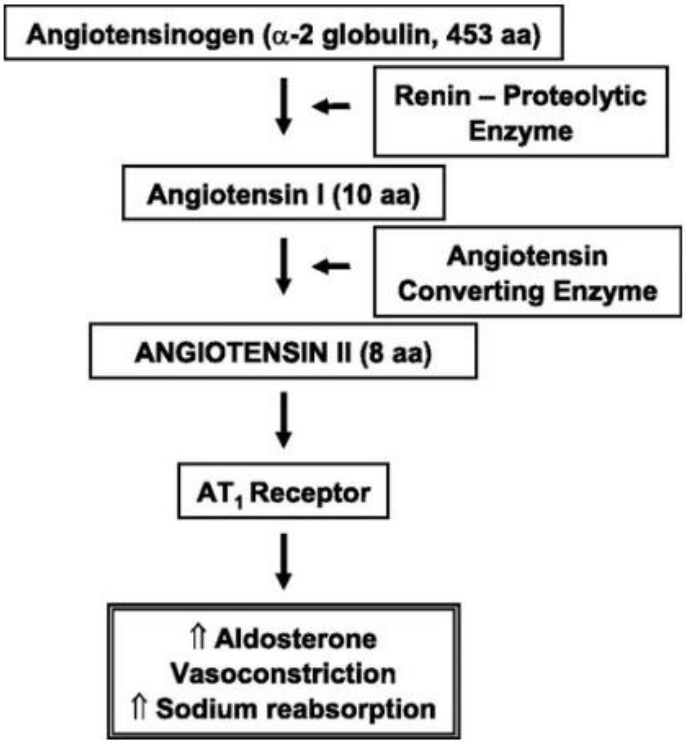

Figure 2.1: The renin-angiotensin system [Adapted from Harrison-Bernard (2009)].

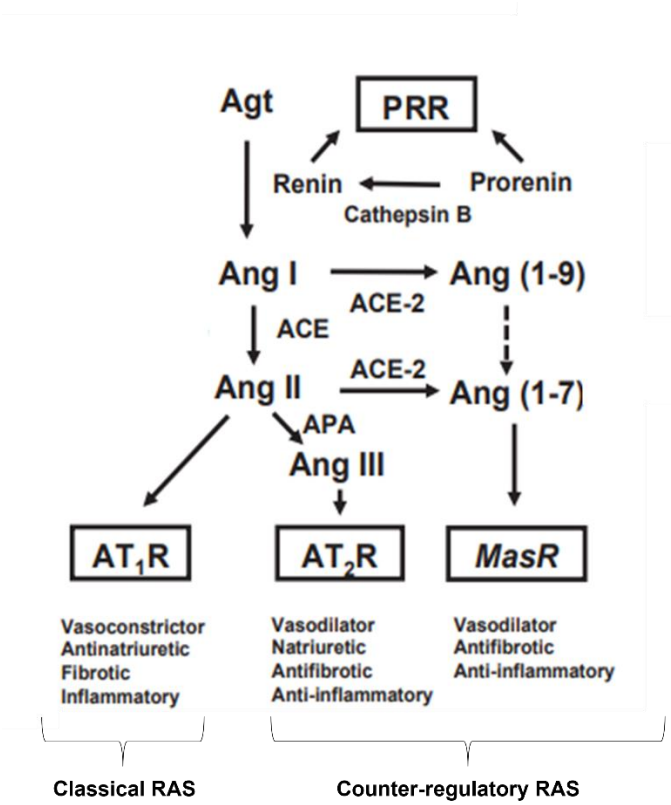

Figure 2.2: The classical and counter-regulatory RAS [Adapted from Carey (2015)].

2.4 Systemic and Local Renin-Angiotensin System

The RAS can operate as an endocrine (commonly knowns as circulating or systemic RAS) and autocrine/paracrine (commonly known as local, or tissue-based RAS) in the vasculature, brain, heart, kidney and etc..

In systemic RAS, AGT is released from the liver and is cleaved by the circulating renin that is released by the juxtaglomerular apparatus (JGA) of the kidney to form Ang I. Ang I is then converted to Ang II by the ACE, a membrane-bound metalloproteinaise that is predominantly expressed on the

surface of the pulmonary endothelial cells. Ang II is the main effector peptide of the RAS and its binding to AT1R or AT2R leads to various cellular responses (Figure 2.3) (Cooper et al., 2021).

Local RAS is an autocrine/paracrine system which has all the RAS components like those in systemic RAS. The regulatory significance of local RAS components like renin, angiotensinogen, ACE, Ang I and Ang II is well recognized (Nehme et al., 2015). It has been demonstrated that systemic RAS is involved in short-term hemodynamic effects regulation while local RAS is involved in structural rebuilding of the vascular wall, brain, heart or kidney (Simko et al., 2021). In recent years, the research focus on the roles of the systemic RAS in the pathophysiology of metabolic syndrome including hypertension and organ damage has shifted to the role of the local RAS in specific tissue (Figure 2.4) (Jahandideh and Wu, 2020).

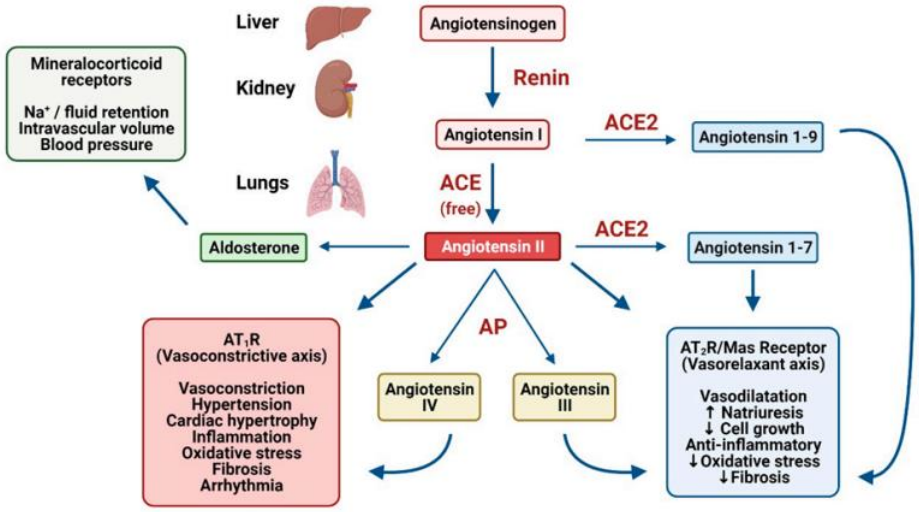

Figure 2.3: Summary action of renin-angiotensin system [Adapted from Cooper et al. (2021)].

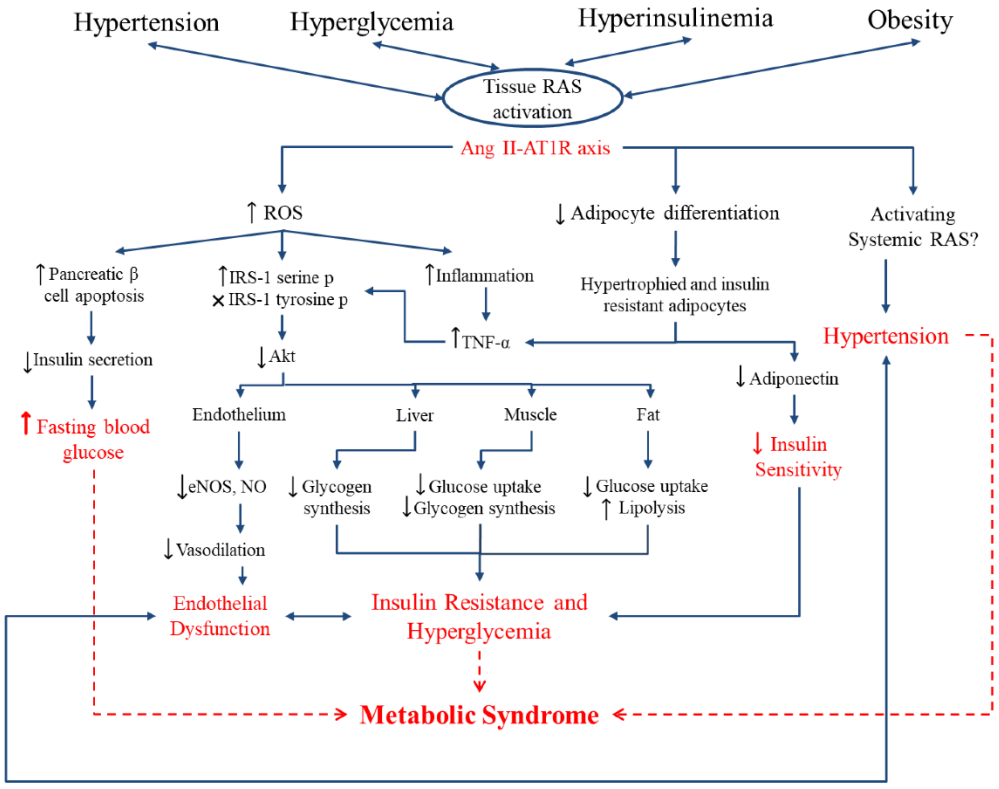

Figure 2.4: Mechanistic diagram on the roles of tissue renin-angiotensin system in metabolic syndrome [Adapted from Jahandideh and Wu (2020)].

2.4.1 Implications of the Intrarenal Renin-Angiotensin System in Pathophysiology of Hypertension

Studies on transgenic animal models have uncovered that intrarenal RAS is implicated in the pathogenesis of hypertension and manipulation of intrarenal RAS mediators is associated with blood pressure reduction. The following review focuses on the implication of the intrarenal RAS components in pathophysiology of hypertension.

All of the RAS mediators are present in the kidney and intrarenal Ang II is formed by independent mechanisms (Vio and Jeanneret, 2003, Prieto et al,,2013).

The intrarenal Ang II is 50 - 100 folds higher than the plasma Ang II levels (Moon, 2013). The high level of intrarenal Ang II suggests the possibility of its production locally in the kidney to acts as a paracrine signaling molecule. The high intrarenal Ang II might also be due to the positive feedback mechanism that stimulates Ang II production upon its binding at AT1R. The binding of Ang II to AT1R exerts negative feedback on the JGA cells to control the balance of renin in circulation. However, a feed-forward mechanism was observed in the intrarenal RAS in which activation of Ang II/AT1R caused by chronic Ang II infusion increased the expression of renin and (P)RR in collecting ducts and ACE and AGT in proximal tubules (Vio and Jeanneret, 2003, Prieto-Carrasquero et al., 2004, Prieto et al., 2013). The co-upregulation of these RAS components maintains the substantially high levels of Ang II in the kidney and amplify the reaction to induce the local vasoconstriction and sodium retention leading to hypertension.

It has been demonstrated that the intrarenal (P)RR, coded by the *Atp6ap2* gene plays significant roles in sodium handling, water homeostasis and implications in hypertension development. It has been demonstrated intrarenal (P)RR is significantly elevated in hypertensive animal models (Ang II-induced hypertensive and two-kidney, one-clip hypertensive rats) (Gonzalez et al., 2011, Prieto et al., 2013). Meanwhile, the intrarenal (P)RR knockout mice showed a diabetes insipidus phenotype with normal blood pressure (Ramkumar et al., 2016).

Intrarenal ACE expression was found higher in various hypertensive animal models including Ang II-induced hypertension, phenylephrine-induced rats, Goldblatt hypertensive rats and hypokalemic rats (Vio and Jeanneret, 2003). Ang-II infusion failed to induce hypertension in the intrarenal ACE-deficient mice (Gonzalez-Villalobos et al., 2013). In another study, N^G-nitro-L-arginine-methyl ester salt challenge failed to induce hypertension in it-ACE mice, a rodent model that lack of ACE expression on the renal tubular epithelial cells (Giani et al., 2017).

Crowley et al. (2006) elucidated that presence intrarenal AT1R is crucial in the pathogenesis of hypertension in Ang II-dependent hypertensive model while overactivation of the extrarenal AT1R is insufficient to induce hypertension in selectively AT1R knockout mice. Another two studies have also concluded that intrarenal AT1R plays the determinant roles in regulating blood pressure in transgenic rodent models (Gurley et al., 2011, Li et al., 2011).

AGT is always recognized as the passive substrate for RAS but emerging evidence suggests that AGT plays a significant role in the pathogenesis of hypertension. Augmentation of the intrarenal AGT and Ang II levels were found in both Ang II and high salt-induced hypertensive models and the researcher concluded that the hypertensive effects is mediated via Ang II/AT1R axis (Kobori et al., 2001a, Kobori et al., 2001b, Kobori et al., 2004). In addition, it has been reported that elevated urinary AGT (that represents intrarenal AGT) is positively correlated with the raised blood pressure especially in the black men population (Kobori et al., 2009, Sato et al., 2018).

Cellular actions of intrarenal AT2R, ACE2 and MasR have been associated with blood pressure reduction in various experimental models. It has been demonstrated that intrarenal AT2R null mice is more susceptible to Ang II-induced hypertension (Siragy et al., 1999). Meanwhile, activation of AT2R causes membrane internalization of sodium transporters [(Na^+/H^+ exchanger 3 (NHE3) and Na^+/K^+-ATPase (NKA)] and results in increased urinary sodium excretion which is associated with blood pressure reduction in SHR. The team further concluded that intrarenal AT2R defect in SHR may results in the pathogenesis of hypertension (Kemp et al., 2019). Patel et al. (2017) has also demonstrated that administration of AT2R agonist results in increased urine flow and urinary sodium excretion in obese Zurker rats.

ACE2 catalyze the degradation of Ang II, a potent vasoconstrictor into Ang (1-7), a vasodilator substance. ACE2-deficient mice showed exaggerated accumulation of Ang II in the kidneys and are more susceptible to the pressor effect of Ang II infusion (Gurley et al., 2006). Meanwhile, ACE2-overexpressed in transgenic stroke-prone SHR showed a lower blood pressure and resistance to the pressor actions of Ang II infusion (Rentzsch et al., 2008).

Activation of Ang (1-7)/MasR is believed to counteract the deleterious effects of Ang II/AT1R by exhibiting natriuresis and diuresis leading to blood pressure lowering effects (Zhuo et al., 2013). Infusion of Ang (1-7) leads to increased blood flow, sodium excretion and decreased renal vascular resistance while these effects were partially abolished by MasR antagonist (Nematbakhsh and Safari, 2014, Patel et al., 2017). Moreover, administration of MasR agonist

results in increased urine flow and urinary sodium excretion in obese Zurker rats (Patel et al., 2017).

2.5 Antihypertensive Treatment

Hypertension is one of the major risk factors for cardiovascular diseases. A systematic review identified that a 10 mmHg reduction of SBP is able to reduce the all-cause mortality by 13% (Ettehad et al., 2016). For those who had elevated blood pressure, lifestyle modification is often initiated instead of medication prescription, including weight loss, increase physical activities, having low sodium diet and alcohol consumption (Minister of Health Malaysia, 2018).

Drug therapy is usually initiated if non-pharmacological intervention is insufficient to improve the hypertensive conditions or the patient with high risk in developing cardiovascular diseases (Minister of Health Malaysia, 2018). Uses of the monotherapy with the first-line antihypertensive drug is the common strategies in initiating treatment. The first-line antihypertensive medications are often described as the acronym ABCD, which represents the ACEi and ARB, beta-blockers, calcium channel blockers (CCB) and diuretics (Table 2.1) (Minister of Health Malaysia, 2018).

2.5.1 Antihypertensive Drugs Targeting the Classical RAS

RAS-targeting drugs treatment is an effective strategy in lowering blood pressure in hypertensive patients. ACEi and ARB are the most commonly used RAS-targeting drugs while uses of renin inhibitors are considered as a newer approach comparing to ACEi and ARB.

Table 2.1: First-line antihypertensive drugs.

Classes	Agents	Mechanism	Contraindications	Adverse effects
ACEi	Captopril Enalapril Lisinopril	Prevent production of angiotensin II through inhibition of ACE	Pregnancy Angioedema Hypersensitivity to ACEIs	Common: cough, headache, dizziness, asthenia Severe: hyperkalemia, AKI, angioedema, fetal toxicity
ARB	Losartan Olmesartan Candesartan	Prevent the action of angiotensin II through binding to AT1R	Pregnancy Hypersensitivity to ARB	Common: headache, dizziness Severe: hyperkalemia, AKI, fetal toxicity
Beta blocker	Atenolol Metoprolol Propranolol	Prevent the action of epinephrine and norepinerphrine through binding to beta-adrenoceptors	Hypersensitivity to beta-blockers	Common: bradycardia, hypotension, dizziness, fatigue Severe: skin rash, first-degree atrioventricular block
CCB	Amlodipine Felodipine	Prevents intracellular Ca2+ influx through binding to calcium channels	Hypersensitivity to CCB	Common: flushing, peripheral edema, dizziness Severe: angioedema
Diuretic	Chlorthalidone Chlorothiazide Hydrochlorothiazide	Decreased fluids reabsorption through inhibition of sodium reabsoprtion	Anuria Hypersensitivity to thiazides or other sulfonamide drugs	Common: dizziness, hypokalemia Severe: cardiac dysrhythmias, cholestatic jaundice, new-onset diabetes mellitus, pancreatitis

Adapted and modified from Ellis et al. (2021).

2.5.1.1 ACE Inhibitors

ACE catalyses the conversion of Ang I to Ang II. Thus, ACEi limits the production of Ang II through inhibition of the action of ACE. ACEi is one of the first-line antihypertensive drugs and is often used to initiate the antihypertensive treatment unless the patient is black or older than 75 years old (Minister of Health Malaysia, 2018).

Clinical uses of ACEi have been well established since decades ago. A clinical trial including 18 hypertensive patients with DBP > 95 mmHg were randomized into a placebo group or one of the four captopril treatment regimens: 25 mg twice daily, 25 mg three times daily, 50 mg twice daily and 50 mg three times daily for 2 weeks (Corea et al., 1983). The SBP and DBP of all captopril-treated groups were significantly lower than the placebo group, with the lowest SBP and DBP found in the group receiving the highest captopril dosage (Corea et al., 1983). Meanwhile, another clinical trial consisting of 495 men with mild to moderate hypertension (DBP between 92 to 109 mmHg) were randomly assigned with placebo (three times daily) or one of the four captopril treatments: 12.5 mg three times daily, 25 mg three times daily, 37.5 mg twice daily and 50 mg three times daily for 7 weeks (Group, 1983). The SBP/DBP reduction were 8.6/5.4, 9.4/6.0, 12.6/6.4, 12.0/7.3 mmHg for 12.5, 25, 37.5 and 50 mg captopril doses respectively. There were only 9 patients (2.6%) who received captopril treatment terminated from the study due to the presence of adverse drug reaction compared with 4 patients (4.8%) receiving placebo (Group, 1983). Thus,

captopril treatment is effective in controlling blood pressure and is well tolerated by the mild to moderate hypertension population.

ACE has two different catalytic domains: one of them cleaves Ang I into Ang II while the other catalyses the degradation of the vasodilator, bradykinin, into an inactive metabolite (Taddei and Bortolotto, 2016). Thus, ACEi can also preserve the plasma bradykinin amount. Several studies identified that ACEi might exert antihypertensive effects by inhibiting the degradation of bradykinin instead of preventing the production of Ang II (Ceconi et al., 2007). A clinical trial identified that 25 mg captopril reduced the MBP of normotensive (n=20) and hypertensive (n=7) subjects which was partially abolished by the addition of icatibant, a bradykinin receptor antagonist. However, the effect of captopril in reducing renal vascular resistance was not affected by icatibant (Straka et al., 2017). Another clinical trial that infused perindoprilat either alone or combined with icatibant in healthy male volunteers identified that perindoprilat lower the MBP of the participant and induced ACE inhibition together with the accumulation of active renin. The icatibant also abolished the blood pressure lowering effect of perindoprilat without altering the effect of perindoprilat on ACE inhibition and active renin concentration (Squire et al., 2000).

The advantages of ACEi were compromised due to the “ACE escape” event in which the plasma Ang II returns to basal levels after chronic ACEi treatment (Lorenz, 2010). ACEi inhibits the production of Ang II and suppresses the negative feedback mechanism which in turn stimulates production of renin and Ang I. Ang I accumulation induces the non-ACE

pathway such as chymase to produce Ang II that might worsen the disease state (Izzo and Weir, 2011). The ACEi treatment also induces some undesirable side effects including cough, hyperkalaemia and angioedema leading to discontinuity of the medicine (Yilmaz, 2019).

2.5.1.2 Angiotensin Receptor Blockers

ARB binds to AT1R and blocks the activation of AT1R by Ang II which results in a reduction of blood pressure. Currently, there are 8 types of ARB but only four of them are used by the government hospital and clinics in Malaysia which are irbesartan, telmisartan, losartan and valsartan (Minister of Health Malaysia, 2010). ARB is often prescribed as an alternative for ACEi when patients cannot tolerate the side effects of ACEi including cough and angioneurotic oedema. ARB does not induce cough as it does not cause an increase in bradykinin levels (Dezsi, 2016).

A clinical trial (n=440) has been conducted to identify the efficacy of losartan in treating mild to moderate hypertensive African Americans with DBP between 95-109 mmHg using titrated dose strategy (Flack et al., 2001). The patients were given 50 mg of losartan for the first 4 weeks while the dose was increased to 100 mg for the second 4 weeks, and 150 mg for the third 4 weeks for those who still had high DBP (> 90 mmHg) after every 4 weeks. The SBP/DBP of the participants was reduced by 4.6/1.8, 4.3/2.2 and 4.1/2.7 mmHg after 4, 8 and 12 weeks of losartan treatment. The most common adverse events were headaches and dizziness but these adverse events may not be drug-related

as a similar frequency of adverse events was observed in the placebo group (Flack et al., 2001).

A recent multinational cohort study identified ARB to be as effective as ACEi in reducing the risk for acute myocardial infarction, cardiovascular events, heart failure and stroke. It was also recognized for having a better safety profile due to its ability to lower the risk for developing adverse events including angioedema, cough, pancreatitis and gastrointestinal bleeding (Chen et al., 2021).

2.5.1.3 Renin Inhibitors

Renin inhibitors inhibit renin activity leading to sequential reduction in AGT, Ang I and Ang II and ultimately blood pressure reduction. It has been suggested that renin inhibitors might be effective in suppressing the entire RAS cascade as renin is the step-limiting enzyme in the production of Ang II (Muller and Luft, 2006).

Aliskiren is the first renin inhibitor that has been used in clinical settings. The blood pressure lowering effect of aliskiren has been well documented in clinical trials. Japanese hypertensive patients (n = 455) who received 75, 150 and 300 mg aliskiren had lower SBP/DBP by 5.7/4.0, 5.9/4.5 and 11.2/7.5 mmHg respectively (Kushiro et al., 2006). Meanwhile, a clinical trial had demonstrated that aliskiren exhibited its blood pressure effect in a dose-dependent manner in Caucasians. The team demonstrated that 150, 300 and 600

mg aliskiren reduced SBP/DBP by 9.2/5.4, 10.9/6.2 and 12.0/7.6 mmHg respectively (Oh et al., 2007). In contrast, another Caucasian predominant hypertensive population (n=652) identified that increasing aliskiren dose to 600 mg did not provide further antihypertensive effect (Gradman et al., 2005).

Aliskiren was found to be less effective in reducing blood pressure in the Black population compared with the Caucasian population (Oh et al., 2007). The 150, 300 and 600 mg aliskiren only reduced the SBP/DBP of the Black patients by 8.5/4.8, 11.2/7.5 and 9.6/8.4 mmHg (placebo: 1.5/2.8 mmHg) despite the greater reduction observed in the Caucasian patients (13.1/11.6, 13.9/11.2 and 14.7/12.5 mmHg; placebo: 1.6/5.5 mmHg) (Oh et al., 2007). This might be due to the Black population often having a lower plasma renin activity (PRA) which causes the renin inhibitor to be less effective (Sagnella, 2001).

Aliskiren is considered safe and exhibits placebo-like tolerability due to its similar adverse events rate in treatment groups ranging from 75 – 600 mg (33-55%) compared with placebo (27-50%) (Gradman et al., 2005, Kushiro et al., 2006, Oh et al., 2007). Headache, nasopharyngitis and dizziness are the most common adverse events found in these clinical trials and are not related to aliskiren treatment due to the presence of similar frequency across the placebo and treatment groups (Gradman et al., 2005, Kushiro et al., 2006, Oh et al., 2007). Nevertheless, it has been demonstrated that withdrawal of aliskiren causes a slight rebound of the PRA (Oh et al., 2007).

2.5.2 PRR Antagonists, ACE2 Activators, AT2R Agonists and MasR Agonists as Novel Antihypertensive Agents

Despite extensive understanding of its pathophysiology and the availability of effective treatment strategies, less than 13% of hypertensive patients achieved satisfactory blood pressure control (<140/90 mm Hg) while on treatment. In Malaysia, only 37.4% of patients receiving antihypertensive treatments achieve desired blood pressure control (<140/90 mmHg) (Minister of Health Malaysia, 2018). A common approach to manage uncontrolled hypertension is through uses of more than one classes of antihypertensive drugs, which frequently results in polypharmacy, drug-drug interaction and poor patient compliance, as well as medical burden.

It is certainly that continuous searching and discovery for new antihypertensive agent is favourable for a more 'tailored" drug treatment for personalised medicine. The sympathetic nervous system, central nervous system, RAS, aldosterone receptors and endothelin receptors are the new molecular targets to treat raised blood pressure. The following review focuses on the discovery of (P)RR antagonist, AT2R agonist, ACE2 agonist and MasR agonists as the new targets for antihypertension treatment.

2.5.2.1 (P)RR Antagonist

(P)RR is a receptor for prorenin and renin while the (P)PR-ligand complex catalyses Ang I production (Nguyen et al., 2002). Prorenin is a precursor of renin, and its plasma concentration is about 10 times greater than

renin. It has been demonstrated that prorenin has a higher (P)RR binding affinity than renin due to the presence of an effective (P)RR binding site called as prosegment. Inhibition or blocking of this pro-segment present in prorenin is believed to be an effective strategy in halting Ang I production (Wang et al., 2020).

PRO20 is synthesized based on the first 20 amino acids of the prosegment of the prorenin and was demonstrated to have antihypertensive effects in various hypertensive animal models including the prorenin and deoxycorticosterone acetate (DOCA) salt-induced hypertensive rodents (Li et al., 2015, Wang et al., 2020).

Intrarenal and intravenous PRO20 infusion were found to reduce the MBP of DOCA salt-induced hypertensive rats through reduction of the water intake and urine volume. However, these effects were more prominent in those received intrarenal PRO20 and the research team concludes that intrarenal RAS plays a crucial role in regulating blood pressure (Wang et al., 2020).

It is worth mentioning that all the current (P)RR antagonists are peptide compounds which make them not suitable for oral administration. Other disadvantages associated with peptide compounds as therapeutic drugs include limited membrane permeability and *in vivo* instability due to the presence of protease (Xu et al., 2016, Lee et al., 2019).

2.5.2.2 AT2R Agonists

Activation of AT2R provides cardioprotective effects through eliciting vasodilation and natriuresis that counteract the actions of AT1R. Thus, the administration of AT2R agonists that stimulates the activation of AT2R is believed to have therapeutic potential in treating hypertension.

Compound 21 (C21) is a selective AT2R agonist. Danyel et al. (2013) concluded that C21 exerts a consistent but weak vasorelaxation effect in the isolated aorta, coronary, mesenteric and iliac arteries in both normotensive and hypertensive rodent models. Another study showed that intravenous administration of C21 increased the renal blood flow and sodium excretion but decreased the renal vascular resistance in rats (Hilliard et al., 2012, Kemp et al., 2014).

Interestingly, it has been demonstrated that C21 treatment induced AT2R translocation to the apical membrane and the internalization of NHE3 and NKA without affecting their protein expressions (Kemp et al., 2014). This research further concluded that C21 exerts its biological action via AT2R as addition of AT2R antagonist reversed all these effects and the changes in the urinary sodium excretion was not observed in AT2R null mice (Kemp et al., 2014).

Despite the action of C21 in inducing vasorelaxation and natriuresis, there were no changes in the MBP in the above mentioned studies (Hilliard et

al., 2012, Kemp et al., 2014). Kemp et al. (2014) further demonstrated that the route of administration of C21 affects its antihypertensive property. Intravenous C21 required co-administration of ARB to lower the blood pressure while intrarenal and intracerebroventricular C21 infusion exerted a direct blood pressure lowering effect in rats (Kemp et al., 2016, Dai et al., 2016, Carey, 2017). In addition, Carey (2017) hypothesized that the blood pressure lowering effect of AT2R depends on (i) activation state of RAS (ii) the abundance of functional AT1R and/or the presence of AT1R blockage.

2.5.2.3 ACE2 Activators

ACE2 catalyses conversion of Ang II to Ang (1-7). Uses of ACE2 activator are found to be effective in increasing the catalytic efficiency of ACE2 as well as ACE2 expression.

IRW (Ile-Arg-Trp) is the first naturally derived ACE2 activator from egg ovotransferrin. *In vitro* studies showed that co-incubation of IRW with ACE2 increased ACE2 catalytic efficiency in a dose-dependent manner (Liao et al., 2018). Molecular docking identified that IRW binds to sub-domain I of ACE2 and further activates ACE2 by preventing the shrinkage of the active site and therefore allowed for subsequent substrates bindings (Liao et al., 2018). On the other hand, the same research team revealed that vascular smooth muscle cells treated with IRW showed higher ACE2 gene and protein abundance (Liao et al., 2018).

Oral administration of IRW reduced blood pressures in SHR and it was associated nitric oxide (NO)-mediated vasorelaxation, lower plasma Ang II and higher bradykinin levels (Liao et al., 2019). It has been shown that addition of MasR antagonist reversed the antihypertensive and vasorelaxation effect of IRW. This phenomenon could indicate the involvement IRW in ACE2/Ang (1-7)/MasR axis (Liao et al., 2019). The same research team further revealed that IRW treatment upregulates protein expression of ACE2 and MasR but not ACE, AT1R and AT2R in the aorta (Liao et al., 2019).

2.5.2.4 MasR Agonists

Ang (1-7) is the substrate for MasR. Thus, exogenous administration of MasR agonist which include Ang (1-7) is a proposed strategy in activating the protecting arm of the RAS with the goal in alleviating raised blood pressure.

Ang (1-7) was found to be involved in the sodium uptake in cultured renal proximal tubule cells (Su et al., 2017). Subcutaneous Ang (1-7) reduced SBP and improved renal function in Akita mice, an animal model for spontaneous type 1 diabetes (Shi et al., 2015). Ang (1-7) also suppressed intrarenal RAS activation by downregulating protein expression of renal AGT and ACE and decreased urinary AGT excretion; but upregulating ACE2 and MasR protein expression. The addition of MasR antagonist eliminated all of these effects, suggesting that the action of Ang (1-7) was mediated through MasR (Shi et al., 2015).

Ang (1-7) has a short half-life as it is susceptible to ACE conversion to angiotensin (1-5) (Jiang et al., 2014). Thus, metabolically stable Ang (1-7), such as encapsulation with hydroxypropyl-β-cyclodextrin or generation of peptidase resistance Ang (1-7) analog has been developed to overcome this problem (Povlsen et al., 2020). The cyclic Ang (1-7) [cAng (1-7)] is an analog for Ang (1-7) which is a thioether bridge that has been induced to link the amino acid residues 4 and 7 to form a lanthionine (Kluskens et al., 2009). The thioether bridging causes the resistance of cAng (1-7) towards the degradation action of ACE without affecting its interaction with the MasR. The proteolytic resistance properties of cAng (1-7) resulted in a 34 folds reduction in plasma clearance compared with Ang (1-7) (Kluskens et al., 2009). An *ex vivo* study has demonstrated that the maximum degree of vasorelaxation that induced by cAng (1-7) in aorta rings is doubled compared to Ang (1-7) (Kluskens et al., 2009). Despite the potential role of cAng (1-7) in lowering blood pressure, no study has been conducted to examine its possible antihypertensive effects using hypertensive animal models. Nevertheless, the effectiveness of cAng (1-7) in treating myocardial infarction and cerebral stroke has been demonstrated in several studies (Durik et al., 2012, Povlsen et al., 2020).

2.6 Epigallocatechin Gallate and Its Potential Uses as a Therapeutic Agent

EGCG is the most abundance catechin present in the green tea plant, *Camellia sinensis* and belongs to the flavonoid family. Green tea extract (GTE) and purified EGCG have been examined thoroughly for their beneficial effects in the prevention and treatment of different diseases namely cancer,

cardiovascular, metabolic and neurodegenerative disorders (Dou et al., 2008, Liu et al., 2014, Wang et al., 2014b).

The therapeutic range and safety profile of EGCG have not been thoroughly assessed, either in preclinical or clinical phases. Nevertheless, GTE and concentrated EGCG have been marketed as herbal supplements and become famous among consumers due to its potential health beneficial effects during recent years (Won et al., 2012, Albassam and Markowitz, 2017). It is alarming when people consider EGCG as a natural product and are not aware of the potential toxicity effects.

Preclinical studies have demonstrated that EGCG exhibits its blood pressure lowering effects via its potent antioxidative activity, vasodilation effects, suppression on systemic RAS and sympathetic system (Potenza et al., 2007, Deka and Vita, 2011, Lorenz et al., 2015, Qian et al., 2018).

Some preclinical studies have suggested that EGCG may possess pro-oxidative activity when administered in high doses while green tea-associated liver injury has been reported in several case reports (Maeta et al., 2007, Lambert et al., 2010, Wang et al., 2015). The following review focuses on (i) EGCG structural property and its biological actions, (ii) blood pressure lowering effects of EGCG in epidemiological data, clinical trials and preclinical studies, (iii) potential uses of EGCG as an agonist/antagonist in RAS axis and (iv) possible toxic effect of EGCG.

2.6.1 Structural Property and Biological Activities of EGCG

EGCG ($C_{22}H_{18}O_{11}$) with a molecular weight of 458.4 g/mol (PubChem CID: 65064) is the most abundant catechin found in green tea (Figure 2.5). EGCG comprises of 4 rings which known as ring A, B, C and D. Antioxidant activity of EGCG results from the transfer of hydrogen atom in hydroxyl groups (-OH) in its aromatic rings. It has been reported that ring A neutralizes malondialdehyde through C6 or C8 (Zhu et al., 2009). Ring B induces apoptosis via autooxidation activities (Ishii et al., 2011). Ring D is believed to be a fatty-acid synthase inhibitor.

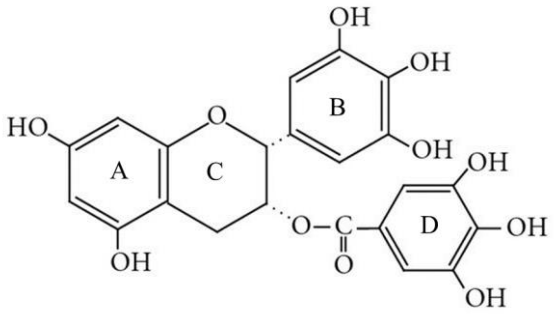

Figure 2.5: Chemical structure of EGCG.

2.6.2 Effects of Green tea and EGCG on Blood Pressure Changes in Humans

Consumption of tea especially green tea has been associated with improved cardiovascular risk factors in several large epidemiological studies. A meta-analysis done by Peng et al. (2014) that included 13 randomized controlled trials had identified green tea consumption can reduce the blood pressure of prehypertensive and hypertensive individuals. Meanwhile, another meta-analysis of randomised controlled trials involving 25 studies with 1476 subjects

reported that long term green tea intake significantly lowered SBP and DBP by 2.1 and 1.7 mmHg respectively (Liu et al., 2014). Another meta-analysis conducted by the Griffith Health Institute, Australia, concluded that green tea and its catechins may ameliorate raised blood pressure and the effect may be greater in those with SBP of more than 130 mmHg. This meta-analysis suggested that the blood pressure lowering effect of green tea might be associated to its total and low-density lipoprotein lowering effect (Khalesi et al., 2014).

In contrast, Hodgson observed an increase in blood pressure 30 minutes after ingestion of green or black tea in normotensive men which disappeared an hour later (Hodgson et al., 1999). A few years later, Hodgson further reported that the acute hypertensive effect of tea consumption was blunted when it was consumed with a meal (Hodgson et al., 2005). Yet, several studies have shown no relation between tea consumption and blood pressure changes (Klatsky et al., 1993, Deka and Vita, 2011).

It is believed that blood pressure lowering effect of green tea is due to its active ingredient, EGCG that possesses several crucial biological activities related to blood pressure regulation. Therefore, EGCG has been frequently recommended as a therapeutic agent in treating hypertension.

2.6.3 Antihypertensive Effects of EGCG in Preclinical Studies

Several *in vivo* studies have demonstrated that EGCG exhibits its antihypertensive effects in various hypertensive animal models including SHR, Dahl salt-sensitive rats and abdominal aortic coarctation-induced heart failure rats (Potenza et al., 2007, Zhang et al., 2018, Luo et al., 2020). The treatment period spans from 14 days (subacute) to 42 days (subchronic) and doses range from 10 mg/kg to 200 mg/kg *b.w.*.

It is believed that EGCG exerts its antihypertensive effects in time- and dose-dependent manners. Administration of 10 mg/kg EGCG for 14 days reduced the SBP of SHR by 2, 6, 11, 14, and 15 mmHg on day 3, 6, 9, 12, and 14 respectively (Tan et al., 2021). This time-dependent antihypertensive effect of EGCG was also shown by Potenza et al. (2007) with a larger SBP lowering effect (22 mmHg). This might be due to the longer treatment duration (21 days) and higher EGCG dose (200 mg/kg). Meanwhile, heart failure rats treated with EGCG ranging from 25 to 100 mg/kg for 28 days showed a dose-dependent antihypertensive effect from 10 mmHg to 22 mmHg on MBP reduction (Zhang et al., 2018).

Interestingly, it has been demonstrated that both EGCG and its metabolites are contributing to the blood pressure lowering effect. A single dose of 300 mg/kg EGCG demonstrated an acute and transient blood pressure lowering effect in SHR (Qian et al., 2018). The SBP dropped significantly at 15 minutes and 60 minutes post-EGCG administration in SHR (Qian et al., 2018).

The team further hypothesized that the first blood pressure reduction was the effect of EGCG while the second blood pressure reduction was the effect of its metabolite.

It has been showed that EGCG is metabolised into gallic acids and epigallocatechin (EGC) by the intestinal flora while EGC can be further degraded into valerolactones which is the dominant metabolite and a small amount of valeric acids (Takagaki and Nanjo, 2015). All these intestinal EGCG metabolites showed ACE inhibitory effects (IC_{50}: 1.51 to 19.59 mM). However, the inhibitory activities were weaker than the parental EGCG compound (IC_{50}: 0.38 mM) (Takagaki and Nanjo, 2015). Administration of 450 mg/kg EGC showed significant blood pressure lowering effect (-13 mmHg) in SHR, but the effect is weaker compared to EGCG (-34 mmHg) (Takagaki and Nanjo, 2015, Qian et al., 2018). Meanwhile, 150 mg/kg 5-(3,4,5-trihydroxyphenyl)-γ-valerolactone and 200 mg/kg 5-(3,5-dihydroxyphenyl)-γ-valerolactone which are the major intestinal EGCG metabolites also reduced the SBP in SHR (Takagaki and Nanjo, 2015).

It has been proposed that antihypertensive effects of EGCG is associated with its potent antioxidative property, suppression of the over activation of RAS, and improve endothelial functions (Potenza et al., 2007, Qian et al., 2018, Luo et al., 2020, Mohd Sabri et al., 2022). The following review focus on the effects of EGCG on renin-angiotensin system. It is worth mentioning that the relationship of EGCG, RAS and blood pressure has not been established in SHR.

2.6.4 Effects of EGCG on the Renin-Angiotensin System

The antihypertensive effects of EGCG has been associated with its inhibitory activity on the classical RAS components. The renin inhibitory assay identified that EGCG is the most potent renin-inhibiting catechin with the lowest IC_{50} followed by epicatechin gallate (ECG) and EGC. The same team further demonstrated that EGCG binds to non-active side of renin and leads to changes in conformation of its active side and leads to renin malfunction. The galloyl moiety was suggested to be responsible for the inhibitory effect since gallated catechins (EGCG and ECG) are more effective in inhibiting renin than non-gallated catechin (EGC) (Li et al., 2013).

On the other hand, molecular docking studies shows that EGCG binds to ACE at position Gln 281, Lys 511 and Tyr 520 through the formation of 3 hydrogen bonds via the steric interaction while *in vitro* study shows that that EGCG results in 82% reduction in ACE activity (Ke et al., 2017). The gallate group might contribute to the inhibitory effect of ACE due to gallated catechins [EGCG, ECG and catechin gallate (CG)] generally having higher ACE inhibitory activity than non-gallated catechins [EGC, epicatechin (EC) and catechin (C)] (Takagaki and Nanjo, 2015).

Other than direct inhibition of renin and ACE activity, it has been showed that EGCG inhibits the classical RAS through the regulation of gene and protein expressions. Aorta from male SHR treated with a single dose of 150 and 300 mg/kg EGCG show an increase in miRNA-150-5p expression through

an increase of the host gene, ribosomal protein S11, which in turn downregulates its target gene, specificity protein 1 (SP1) expression (Qian et al., 2018). Meanwhile, SP1 is a transcription factor that regulates the AT1R expression. Reduction in SP1 expression leads to a decrease in AT1R and results in blood pressure reduction in SHR (Qian et al., 2018). *In vitro* study has identified that EGCG attenuates the increase in serum Ang II levels, hepatic ACE and AT1R mRNA expression in the high-fat diet and carbon tetrachloride-treated spontaneously hypertensive stroke-prone rats Z-Leprfa/IzmDmcr (Kochi et al., 2014).

To the best of our knowledge, the effect of EGCG on the counter-regulatory RAS in SHR has not been explored.

2.7 Safety Concern in Using EGCG

Despite of extensive clinical trials in examining therapeutic effects of EGCG in various diseases, still, there is lack of systemic investigation on the safety use of EGCG in different pathological conditions. The existing findings in *in vitro*, *in vivo* and clinical studies/epidemiological observation are inconsistent and sometimes conflicting (Mazzanti et al., 2009, Mereles and Hunstein, 2011, Younes et al., 2018).

A phase IV clinical trial (n = 27) identified that a single 135 and 270 mg EGCG dose given in the form of oral supplement is well tolerated with absence of severe side effects in health participants (Wightman et al., 2012). Moreover,

EGCG dosage up to 800 mg daily for 4 weeks is well tolerated in healthy individuals (Chow et al., 2003). Nevertheless, consumption of EGCG is found to be associated with several side effects including hepatic gluconeogenesis suppression, spontaneous excitatory synaptic transmission inhibition, hypochromic anaemia induction mediated by iron absorption inhibition in addition to gastrointestinal bleeding, liver and kidney damage (Vignes et al., 2006, Collins et al., 2007, Ryan and Hynes, 2007, Mazzanti et al., 2009, Younes et al., 2018). Drug or herbal-induced liver injury may lead to acute liver failure, resulting in either liver transplant or death. Furthermore, liver toxicity is one of the most frequent reasons for pharmacovigilance safety reports and the withdrawal of an approved medicinal product or dietary supplement from the market. As EGCG-induced liver and kidneys damage are the specific research interest of this present study, the following literature review focuses on the potential impact of EGCG on liver and kidney injury.

2.7.1 EGCG-Induced Liver Damage in Epidemiological Data

Case studies had identified that consumption of high doses of green tea-containing dietary supplements have been associated with hepatotoxicity. The increased serum bilirubin and ALT levels is presented in most of the cases while inflammation of the periportal and portal regions were also observed in liver biopsies (Mazzanti et al., 2009, Younes et al., 2018). In 2003, the regulatory agencies in France and Spain suspended market authorization of a weight-loss product containing green tea extract due to multiple incidences of hepatotoxicity cases (Sarma et al., 2008). Mazzanti et al. (2009) conducted a literature search

of publication between 1999 and October 2008 and had successfully retrieved 34 cases of hepatitis related to green-tea consumption. They concluded that there is a causal association between green tea and liver damage; and the hepatotoxicity is probably due to EGCG.

The Norwegian Institute for Public Health systematically reviewed and re-assessed the safety levels of EGCG used in dietary supplements upon a several hepatitis cases that were associated with consumption of health supplement that contains high level of GTE (Norwegian Institute of Public Health, 2015). The panel concluded that bolus administration of more than 0.4 mg EGCG/kg body weight per day may cause adverse effects. In addition to bolus administration, the panel has concluded that GTE given at fasting state may result in higher susceptibility to toxic effect. The panel has concluded that these hepatitis cases may or may not cause by EGCG, as the commercial preparations containing EGCG differ widely, and the composition of the GTE, purity of EGCG, as well as presence of contaminants in the GTE are not known (Norwegian Institute of Public Health, 2015).

The European Food Safety Authority reviewed the safety use of green tea catechins and the panel has concluded that (i) catechins from the traditionally prepared green tea infusion are generally considered to be safe (ii) liver injury reported after consumption of green tea infusions are rare and most probably are due to an idiosyncratic reaction (iii) intake of doses equal or above 800 mg EGCG/day as a food supplement has been shown to increase serum

transaminases in EGCG treated subjects compared to control (Younes et al., 2018).

A systematic review on 159 human intervention studies revealed that the GTE-associated adverse effects is in a dose-dependent manner, and ingestion in large bolus doses leads to increased susceptibility to adverse effects. This systematic review concluded that it is safe to consume up to 338 mg EGCG/day for tea preparation ingested as a solid bolus dose; while it is safe to consume up to 704mg EGCG/day for tea preparation in beverage form (Hu et al., 2018).

Nevertheless, these reports did not elucidate the mechanism underlying the hepatoxicity effect of EGCG and there is still lack of scientific evidence in addressing the association of EGCG ingestion with liver damage in human subjects.

2.7.2 EGCG-Induced Liver Damage in Clinical Trials

A systematic review that included 34 randomized controlled trials identified that the hepatotoxicity effect of EGCG is rare with an odds ratio of 2.1% (Isomura et al., 2016). A systematic review on 159 human interventions conducted by Hu et al. (2018) reported that the (i) majority of studies (104/159) demonstrated that green tea or GTE is generally well tolerated without adverse effects, (ii) 38 studies reported the occurrence of adverse effects that is

associated with consumption of green tea beverage (5/38), GTE (28/38) and EGCG (5/38). Elevated liver enzymes were reported in 11 studies using solid dosage form and largely associated with uses of Polyphenon E® (a commercialised GTE capsules); while only one study was associated with EGCG ingestion (Hu et al., 2018). In conclusion, it seems EGCG-induced liver damage in human intervention studies is rare.

2.7.3 EGCG-Induced Liver and Kidney Damage in Preclinical Studies

Previous pre-clinical study has shown that chronic EGCG treatment (25 mg/kg *b.w.*/day) via drinking water for 108 weeks extends the lifespan of Wistar rats through reduction of aging-associated hypertension, liver and kidney injury. It also inhibits ageing-associated inflammation and oxidative stress through the inhibition of NF-kappaB signaling (Niu et al., 2013).

The Sprague-Dawley (SD) rats fed with EGCG (77% purity) up to 500 mg/kg for 13 weeks showed no treatment-related death nor signs of toxicity (Isbrucker et al., 2006). All the rats had no difference in body weight, food intake, organ weight and clinical biochemistry, with absence of lesions and gross pathological changes during treatment and recovery period.

In contrast, a high dose of oral 1500 mg/kg EGCG increased plasma ALT level by 138-fold and killed 85% of the mice (Lambert et al., 2010). Moreover, 2 – 7 days of repeated dose of 75 mg/kg *b.w.* EGCG resulted in moderate to severe necrosis of the liver of CF-1 mice (Lambert et al., 2010).

Wang et al. (2015) demonstrated that administration of 75 mg/kg EGCG for 5 day through intraperitoneally significantly evoke hepatoxicity in mice. Interestingly, both research teams concluded that the liver injury likely due to EGCG-induced oxidative stress damage.

Oral administration of EGCG at 108 mg/kg for 14 days increases serum ALT activity in female Swiss albino mice. Increased serum ALT and AST activities were more prominent in mice administrated with 67 and 108 mg/kg EGCG intraperitoneally (Ramachandran et al., 2016).

EGCG treatment at 100 mg/kg, *i.p.* for 4 days caused 60% mortality which is associated with EGCG-induced nephrotoxicity (Rasheed et al., 2017). EGCG treatment increased early kidney damage marker, serum neutrophil gelatinase-associated lipocalin and cystatin c levels but not blood urea nitrogen (BUN), a marker for late kidney damage. Histological examination identified the presence of vacuolated renal tubular epithelial with severe congestion in the blood vessel while Rasheed et al. (2017) concluded that diabetic mice were more susceptible to EGCG-induced nephrotoxicity compared with non-diabetic mice.

2.7.4 The Hormetic Properties of EGCG: Antioxidative or Pro-oxidative?

Compounds with hormetic property exert a beneficial effect at low dosages but deleterious effects at high dosages. EGCG exerts antioxidative and pro-oxidative effects at low and high dosages respectively and it is one of the

well-known hormetic phytochemicals that exerts this biphasic dose-response effect (Calabrese et al., 2020).

An *in vitro* study showed that 24-hour incubation with 10 µmol/L EGCG led to ROS formation and subsequently results in hepatocyte injury while lower doses of EGCG decreased ROS production (Kucera et al., 2015). A few studies have demonstrated that EGCG undergoes autoxidation and results in productions of hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and EGCG dimer (Figure 2.6) (Nakagawa et al., 2002, Nakagawa et al., 2004, Elbling et al., 2010).

In addition, EGCG generates hydroxyl radical through the Fenton reaction. *In vitro* study shows that EGCG reduces Fe(III) to Fe(II) state (Nakagawa et al., 2002). The generated Fe(II) reacts with H_2O_2, either present physiologically in the cell or generated from the auto-oxidation of EGCG, to produce hydroxyl radicals. The hydroxyl radicals can activate caspase-3 activity and induce DNA breakage, resulting in EGCG-induced cell death.

Fenton reaction: $H_2O_2 + Fe^{2+} \rightarrow OH^{\bullet} + OH^- + Fe^{3+}$

These findings are supported by studies treating osteoclast-like multinucleated cells and Jurkat cells with increasing doses of EGCG. EGCG treatment stimulats H_2O_2 production and caspase-3 activity leading to the reduction of cell viability in a dose-dependent manner while these effects are attenuated with the presence of catalase, Fe(II) and Fe(III) chelating agents

(Nakagawa et al., 2002, Nakagawa et al., 2004). The results show that the Fenton reaction is involved in the apoptotic effect of EGCG.

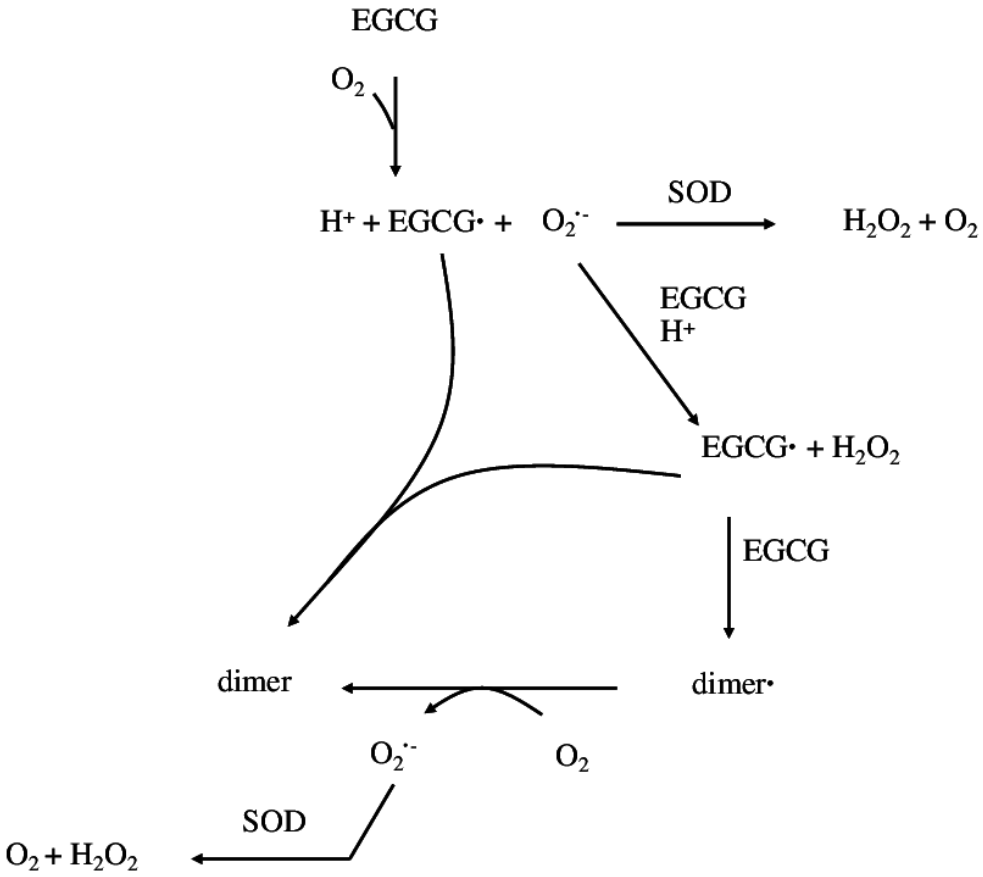

Figure 2.6: The auto-oxidation of EGCG. *EGCG reacts with oxygen and leads to production of EGCG radical and superoxide anion. Then, the generated superoxide anion reacts with another EGCG molecule to produce EGCG radicals and H_2O_2 with the addition of a hydrogen ion. Dimerization of EGCG occurs between two EGCG radicals or an EGCG radical with an EGCG molecule. Then, the dimer radical is neutralized through O_2 and produce superoxide anion as the by-product. The H_2O_2 is also produced from superoxide anion through the presence of SOD* [Adapted from Legeay et al. (2015)].

Numerous studies utilized the biphasic properties of EGCG to induce toxicity or act as protective agents against toxicant-induced toxicity. Elbling et al. (2010) demonstrated that the antioxidative effect of a low dose of EGCG occurs through auto-oxidation. HaCat cells treated with a high dose of EGCG

(50 – 100 uM) or H_2O_2 (60 – 100 uM) shows reduced survival rate which is attenuated in the pre-treated cells with a low dose of EGCG (10 – 20 uM) or H_2O_2 (10 uM). The team concluded that low amount of H_2O_2 generated through auto-oxidation from the low amount of EGCG can provide protection against subsequent oxidation damage.

Male CF-1 mice pre-treated with 500 mg/kg dietary-enriched EGCG for 14 days are protected from the hepatoxicity induced by the subsequent high dose of EGCG (750 mg/kg) that given in a bolus via oral gavage. The team concluded that administration of EGCG through diet allows low and continuous dosing throughout the day and it is effective in preventing the oxidative stress-related DNA breakage and reduction of antioxidant protein levels induced by EGCG. This study provides a novel insight in which acute EGCG toxicity might be less prominent in regular green tea drinkers (James et al., 2015).

CHAPTER 3

METHODOLOGY

3.1 Drug and Chemicals

EGCG (purity>94%) was purchased from Taiyo GmbH (Gevelsberg, Germany). The 2,7-Dichlorofluoroscin Diacetate (DCFDA) dye was purchased from Canvax (Córdoba, Spain). Bradford Reagent was purchased from HiMedia (Maharashtra, India). All the chemicals, consumables, equipment, and software used in this study are listed in the Appendix A.

3.2 Ethical Approval

The experiments involving animal subjects were conducted according to the Universiti Tunku Abdul Rahman (UTAR) Code of Practice for the Care and Use of Animal for Scientific Purposes. The experiment protocols were reviewed and approved by the UTAR Scientific and Ethical Review Board (U/SERC/36/2020 and U/SERC/44/2021).

3.3 Experimental Animals

Male 8-weeks-old SHR were obtained from the University of Malaya, Malaysia, and acclimatized for at least 2 weeks in the animal housing facility, UTAR, Sungai Long Campus prior to commencement of the experiment. The

animals were housed in a facility with 12 hours light-dark cycle at 22℃ ± 2℃ with *ad libitum* access to food pellets and water. The SBP of SHR was screened using an indirect tail-cuff volume pressure recording (VPR) method (CODA, Kent Scientific) and SHR with SBP > 160 mmHg was included in this study.

3.4 Study Design

This study was designed as a two-phase experiment namely Phase I and Phase II. Phase I determined the NOAEL of EGCG treatment while the Phase II investigated the roles of EGCG as a renin-angiotensin-system modulator in ameliorating raised blood pressure in SHR. Figure 3.1 shows the experimental protocol.

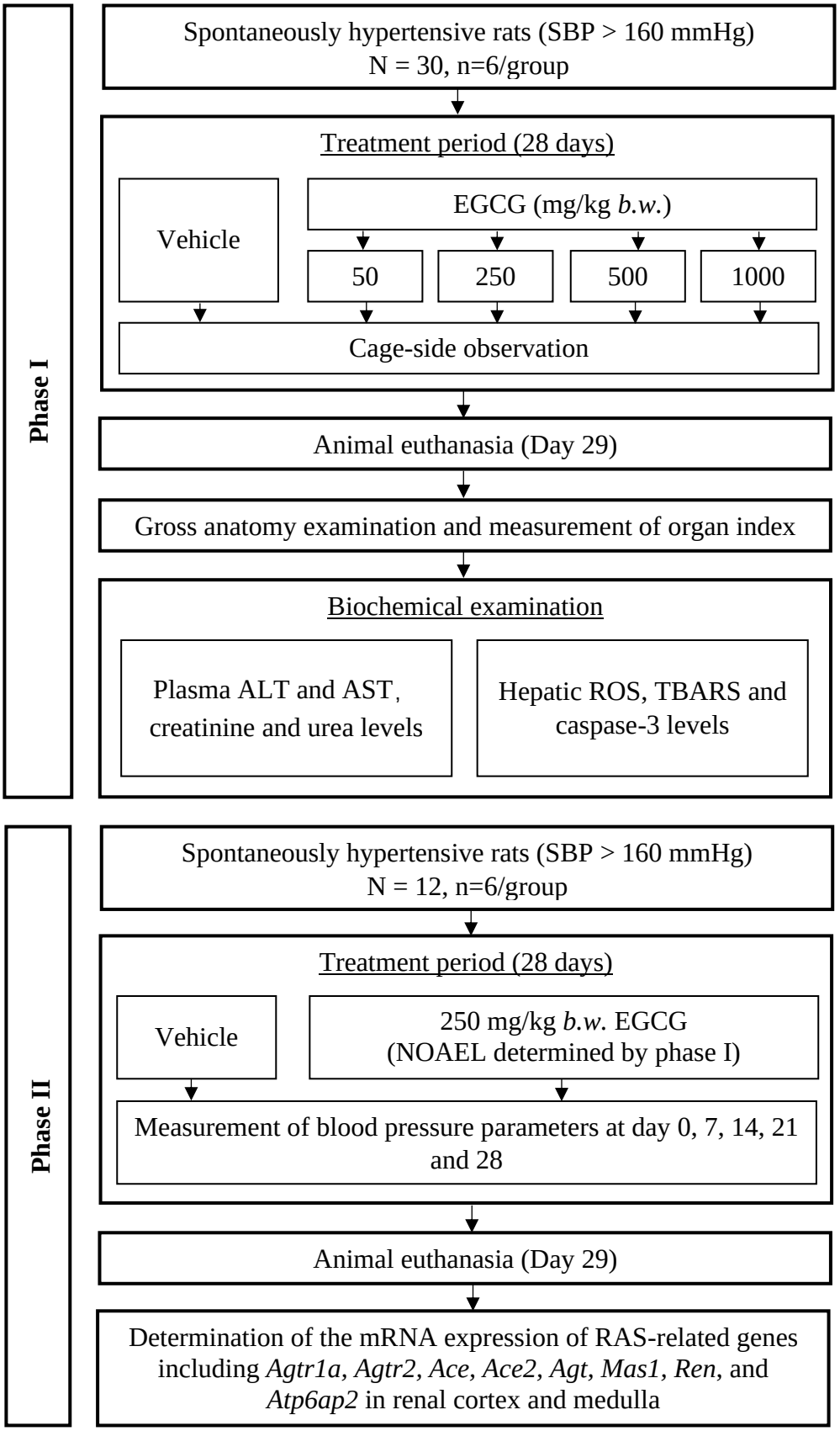

Figure 3.1: Schematic diagram of the study design for the experiment.

3.4.1 Phase I: Overview of the Study Design

Male SHR aged 12 weeks (N=30, n=6 per subgroup) was randomized into 5 groups and were given phosphate-buffered saline (PBS), 50, 250, 500 or 1000 mg/kg, *b.w. i.g.* of EGCG for 28 days between 7 a.m. to 8 a.m.. Body weight, food and water intake were recorded daily. The toxicity assessment was performed throughout the experimental according to the Organisation for Economic Co-operation and Development (OECD) guidelines 423. On day-29, rats were sacrificed for necropsy examination and tissue/blood collections (Figure 3.2).

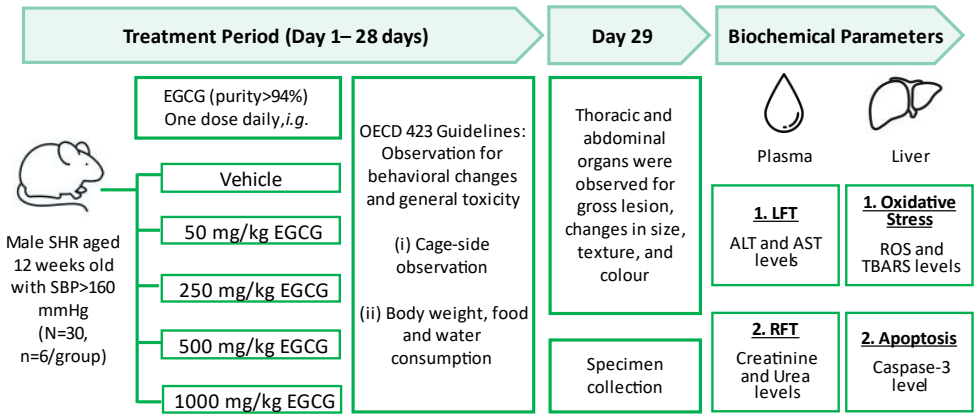

Figure 3.2: Experiment flowchart of the phase I study.

3.4.2 Preparation of PBS Buffer

The 1X PBS buffer was prepared according to the recipe (Table 3.1) and autoclaved before use. All salts were added to 500 mL of distilled water and made up to 1 L as the final volume.

Table 3.1: Recipe for 1 L of 1X PBS buffer (pH 7.4).

Components	**Molecular weight (g/mol)**	**Amount (g)**	**Final molarity (mM)**
Sodium Chloride, NaCl	58.44	8.00	137.00
Potassium Chloride, KCl	74.55	0.20	2.70
Di-Sodium Hydrogen Phosphate, Na_2HPO_4	141.96	1.78	10.00
Potassium Dihydrogen Phosphate, KH_2PO_4	136.09	0.24	1.80

3.4.3 Toxicity Assessment during Treatment

The body weight, food and water intake of SHR were measured daily. Daily food and water intake were calculated through normalization of the body weight. The body weight, food and water consumption were expressed as the 7-day average values.

After each vehicle/EGCG dosing, cage observation was conducted to monitor for any abnormal behaviors which including changes in gait, posture, response to handling, grooming pattern and locomotive pattern. Possible general and systemic toxicity signs were also observed and recorded accordingly (Table 3.2).

Table 3.2: Detail for the category of the observation for behaviour changes and clinical signs.

Observations	Category	Explanation
General condition	Normal	Awake, active, reacts to stimulation
	Mild	Burrows in litter, hides, lies still but is startled when handled
	Severe	Immobile, little or no voluntary movement. Burrows/hides. Presses head against cage bottom. Vocalizes. Extremely afraid and/or aggressive when handled
Porphyrin staining and/ or eye inflammation	Absent	No discolouration, clean and clear eyes
	Mild	Some porphyrin and/or discharge around the eyes and nose
	Severe	Obvious porphyrin on 'face' and/or on legs and paws. Eye(s) closed, squints and/or discharge around eye(s)
Movements and posture	Normal	Normal coordination without any difficulty in movements
	Mild	Moderate in-coordination when the animal is stimulated; hunched posture
	Severe	Marked in-coordination, head held at angle, hunched posture and/or back, does not support itself on all four limbs and/or paralysis
Piloerection	Absent	Fur smooth and well-groomed
	Mild	Moderate piloerection
	Severe	Severe piloerection, sticky and poorly groomed fur
Skin	Normal	Skin covered entirely with fur. No sores or other signs of injury
	Mild	Small sores or scabs, no infection; scratching (signs of itching)
	Severe	Bites or scratches itself or trauma from others. Signs of infection such as redness and/or pus or serious discharge; sticky and poorly groomed fur. Non-healing operation wounds or broken sutures

Table 3.2 (cont): Detail for the category of the observation for behaviour changes and clinical signs.

Appetite/ Food and water intake	Normal	Normal appetite, eating and drinking regularly
	Mild	Reduced appetite, consume less food and water
	Severe	No interest in food and appears dehydrated
Defecation	Normal	Firm faecal boli with brown colour
	Mild	Faeces looser or harder than normal and/or abnormal colour
	Severe	Diarrhoea (excessive watery stool)/ Constipation (no stool or very hard stool) and/or abnormal colour
Urination	Normal	Normal urine colour (pale yellow to yellowish) without any odour
	Abnormal	Abnormal urine colour and/or a strong odour
Breathing Difficulties	Absent	Normal respiration, not strained or wheezy
	Present	Breathes with open mouth, abdominal breathing or panting, crackle and/or gasping noises

3.4.4 Animal Euthanasia, Necropsy and Specimens Collections

The rats were fasted overnight after the last dose of EGCG on day-28. On day-29, the rats were euthanized via excess inhalation of carbon dioxide in a closed chamber followed by cervical dislocation. The death of animals was confirmed through the absence of corneal reflex, heartbeat and response to toe pinch.

Upon dissection, the thoracic (heart and lungs) and abdominal organs (liver, stomach, spleen, small intestine, large intestine and kidneys) were observed macroscopically for any gross pathological changes in terms of size,

colour, texture and abnormality (redness, pus, ulceration, swollen, spots and necrosis). The organs were then removed, washed three times using PBS and dried with tissue paper before weighing. Organ index was calculated using the following formula: *organ index* (g/ 100 g *b.w.*) = *organ weight*/*body weight* x 100. Liver tissues were cryopreserved using liquid nitrogen and stored at –80℃.

The whole blood sample was obtained through the inferior vena cava and was quickly collected in a lithium heparinized tube. The whole blood sample was stored at 4℃ before centrifugation for plasma collection. The liver was harvested, washed and snap-frozen in liquid nitrogen, and kept at –80℃

3.4.5 Preparation of Plasma Sample

Plasma was obtained through centrifugation of whole blood sample at 1,000 x g, 4℃ for 10 minutes. Plasma was collected, aliquoted and stored at –80℃ for determination of ALT, AST, creatinine and urea levels.

3.4.6 Liver Function Test

Liver is the major organ responsible for the metabolism in our body. Thus, numerous enzymes are produced by the hepatocytes to carry out the metabolism function. Liver injury disrupts cell membrane, leading to leakage of enzymes into circulation. Therefore, the presence of liver injury can be identified through measurement of the amount of hepatic enzymes in blood circulation (Lala et al., 2022). ALT and AST are two of the hepatic enzymes

which had been widely used for the determination of liver injury. ALT is more specific than AST since ALT is highly expressed by the liver only while high amount of AST also can be found in the heart and muscle other than liver.

3.4.7 Determination of Plasma ALT Level

Plasma ALT level was determined using the alanine transaminase colorimetric activity assay kit (Cayman, USA) according to the manufacturer's instructions.

3.4.7.1 Assay Procedure

A volume of 150 µL of ALT substrate solution which contains L-alanine was added to a 96-well plate followed by 20 µL of ALT cofactor solution containing nicotinamide adenine dinucleotide (NADH) and lactate dehydrogenase (LDH) and 20 µL of the sample. The plate was covered and incubated at 37℃ for 15 minutes before the addition of 20 µL of ALT initiator solution consisting of α-ketoglutarate. The absorbance at 340 nm was measured upon adding of ALT initiator once every minute for 10 minutes at 37℃.

3.4.7.2 Calculation

A graph of absorbance value versus time was plotted to obtain the slope of the linear portion of the curve. ALT level was calculated using the following formula:

$$ALT\ activity\ (U/L) = \left(\frac{\Delta A340/\min x\ 0.21mL}{4.11\ mM^{-1}\ x\ 0.02\ mL}\right) * 1000$$

3.4.8 Determination of Plasma AST Level

The plasma AST level was determined using aspartate transaminase colorimetric activity assay kit (Cayman, USA) according to the manufacturer's instructions.

3.4.8.1 Assay Procedure

A volume of 150 µL of AST substrate solution which contains L-aspartate, 20 µL of AST cofactor solution containing NADH, LDH and malate dehydrogenase, and 20 µL of the sample were added accordingly into a 96 well plate. The plate was covered and incubated at 37℃ for 15 minutes before being added with 20 µL of AST initiator solution which consists of α-ketoglutarate. The absorbance at 340 nm was measured upon adding of AST initiator once every minute for 10 minutes at 37℃.

3.4.8.2 Calculation

A graph of absorbance value versus time was plotted to obtain the slope of the linear portion of the curve. AST level was calculated using the following formula:

$$AST\ activity\ (U/L) = \left(\frac{\Delta A340/\min x\ 0.21mL}{4.11\ mM^{-1}\ x\ 0.02\ mL}\right) * 1000$$

3.4.9 Kidney Function Test

Kidney function tests refer to a panel of biochemical tests that are used to evaluate the function of kidneys. One of the major functions of kidneys is to excrete waste products from our bodies. Therefore, measuring the circulating levels of waste products can be used to identify whether the kidneys are working well. The levels of waste products are inversely proportional to the function of kidneys. Impairment in kidney function will reduce its efficiency in the excretion process, leading to the accumulation of waste products. Creatinine and urea are the two most common waste products for the assessment of kidney function.

3.4.10 Determination of Plasma Creatinine and Urea Levels

The plasma samples were outsourced to Prima Nexus, Malaysia for the determination of plasma creatinine and urea levels using Biolis 24i Premium automated biochemical analyzer (Tokyo, Japan).

3.4.11 Preparation of 10% Liver Tissue Homogenate

The 10% liver tissue homogenate was prepared in Tris-HCl buffer.

3.4.11.1 Preparation of Lysis Buffer, Tris-HCl

Tris-HCl buffer, 40 mM was prepared by dissolving 4.8456 g Tris base (Fisher Scientific, USA) in 800 mL of reverse osmosis (RO) water. The pH of the buffer was adjusted to 7.4 using concentrated 2M HCl acid (Chemiz, Malaysia) and topped up to 1 L using RO water.

3.4.11.2 Procedure

A volume of 30 – 50 mg liver tissues were added with ice-cold 40 mM Tris-HCl buffer, pH 7.4 at a weight: volume ratio of 1:9, and homogenated on ice using a tissue homogenizer equipped with a 5 mm X 75 mm flat bottom generator probe at 5,000 rpm for 1 minute (Omni International, USA). The 10% tissue homogenates were then centrifuged at 10,000 x g, 15 minutes, at 4℃ and the supernatant were collected, aliquoted and stored at –80℃ until further analysis (ROS, TBARS and caspase-3 levels).

3.4.12 Oxidative Stress

Oxidative stress occurred when there is an imbalance between the production and removal of free radicals, which are known as reactive oxygen species in our body. Various biological reactions including respiratory chain, mitochondria respiration and cytochrome P450 system generate ROS as a by-product (Pizzino et al., 2017).

Oxidative stress can be measured through direct and indirect measurements. The direct measurement involved quantifying the ROS using fluorescence probes. DFCA-DA is the most widely used dye for the determination of total intracellular ROS. The probe is able to penetrate the cell and cleave by intracellular esterase to produce DCFH (Katerji et al., 2019). Then, DCFH will react with ROS through a cascade redox event to generate fluorescence DCF (Zielonka and Kalyanaraman, 2008).

Direct measurement of ROS levels is often less accurate due to the short half-life of the ROS and is highly regulated by the intracellular redox homeostasis mechanism (Katerji et al., 2019). Thus, indirect measurement of oxidative stress provides a promising alternative approach through examining the oxidative damage caused by ROS. Lipid peroxidation occurs when the ROS attack polyunsaturated fatty acids, the main constituent of the cell membrane and produces MDA as one of the end products (Ayala et al., 2014). Thus, MDA can be used as a biomarker to identify the degree of lipid peroxidation and quantified the amount of ROS indirectly.

3.4.13 Determination of Liver ROS Level

DCFDA dye (Canvax, Spain) was used to quantify the levels of ROS.

3.4.13.1 Preparation of Working DCFDA Solution

Working DCFDA solution (10 µM) was prepared by diluting 10 µL of concentrated 20 mM DCFDA solution in 20 mL Tris-HCl buffer.

3.4.13.2 Assay Procedure

A volume of 100 µL of 10% liver tissue homogenate were added with 100 µL of working DCFDA solution and incubated at 37℃ for 30 minutes. The fluorescence intensity was measured at Ex/Em = 485/530 nm using a fluorescence microplate reader (Tecan, Switzerland).

3.4.13.3 Calculation

The liver ROS level was normalized to the vehicle control group and expressed as the relative fold change of control.

3.4.14 Determination of Liver Total Protein Concentration

The total protein concentration of liver samples was determined using the Bradford assay method. The TBARS and caspase-3 levels were normalized with the total protein level.

3.4.14.1 Preparation of Protein Standard Curve

The bovine serum albumin (BSA) stock solution (1,000 ng/µL) was prepared by dissolving 1 g BSA (Nacalai Tesque, Japan) in 1 mL Tris-HCl buffer (40 mM). The protein standard curve was constructed using protein concentrations ranging from 0 to 500 ng/µl (Table 3.3).

Table 3.3: Preparation of protein standard solution through serial dilution.

Tube	Standard solution (µL)	Tris-HCl buffer, 40 mM (µL)	Final Concentration (ng/µL)
1	500 µL from stock solution	500	500.00
2	500 µL from solution 1	500	250.00
3	500 µL from solution 2	500	125.00
4	500 µL from solution 3	500	62.50
5	500 µL from solution 4	500	31.25
6*	0	500	0

*Tube 6 is the blank solution

3.4.14.2 Assay Procedure

The 10% tissue homogenate was further diluted with two serial 10-fold dilutions. A volume of 20 µL of standard solution or diluted sample was added to the 96-well plate followed by 200 µL of Bradford's reagent (HiMedia, India). The mixture was incubated at room temperature for 10 minutes. The plate was read at 595 nm using a microplate reader (Tecan, Switzerland).

3.4.14.3 Calculation

The absolute absorbance of the standard/sample was calculated through subtraction of the absorbance value of the blank solution. The standard curve was plotted with absorbance value on the X-axis and protein concentration on the Y-axis. A curvilinear regression was performed, and a 3-parameter polynomial equation was generated, e.g., $y = ax^3 + bx^2 + cx + d$ using Microsoft Excel software. The protein concentration was determined by substituting the absorbance value into the equation and multiple with the dilution factor (100).

3.4.15 Determination of Liver TBARS Level

The liver TBARS level was determined using the TBARS colorimetric assay kit (Elabscience, China).

3.4.15.1 Preparation of TBARS Standard Solution

A serial concentration of TBARS standard solutions ranging from 0 to 100 µmol/L were prepared through dilution of the 200 µmol/L stock standard solution using RO water (Table 3.4).

Table 3.4: Preparation of TBARS standard solution through dilution.

Tube	Standard solution (µL)	RO water (µL)	Final Concentration (ng/µL)
1	250 µL from stock solution	250	100
2	200 µL from stock solution	300	80
3	150 µL from stock solution	350	60
4	200 µL from stock solution	800	40
5	500 µL from solution 4	500	20
6	500 µL from solution 4	500	10
7	500 µL from solution 4	500	5
8*	0	500	0

*Tube 8 is the blank solution

3.4.15.2 Assay Procedure

A 0.1 mL of 10% tissue homogenate was added into a 15 mL centrifuge tube followed by 0.1 mL clarificant reagent and 4 mL chromogenic agent. The tube was sealed and incubated at 95℃ in a water bath for 1 hour. The tubes were then transferred into an ice box for 10 minutes to terminate the reaction. The tubes then were centrifuged at 1600 x g for 10 minutes. A volume of 250 µL of supernatant was transferred into a 96 well plate and read at 532 nm using a microplate reader (Tecan, Switzerland).

3.4.15.3 Calculation

The absolute absorbance of the standard was calculated through subtraction of the absorbance value of the blank solution. The standard curve was plotted with TBARS concentration on the X-axis and absorbance on the Y-

axis to identify the Y-intercept and slope. The liver TBARS level was calculated using the formula:

$$TBARS\ (\mu mol/L) = \frac{\left((ODsample - ODblank) - Y\ intercept\right)}{slope}\ x\ \frac{dilution\ factor}{protein\ concentration\ (gpro/L)}$$

The liver TBARS level was normalized to the liver protein concentration and expressed as nmol/mg protein.

3.4.16 Apoptosis

Apoptosis is a form of programmed cell death which is mediated by proteolytic enzymes known as caspases. The apoptosis can be activated through either intrinsic or extrinsic pathways depending on the location of the apoptotic signals. Both intrinsic and extrinsic pathways activate caspase 3 which is the executioner caspase to induce apoptosis. Thus, measurement of the caspase-3 activity can provide information on the magnitude of the cellular apoptosis.

3.4.17 Determination of Liver Caspase-3 Level

The caspase-3 level in the liver was determined using the caspase-3 activity assay kit (Elabscience, China).

3.4.17.1 Assay Procedure

The 50 µL of 2X reaction working solution, 45 µL of 10% tissue homogenate and 5 µL of Ac-DEVD-pNA (substrate of caspase-3) were added accordingly to a 96 well. The Tris-HCl buffer was used to replace tissue homogenate which acts as the blank solution. The solutions were incubated at 37°C for 90 minutes. The absorbance at 405 nm was measured with a microplate reader.

3.4.17.2 Calculation

The absolute absorbance of the liver sample was calculated through subtraction of the absorbance value the of blank solution. The liver caspase-3 activity was normalized to protein concentration followed by the vehicle control group and expressed as relative fold change of control.

3.4.18 Phase II: Overview of the Study Design

Based on the findings of Phase I, the NOAEL of EGCG is determined at 250 mg/kg EGCG.

Male SHR aged 12 weeks old with SBP > 160 mmHg were treated with either PBS or 250 mg/kg *b.w.* EGCG via oral gavage (n=6 per subgroup) for 28 days. The blood pressure parameters including SBP, DBP and MBP of the rats were measured using the indirect tail-cuff VPR method (CODA, Kent Scientific,

CT, USA). All the blood pressure readings (SBP, DBP and MBP) were retrieved from the blood pressure analyzer directly. After the last dose of EGCG on day-28, the rats were fasted overnight. On day-29, the rats were euthanized via excessive inhalation of carbon dioxide followed by a cervical dislocation. Kidneys were harvested and washed with PBS. Renal cortex and medulla regions were collected separately and stored in RNAlater^{TM} solution. Cortical and medullary total ribonucleic acid (RNA) was extracted using commercially available kit (New England Biolabs, United State). The total RNA was reverse transcribed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, United State). Figure 3.3 shows the experimental protocol of the phase II study.

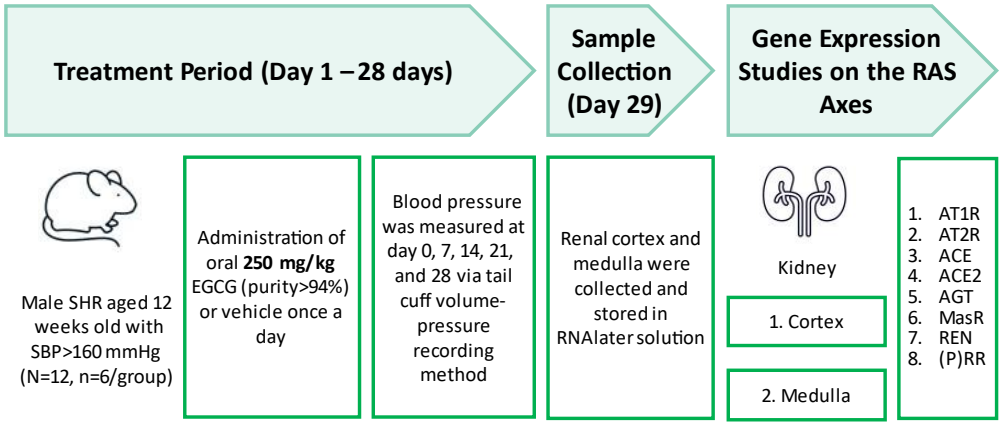

Figure 3.3: Experiment flowchart of the phase II study.

3.4.19 Blood Pressure Measurement

The blood pressure parameters including SBP, DBP and MBP of the rats were measured using the indirect tail-cuff VPR method (CODA, Kent Scientific, CT, USA). The CODA system is able to measure SBP and DBP directly and

calculate the MBP based on the measured SBP and DBP values. The rats were trained for the blood pressure measuring procedure for four 30-minutes sessions during the 2 weeks acclimatization period. The rats were restrained with a transparent restrainer and warmed with a warming pad at 33℃ for 15 minutes before the blood pressure measurement. A rectal temperature probe was inserted into the rectal of the rats for body temperature monitoring. All blood pressure measurements were conducted during 0800 – 1200 to avoid the influence of circadian rhythm on blood pressure. Three consecutive readings with not more than 10 mmHg difference were taken for data analysis.

3.4.20 Animal Euthanasia

On day-29, the rats were euthanized via excess inhalation of carbon dioxide in a closed chamber followed by a cervical dislocation. Kidneys were harvested, washed thoroughly with PBS and sliced at the coronal plane into equal half. The renal cortex and medulla regions were identified and separated accordingly. Medullary and cortical tissues were stored in RNAlater^{TM} solution separately at 4℃ overnight. Thereafter, the preserved tissues were removed from RNAlater^{TM} solution and stored at –80℃ until further gene expression studies.

3.4.21 Measurement of mRNA Levels of the RAS-related Genes

3.4.21.1 Total RNA Extraction

The total RNA of the renal cortex and medulla were isolated using Monarch® Total RNA Miniprep Kit (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's protocol. Briefly, 25 to 30 g of the frozen tissue prefixed with RNAlater™ was added into 300 uL of DNA/RNA protection reagent and homogenized on ice using tissue homogenizer (Omni International, USA) at 5,000 rpm for 1 minute. The homogenate was added with proteinase K and incubated at 55℃ for 5 minutes. The supernatant was collected and subjected to further genomic deoxyribonucleic acid (gDNA) removal and RNA purification procedures according to the manufacture's protocol. An additional on-column DNase I treatment step was performed to remove residual gDNA. Total RNA was eluted using 50 uL of nuclease-free water. The eluted total RNA was assessed for its concentration, purity, and integrity.

3.4.21.2 Determination of RNA Concentration, Purity, and Integrity

The purity and concentration of extracted RNA were determined using preprogrammed Nanodrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, NC, USA). RNA integrity was examined using the bleach gel method (Aranda et al., 2012). The RNA sample which contains 1000 ng RNA was loaded on 1% (w/v) agarose gel added with 1% (v/v) Chlorox bleach and pre-stain with RedSafe™ nucleic acid staining solution (iNtRON,

South Korea). The gel was electrophoresed at 100 V for 35 minutes before visualized using UVP BioSpectrum® 410 imaging system (Analytik Jena, USA).

3.4.21.3 Preparation of cDNA Sample

The cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). A total of 2 ug of RNA was added into the mixture consisting of random primers, dNTPs, reverse transcriptase and nuclease-free water (Table 3.5). The reaction was prepared in a final volume of 20 uL to yield 100 ng/µL of cDNA. The mixture was put into a thermal cycler with programmed cycling conditions (Table 3.6).

Table 3.5: Components of a 20 µL reverse transcription reaction mixture.

Components	**Stock concentration**	**Final concentration**	**Volume (µL)**
RT buffer	10X	1X	2.0
dNTPs mix (100 mM)	25X	1X	0.8
RT random primers	10X	1X	2.0
MultiScribe™ reverse transcriptase	NA	NA	1.0
Nuclease-free water	NA	NA	4.2
Diluted RNA sample	200 ng/µL	2 ug	10.0
		Total volume	20.0

Table 3.6: Thermal cycling conditions for reverse transcription.

Cycle step	Temperature (°C)	Time (min)
Primer annealing	25	10
DNA polymerization	37	120
Enzyme deactivation	85	5

3.4.21.4 Determination of mRNA level through RT-qPCR

The mRNA expression of RAS-related genes (*Agtr1a, Agtr2, Ace, Ace2, Agt, Mas1, Ren, Atp6ap2)* and reference gene (*Gapdh)* were determined using the RT-qPCR technique. Primer sequences were obtained from previous studies (Williamson et al., 2017, Tan et al., 2021) (Table 3.7). Real-time PCR was performed using Luna® Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA) (Table 3.8) on the StepOnePlus^{TM} Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) according to manufacturer's protocol (Table 3.9). The primer efficiency (90 – 110%, R^2 value > 0.98) was validated by using cDNA concentrations ranging from 3.125 to 50 ng (Appendix D). The amplicon size of the PCR product was validated through primer BLAST software and the PCR product was resolved on 2% (*w/v*) agarose gel at 80 V for 40 minutes (Appendix E). The melt curve analysis was conducted to identify the specificity of the PCR reaction (Appendix F). The mRNA expression level was normalized to *Gapdh* expression level. The relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method and expressed as fold change relative to control group.

Table 3.7: Primer sequences and amplicon size for reference and RAS-related genes.

Gene	Accession number	Forward Primer	Reverse Primer	Amplicon size (bp)
Gapdh	NM_017008.4	ATGGGAAGCTGGTCATCAAC	GTGGTTCACACCCATCACAA	221
Agtr1a	NM_030985.4	CTGCCACATTCCCTGAGTTAAC	ATCACCACCAAGCTGTTTCC	302
Agtr2	NM_012494.3	TAATCTCAACGCAACTGGCACC	GCCAAAAGGAGTAAGTCAGCCA	222
Ace	NM_012544.1	GCCCCCTGTACAAGTGTGAT	TAGGAAGAGCAGCACCCACT	347
Ace2	NM_001012006.1	CAGGAAGCTGAAGACCTGTCT	TTCAACTGTTTGTTCTTGTCTG	251
Agt	NM_134432.2	TTCAGGCCAAGACCTCCC	CCAGCCGGGAGGTGCAGT	309
Mas1	NM_012757.2	GGCGGTCATCATCTTCATAGC	CTTCTTCTTACTGCTGCCCAC	313
Ren	NM_012642.4	CACTCTTGTTGCTCTGGACCT	GGGGTACCAATGCCGATCTC	250
Atp6ap2	NM_001007091.1	CCGTGGCACCATGGCTGTGCT	GCAAGCCCTGGCCAAGACAG	204

Table 3.8: Components of a 10 µL real-time PCR reaction mixture.

Components	Stock concentration	Final concentration	Volume (µL)
Master Mix	2X	1X	5.00
Forward primer	10 µM	0.25 µM	0.25
Reverse primer	10 µM	0.25 µM	0.25
Diluted DNA template (10X dilution)	10 ng/µL	10 ng	1.00
Nuclease-free water	NA	NA	3.50
		Total volume	10.0

Table 3.9: Thermal cycling conditions for real-time PCR.

Cycle step	Temperature (°C)	Time (s)	Cycles
Initial denaturation	95	60	1
Denaturation	95	15	40
Extension	60	30	
Melt curve*	60 - 95	NA	1

*The melt curve analysis was conducted according to the default setting of the StepOnePlus™ Real-Time PCR System

3.5 Statistical Analysis

The data were presented as mean ± standard deviation (SD). Statistical analyses were conducted using IBM SPSS Statistics for Windows, version 26 (IBM Corp., USA). Independent t-test was used for 2-group comparison while one-way or two-way ANOVA followed by Dunnett's post-hoc test was performed for multiple group comparisons. Pearson correlation test was conducted to examine the association of two variables. A p-value < 0.05 was considered statistically significant.

CHAPTER 4

RESULTS

PHASE I STUDY RESULTS

4.1 Effects of EGCG Treatment on Body Weight, Food, and Water Intakes

There were no changes in the body weight, food, and water intakes among the vehicle and EGCG groups (Table 4.1).

Table 4.1: Effects of EGCG on body weight, food intake, and water intakes.

Parameters	**EGCG (mg/kg *b.w.*)**				
	0	**50**	**250**	**500**	**1000**
Body weight (g)					
D0	262.9 ± 18.7	269.3 ± 17.2	273.8 ± 21.1	267.2 ± 18.7	266.2 ± 18.1
D7	275.4 ± 14.0	280.2 ± 19.1	283.9 ± 16.8	272.5 ± 16.6	275.1 ± 15.0
D14	285.2 ± 11.7	290.6 ± 18.7	291.8 ± 20.2	280.8 ± 18.4	282.5 ± 19.1
D21	293.6 ± 14.9	301.6 ± 21.4	302.6 ± 22.4	286.3 ± 13.9	295.4 ± 19.8
D28	305.8 ± 15.4	306.7 ± 23.7	309.6 ± 22.7	289.0 ± 13.3	296.4 ± 23.7
Food intake (g/ 100 g b.w.)					
D0	7.0 ± 1.6	7.8 ± 1.3	6.1 ± 1.2	6.2 ± 1.4	6.2 ± 0.8
D7	6.8 ± 1.3	7.7 ± 0.6	7.4 ± 1.2	6.8 ± 0.6	6.6 ± 0.3
D14	6.6 ± 1.2	7.0 ± 1.1	7.1 ± 0.8	6.9 ± 1.2	6.5 ± 0.7
D21	6.3 ± 0.6	6.5 ± 0.4	6.1 ± 0.5	6.4 ± 0.8	6.7 ± 0.2
D28	6.3 ± 1.3	6.4 ± 1.3	6.4 ± 1.1	6.2 ± 1.1	5.3 ± 0.6
Water intake (g/ 100 g b.w.)					
D0	8.6 ± 0.5	7.9 ± 1.9	8.8 ± 0.3	7.0 ± 0.1	9.8 ± 0.8
D7	8.5 ± 0.5	9.6 ± 0.2	9.4 ± 0.4	8.7 ± 0.2	8.5 ± 0.7
D14	8.6 ± 0.1	9.3 ± 0.9	9.5 ± 0.6	8.8 ± 0.1	9.2 ± 0.4
D21	8.3 ± 0.1	8.8 ± 0.4	9.1 ± 1.1	8.8 ± 0.7	9.5 ± 0.8
D28	8.0 ± 0.4	9.2 ± 1.3	9.6 ± 1.5	8.4 ± 1.1	7.8 ± 0.6

Food and water intake were calculated through normalization to the body weight as the 7-day average. Data were expressed as mean ± SD.

4.2 Effects of EGCG on Mortality rate, Behavioral Changes, Observable Clinical Signs, Gross Anatomy of Thoracic and Abdominal Organs and Relative Organ Weights

All the treated and untreated rats survived until the end of the experimental period.

No toxic signs were observed in all the groups (Table 4.2). There were neither changes in the gross anatomy of the thoracic and abdominal organs nor the relative organ weights between vehicle and EGCG groups (Table 4.3/ Appendix G).

Table 4.2: Cage-side observation for behaviour changes and clinical signs.

Observations	**EGCG (mg/kg *b.w.*)**				
	0	**50**	**250**	**500**	**1000**
General condition	Normal	Normal	Normal	Normal	Normal
Porphyrin staining and/ or eye inflammation	Absent	Absent	Absent	Absent	Absent
Movements and posture	Normal	Normal	Normal	Normal	Normal
Piloerection	Absent	Absent	Absent	Absent	Absent
Skin	Normal	Normal	Normal	Normal	Normal
Appetite/ Food and water intake	Normal	Normal	Normal	Normal	Normal
Defecation	Normal	Normal	Normal	Normal	Normal
Urination	Normal	Normal	Normal	Normal	Normal
Breathing Difficulties	Absent	Absent	Absent	Absent	Absent

Table 4.3: Summary of the examination of the gross anatomy of various organs in SHR treated with different concentrations of EGCG.

Organs	**EGCG (mg/kg *b.w.*)**				
	0	**50**	**250**	**500**	**1000**
Heart	Normal	Normal	Normal	Normal	Normal
Lungs	Normal	Normal	Normal	Normal	Normal
Liver	Normal	Normal	Normal	Normal	Normal
Stomach	Normal	Normal	Normal	Normal	Normal
Spleen	Normal	Normal	Normal	Normal	Normal
Small intestine	Normal	Normal	Normal	Normal	Normal
Large intestine	Normal	Normal	Normal	Normal	Normal
Kidneys	Normal	Normal	Normal	Normal	Normal

Normal: There are no changes in the colour and texture of the organ, absence of enlargement of the size, lesion, and abnormality including redness, pus, ulceration, swollen, spots, and necrosis.

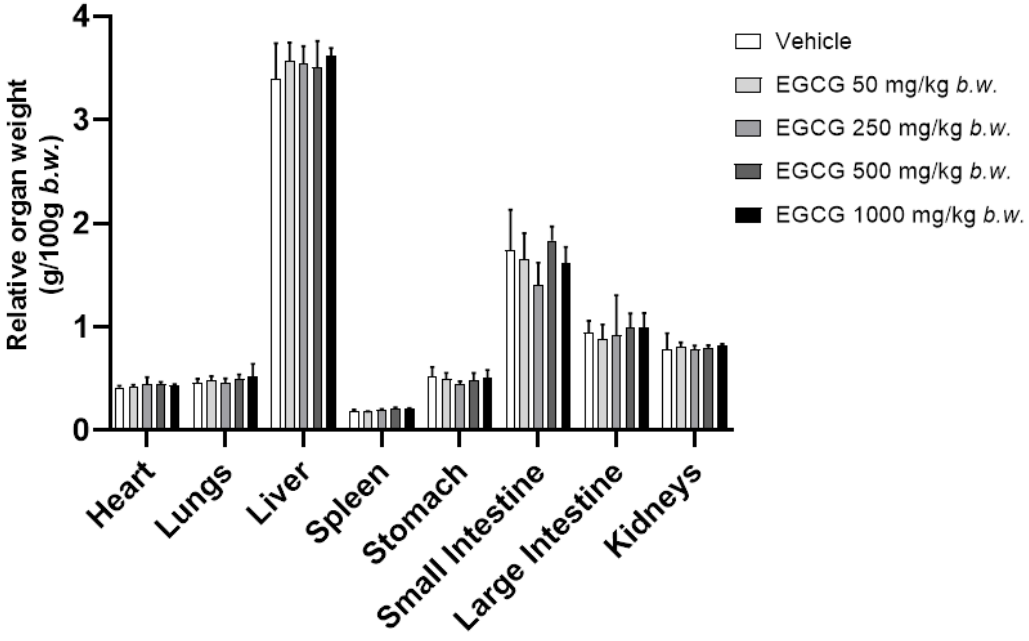

Figure 4.1: Effects of EGCG on relative organ weight. Relative organ weights were calculated through the normalization of organ weight to body weight. Data were expressed as mean ± SD, $n = 6$.

4.3 Effects of EGCG on Plasma ALT, AST and Hepatic ROS, TBARS and Caspase-3 Levels

Elevated plasma ALT and AST levels were found in SHR treated with 1000 mg/kg EGCG compared to the vehicle group (Figure 4.2 & 4.3). The changes in ALT and AST levels were significant associated (Figure 4.4). EGCG did not cause significant changes in the plasma levels of creatinine and urea (Figure 4.5 & 4.6). Increased hepatic TBARS level was noticed in SHR treated with 500 and 1000 mg/kg of EGCG (Figure 4.8). No changes in hepatic ROS and caspase-3 levels among the vehicle and EGCG treated groups (Figure 4.7 & 4.9). The effect of EGCG on the parameters mentioned above had been tabulated in Appendix H.

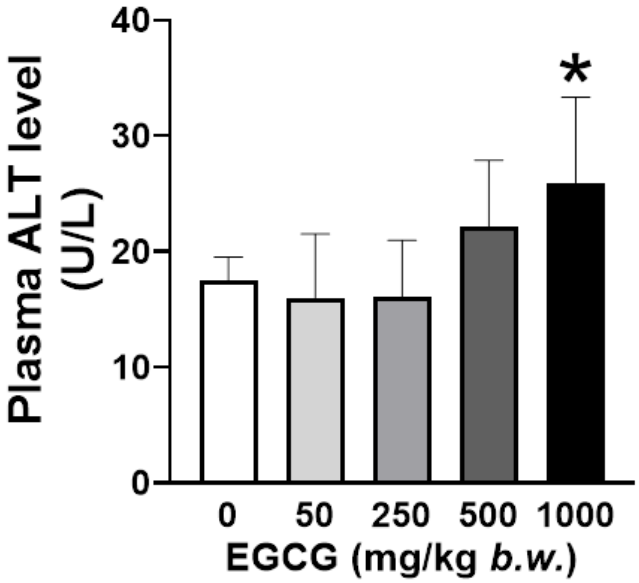

Figure 4.2: Effect of EGCG on plasma ALT level. *$p < 0.05$ vs vehicle, one-way ANOVA followed by Dunnett's test, $n = 6$.

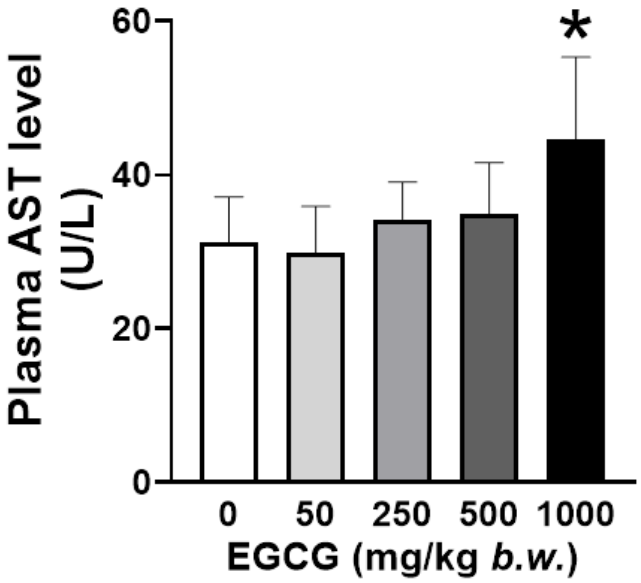

Figure 4.3: Effect of EGCG on plasma AST level. *$p < 0.05$ vs vehicle, one-way ANOVA followed by Dunnett's test, $n = 6$.

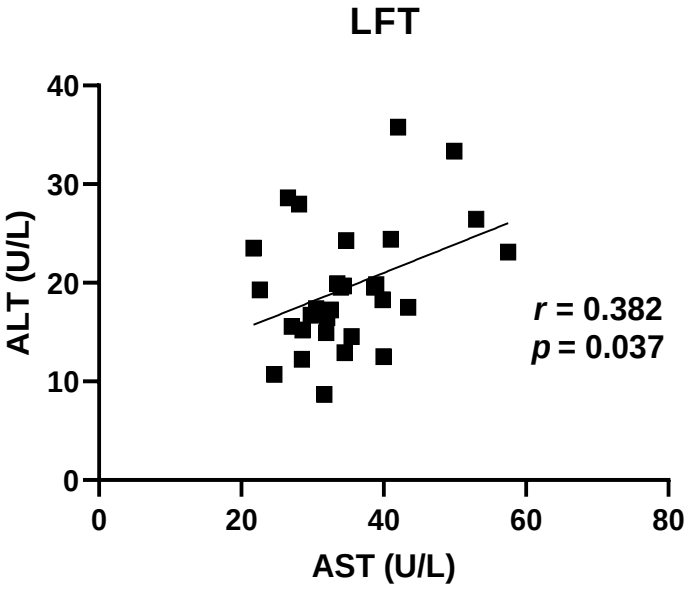

Figure 4.4: Pearson correlation between plasma ALT and AST levels, $n = 36$.

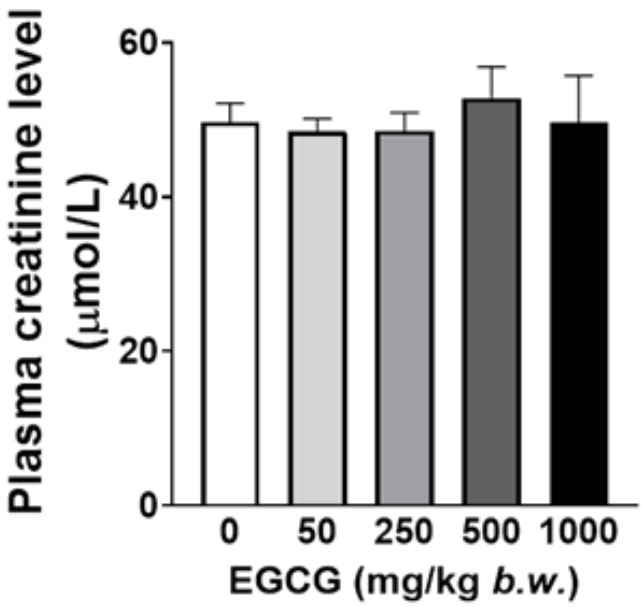

Figure 4.5: Effect of EGCG on plasma creatinine level, *n* = 4-6.

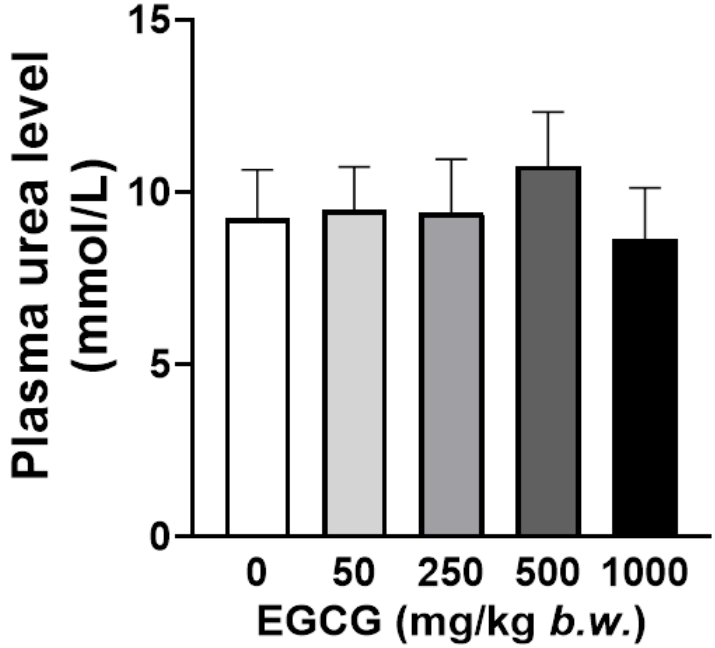

Figure 4.6: Effect of EGCG on plasma urea level, *n* = 4-6.

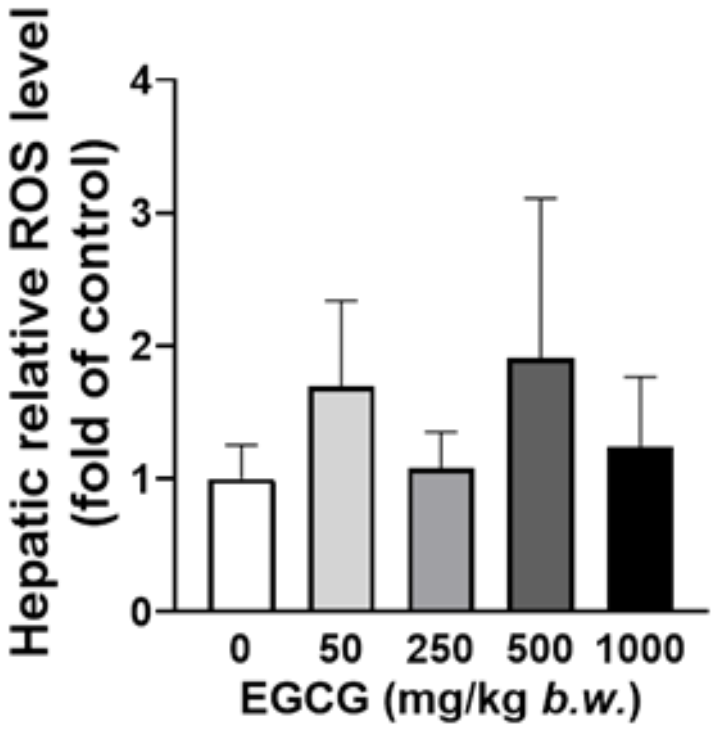

Figure 4.7: Effect of EGCG on hepatic ROS level, $n = 6$.

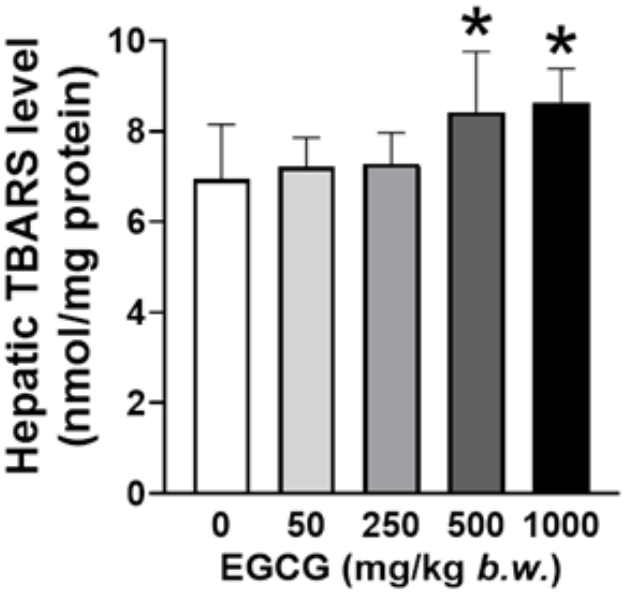

Figure 4.8: Effect of EGCG on hepatic TBARS level. *$p < 0.05$ vs vehicle, one-way ANOVA followed by Dunnett's test, $n = 6$.

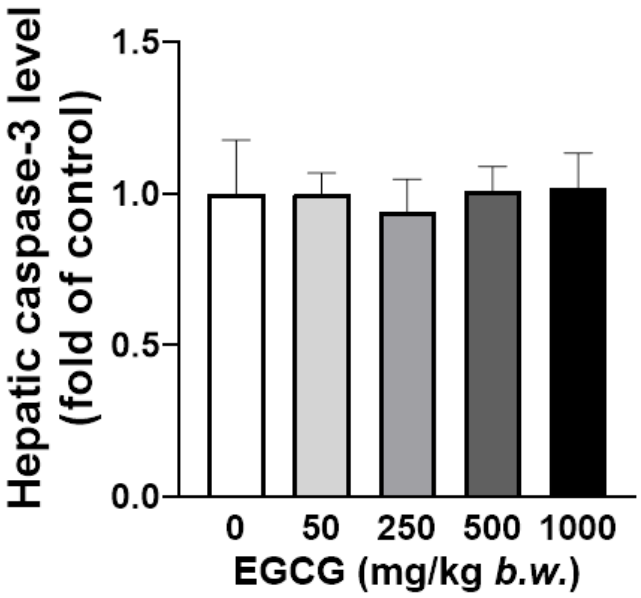

Figure 4.9: Effect of EGCG on hepatic caspase-3 level, $n = 6$.

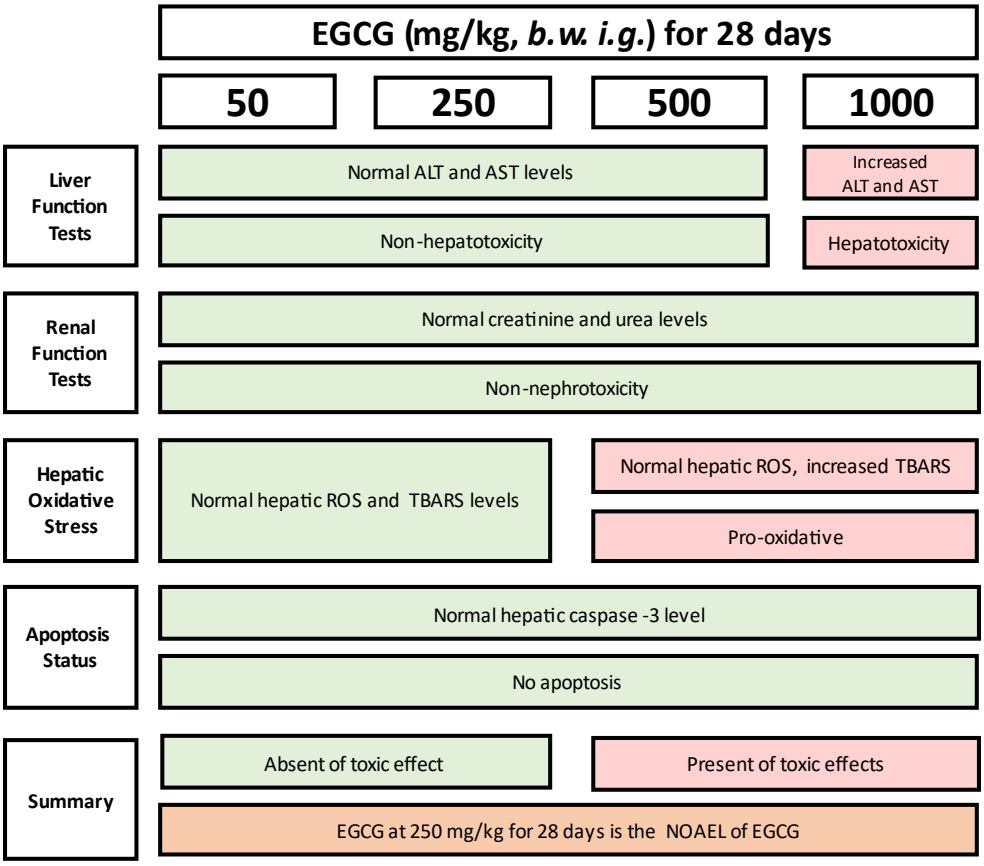

Figure 4.10: Summary results of the Phase I study.

PHASE II STUDY RESULTS

4.4 Effects of EGCG on Blood Pressures

SHR treated with 250 mg/kg EGCG showed a significant reduction in SBP, DBP and MBP by 23, 21, and 22 mmHg respectively at day-28 compared with the untreated control (Figure 4.11/ Appendix I). The SBP and MBP reduction was evident from day-7 onwards while DBP reduction was apparent on day-21 and day-28

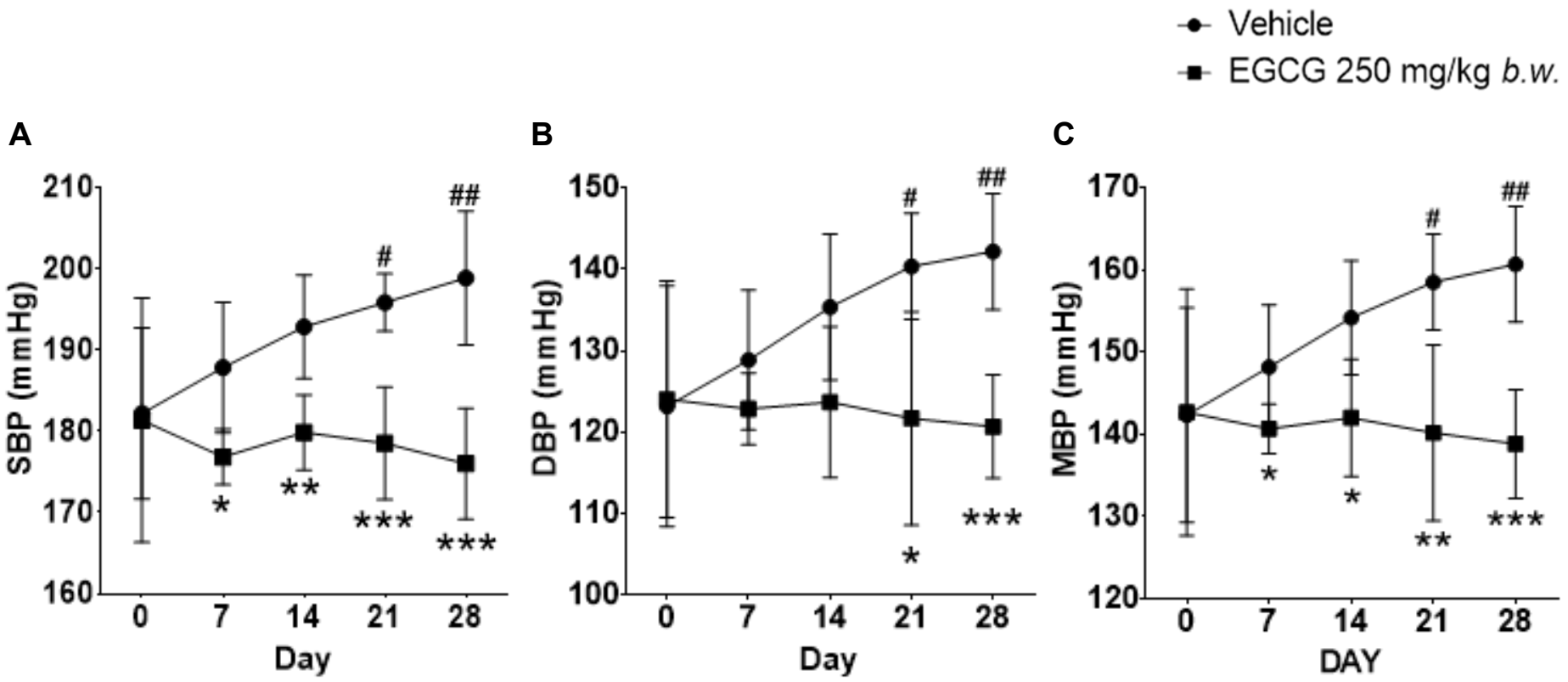

Figure 4.11: Effects of 28-days EGCG treatment on the (A) SBP, (B) DBP, and (C) MBP. Data were expressed as mean ± SD. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs day 0; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs vehicle, two-way ANOVA followed by Dunnett's test, $n = 6$.

4.5 Effects of EGCG on mRNA Expression of RAS-related Genes in Renal Cortex and Medulla

EGCG treatment significantly increased mRNA expression of renal cortical *Agtr2* and *Ace2* by 2.4 and 1.3 folds, but had no effect on *Agtr1a*, *Ace*, *Agt*, *Mas1*, *Ren* and *Atp6ap2* mRNA expression compared with vehicle (Figure 4.12/ Appendix J). The SHR administrated with EGCG demonstrated a significant increase in the renal medullary *Agtr2* (1.9 folds), *Ace* (1.4 folds), and *Mas1* (2.0 folds) mRNA expression, and significantly decreased renal medullary *Ren* (2.7 folds) mRNA expression in comparison with vehicle control (Figure 4.13). There were no changes in the *Agtr1a*, *Ace2*, *Agt* and *Atp6ap2* mRNA expression between vehicle and EGCG treated groups (Figure 4.13/ Appendix J).

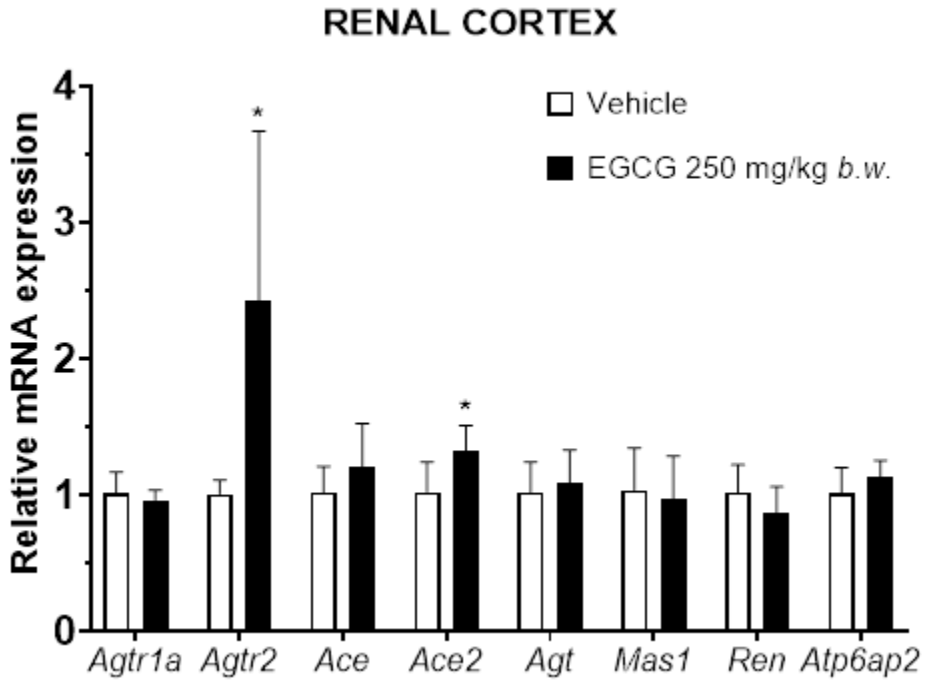

Figure 4.12: Relative mRNA expression of RAS-related genes in renal cortex. Data were normalized to the *Gapdh* gene expression and expressed as mean ± SD. *$p < 0.05$ vs vehicle, independent t-test, $n = 6$.

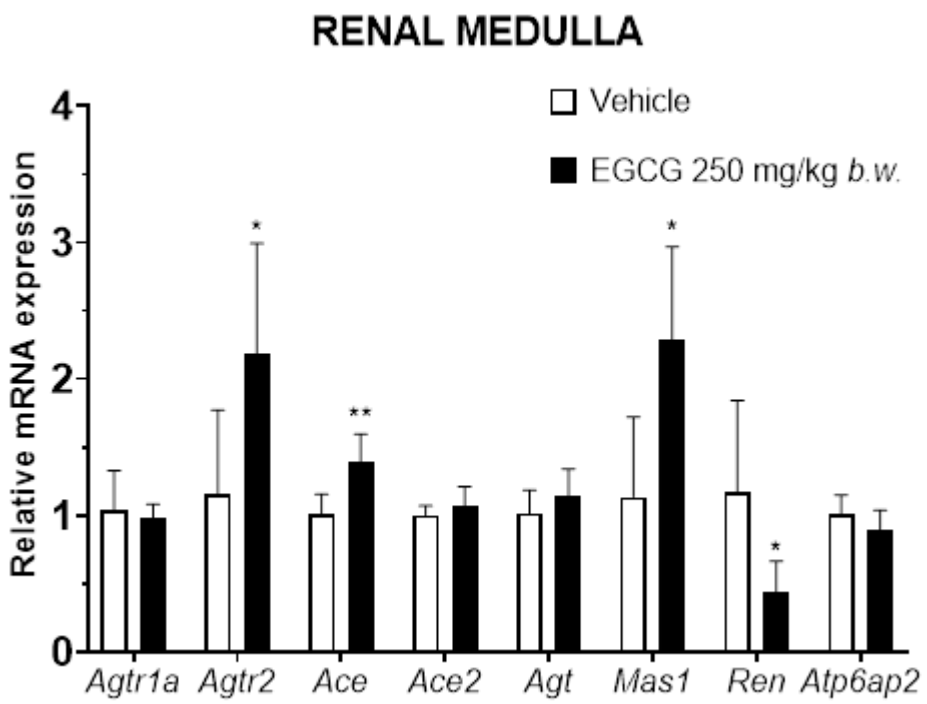

Figure 4.13: Relative mRNA expression of RAS-related genes in the renal medulla. Data were normalized to the *Gapdh* gene expression and expressed as mean ± SD. *$p < 0.05$, **$p < 0.01$ vs vehicle, independent t-test, $n = 6$.

4.6 Correlation between Blood Pressures and RAS-related Genes

The SBP reduction in EGCG treated SHR was significantly associated with the renal medullary *Agtr2*, *Ace* and *Ren* mRNA expression but not the expression of other genes (Table 4.4). Meanwhile, no significant correlation was found between the DBP and MBP of SHR with the RAS-related genes.

The SBP reduction was correlated moderately with the upregulation of *Agtr2* and *Ace* and the downregulation of *Ren* mRNA expression (Table 4.4). It is worth mentioning that a trend of upregulation of *Agtr2*, *Ace* and *Mas1* was observed and downregulation of *Ren* correlated moderately ($0.4 \leq r < 0.8$) with DBP and MBP reduction although the significant level was not be achieved. Notably, *Ace2* expression showed an exceptional case of correlating weakly ($0.0 \leq r < 0.4$) with the reduction of blood pressure.

Table 4.4: Pearson correlation analysis between RAS-related genes affected by EGCG treatment and blood pressure parameters.

RAS-related genes	**SBP**		**DBP**		**MBP**	
	r	***p***	***r***	***p***	***r***	***p***
Renal Cortex						
Agtr2	-0.497	0.100	-0.323	0.297	-0.400	0.198
Ace2	-0.283	0.373	0.084	0.794	-0.024	0.942
Renal Medulla						
Agtr2	-0.586*	0.045	-0.545	0.067	-0.563	0.057
Ace	-0.585*	0.046	-0.429	0.164	-0.494	0.102
Mas1	-0.506	0.093	-0.536	0.072	-0.521	0.082
Ren	0.584*	0.046	0.349	0.267	0.441	0.151

SBP: systolic blood pressure; DBP: diastolic blood pressure; MBP: mean blood pressure. *$p < 0.05$.

CHAPTER 5

DISCUSSION

5.1 Study Key Findings and Highlights

A 4-week of subacute oral administration of EGCG at 500 mg/kg *b.w.* increases the hepatocellular lipid peroxidation which reflected by the elevated TBARS levels. When EGCG is given at a higher dose (1000 mg/kg *b.w.*), it causes further hepatocellular injury indicated by the increased level of plasma transaminases (ALT & AST). No evidence of hepatocyte apoptosis via observation of the unchanged level of the apoptosis biomarker (caspase-3) suggests that the liver damage could be at a mild stage. Therefore, this study concludes that 4 weeks of EGCG at a dose of 250 mg/kg *b.w.* [human equivalent dose = 2430 mg] does not cause statistically significant increase in the frequency of adverse effects in SHR. Further molecular study shows that the blood pressure-lowering effect of EGCG is associated with the upregulated renal *Ace* and *Agtr2* expressions. Increased activation of AT2R further suppresses renin expression via the negative feedback loop, inhibits the entire systemic and local classical arm of RAS, and results in blood pressure reduction (Figure 5.1).

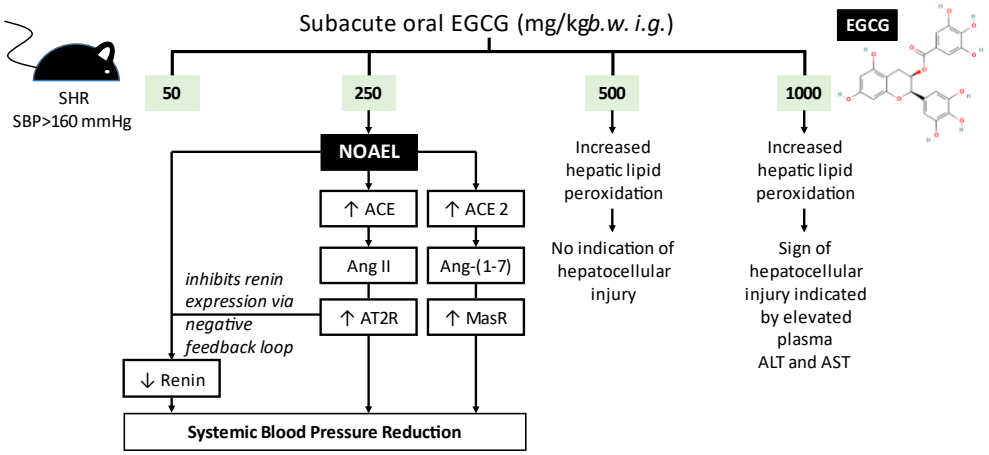

Figure 5.1: Safety and efficacy of EGCG in modulating raised blood pressure and intrarenal RAS in SHR.

5.2 Phase I: Determination of the NOAEL of EGCG Treatment

Toxicity screening of new compound is crucial for drug discovery and development process. *In vitro* and *in vivo* toxicity testing on various biological systems discovers dose-, species- and organ- specific toxic effect of a new compound and helps to determine the NOAEL which is needed to initiate the clinical evaluation of new compounds (Parasuraman, 2011). The therapeutic effects of EGCG in various diseases have been extensively studied; unfortunately, the evidence on its toxic effect and determination of NOAEL for different disease models are very limited. To the best of our knowledge, this study is the first study determining NOAEL of subacute EGCG in a rodent model of primary hypertension, SHR.

This study utilized body weight, food and water intakes, cage-side observation, necropsy findings and clinical chemistry in assessing possible toxic effects of 28-day of oral 50 – 1000 mg/kg *b.w* EGCG administration in SHR.

The EGCG compound used in this study is highly purified (>94%) despite the presence of other naturally occurring catechins at low concentration (Isbrucker et al., 2006). The rationale of the EGCG dose selection and treatment duration were determined based on the previous literatures (Isbrucker et al., 2006, Potenza et al., 2007, Chan et al., 2010, Ramachandran et al., 2016), along with the OECD guidelines 423. It has been demonstrated that EGCG > 1000 mg/kg *b.w.* was lethal to normotensive mice (Chan et al., 2010, Lambert et al., 2010), while EGCG at 50 – 200 mg/kg. b.w. was generally safe (Potenza et al., 2007, Wang et al., 2012). Therefore, this study hypothesized that the NOAEL for EGCG could be ranged from 50 – 1000 mg/kg *b.w.*. With consideration of research feasibility and cost-effectiveness along with the existing evidence, this study concluded that a 28-day of EGCG treatment would be an ideal duration as a preliminary study in testing possible toxic effect of EGCG in SHR.

This study prioritized possible toxic effects of EGCG on liver and kidneys as these are the main organs susceptible to toxicants. The liver is highly susceptible to toxicants because of its detoxification function. It processes/detoxifies almost all exogenous substances that enter the blood stream, and later these detoxified products are being excreted by the kidneys. Plasma ALT and AST are the common biomarkers for liver injury, while urea and creatinine are the common biomarkers for kidney injury. Other than these systemic biomarkers, this study further examined several tissue-based biomarkers for hepatic injury, which included ROS, TBARS and caspase-3 levels.

5.2.1 Effects of EGCG on Body Weight, Food and Water Intakes

Body weight, food and water intakes are the common indicators in assessing the general health conditions of laboratory animals (Ghasemi et al., 2021). The findings of the study suggest that EGCG treatment did not cause changes in energy expenditure (reflected by unchanged body weight and food intake) and fluid homeostasis (reflected by unchanged water intake) in SHR.

Body weight reduction and lack of appetite always associated with abnormalities and diseases (Ghasemi et al., 2021). In addition, these are common indicators in monitoring presence of any distress, discomfort or/and pain (Ghasemi et al., 2021). The findings of this study suggest that the 28-day of oral 50 – 1000 mg/kg *b.w*. EGCG did not resulted in any abnormalities, diseases, distress, discomfort or pain in SHR.

To the best of our knowledge, this present study is the first study examined the effect of EGCG in SHR via cage-side observation, as well as the changes in body weight, food and water intakes. As there are no existing data to compare with, therefore, the following discussion focused on the effect of GTE/EGCG on body weight in other rodent models.

In obesity studies, it has been showed that EGCG treatment reduced body weight along with the lipid profiles in rodent model of obesity (Bose et al., 2008, Friedrich et al., 2012). Some studies suggest that anti-obesity effect of EGCG could be mediated via appetite suppression, inhibitory effect on

intestinal absorption and increased energy expenditure (Wang et al., 2014a). Meanwhile Ashida et al. (2004) showed that GTE suppressed adipogenesis-related transcriptional factor, with no body weight changes in Wistar rats. Potenza et al. (2007) showed that 3 weeks of 200 mg/kg b.w. EGCG treatment leads to body weight reduction in pre-obese SHR/NHsd, haplotype $RT1^{k}$. It is uncertain that the EGCG treatment given in this present study exhibits any effects on lipid-related parameters; this study re-affirmed that 28-day of 50 – 1000 mg/kg *b.w*. EGCG through orally did not lead to significant body weight changes in typical strain of SHR.

5.2.2 Effects of EGCG on Mortality rate, Behavioral Changes, Observable Clinical Signs, Gross Anatomy and Relative Organ Weights of Thoracic and Abdominal Organs

Other than body weight, food and water intake, cage-side observation is an effective strategy in monitoring health condition of laboratory animals. Behavioral changes (movement, posture) and presence of clinical signs (abnormal eye discharge, signs of skin infection, reduced appetite, abnormal amount/colour of feces or urine, and breathing pattern) are also the essential checklist for cage-side observation according to the OECD guidelines 423. Changes in organ weights might indicate hypotrophy, hypertrophy or abnormal accumulation of biomolecules.

Previous toxicology studies revealed that a single dose of oral 2000 mg/kg *b.w.* EGCG (93% purity) was lethal to Wistar rats while 13 weeks of repeated dose of 500 mg/kg *b.w.* EGCG-containing diet (77% purity) did not

cause deaths or observable clinical signs in SD rats (Isbrucker et al., 2006). This study further demonstrated that oral EGCG up to 1000 mg/kg *b.w.* did not cause any unwanted effects on observable clinical signs, gross anatomy and relative organ weights of the thoracic and abdominal organs in SHR.

5.2.3 Effect of EGCG on Plasma ALT, AST, Creatinine and Urea and Hepatic ROS, TBARS and Caspase-3 Levels

A 4-week of oral administration of EGCG at 500 mg/kg *b.w.* increases the hepatocellular lipid peroxidation which reflected by the elevated TBARS levels. When EGCG is given at a higher dose (1000 mg/kg *b.w.*), it causes further hepatocellular injury indicated by the increased level of plasma transaminases (ALT & AST). This is undeniable that elevated plasma AST could indicate cardiac injury as well. Therefore, a correlation graph of AST and ALT was plotted to re-confirm the relationship of ALT and AST found in this study (Figure 4.4). No evidence of hepatocyte apoptosis via observation of the unchanged level of the apoptosis biomarker (caspase-3) suggests that the liver damage could be at a mild stage. Unchanged plasma creatinine and urea levels indicates that this EGCG regimen is not nephrotoxic to SHR.

Inadequate evidence on EGCG hepatotoxicity in previous literature could be due to EGCG induces mild liver injury that could not be identified via cage-side observation or necropsy and/or lack of awareness among the researchers on the potential toxic effect of EGCG. The present study shows that the 500 - 1000 mg/kg *b.w. i.g.* EGCG-induced hepatotoxicity is at a mild stage and not detectable through cage-side observation and necropsy findings. A 13

weeks of repeated dose of 500 mg/kg *b.w.* EGCG-containing diet (77% purity) did not cause deaths or observable clinical signs, as well as changes in hematology profile, liver (ALT, AST) and kidney (creatinine, BUN) injury biomarkers at the end of the supplementation period and 4-week of recovery period in Sprague-Dawley rats (Isbrucker et al., 2006). Our study reveals that 1000 mg/kg *b.w.* EGCG with purity > 94% for 4 weeks leads to augmented ALT and AST levels in SHR. Non-dosing recovery phase was not included in this present study mainly to increase the sensitivity in detecting EGCG-related injury as well as to avoid the "masking effect" of cellular self-recovery as noticed in a previous study (Ramachandran et al., 2016). A lower dose of EGCG (150 mg/kg *b.w.*) administered via intraperitoneal route showed significant nephrotoxicity in male Swiss albino mice (Rasheed et al., 2017) while this present study showed that orally administered EGCG up to 1000 mg/kg *b.w.* did not cause changes in kidney injury biomarkers in genetically hypertensive SHR. Inconsistent findings on the safety profile of EGCG among studies could be due to variation in (i) purity, duration and route of administration of the administered EGCG (ii) degree of susceptibility to EGCG due to different genetic make-up, pathological conditions or feeding status (Isbrucker et al., 2006, Lambert et al., 2010, Ramachandran et al., 2016, Rasheed et al., 2017).

The underlying mechanisms on how EGCG causes organ damage particularly hepatoxicity are not well studied. Lambert et al. (2010) have demonstrated that EGCG (500-1500 mg/kg, *i.g.*) induced hepatoxicity was associated with induction of hepatic oxidative stress (with elevated oxidative stress markers, TBARS, ALT, AST) and these effects are in a time- and dose-

dependent manner in mice. Wang et al. (2015) reported that EGCG triggered hepatoxicity through its transcriptional inhibitory activity on the major intracellular antioxidant enzymes i.e superoxide dismutase, catalase and glutathione peroxidative and subsequently results in excessive buildup of ROS and cellular damage, in addition to suppression of nuclear factor erythroid 2-related factor 2 pathway via downregulation of DJ-1 in mice.

5.2.4 Determination NOAEL of EGCG in SHR

As the NOAEL is species- and disease-specific, and there is lack of data on NOAEL of EGCG in rodent models especially SHR, the following discussion focus on NOAEL of EGCG as well as GTE on different rodent species/disease models. Chan et al. (2010) concludes that NOAEL of 14-week GTE treatment (contains 48% of EGCG) for both F344/NTac rats and B6C3F1 mice is 500 mg/kg *b.w.* (equivalent to 240mg/kg b.w. of EGCG). Isbrucker et al. (2006) concludes that 13-week of 500 mg/kg *b.w.* is safe to SD rats. It seems the recommendations on NOAEL for EGCG in rodents is ≤ 500 mg/kg *b.w.*, albeit the study design and the examined parameters are not completely identical.

In conclusion, with all the findings discussed above, the NOAEL of 28-day oral EGCG in SHR was established at 250 mg/kg *b.w.* (Figure 4.10), and this dose was applied to the Phase II of the study in examining its effects on blood pressure and intrarenal RAS in SHR.

5.3 Phase II: Investigation of the Roles of EGCG as a Renin-Angiotensin System Modulator in Ameliorating Raised Blood Pressure in SHR

This present study shows that antihypertensive effects of EGCG in SHR is in a cumulative time-dependent manner. Greater blood pressure reduction is observed in those SHR treated with longer periods (Figure 4.11). These blood pressure lowering effects are statistically associated with the changes in the transcriptional levels of intrarenal *Ren*, *Ace* and *Agtr2*. Nevertheless, the causal relationship between changes in blood pressure and RAS gene expression cannot be established in this present study as it involves complex mechanisms. This study further postulates that activation of local ACE/Ang II/AT2R results in systemic blood pressure reduction and the Ang II production is independent from the actions of the decreased renin shown in this study (Figure 5.1).

5.3.1 Effects of EGCG on Blood Pressures

This present study reveals that EGCG suppressed the elevation of systolic blood pressure progressively at -11, 13, 17 and 23 mmHg (5.9%, 6.7%, 8.7%, and 11.6%) following 7, 14, 21 and 28 days of treatment. Meanwhile, the reduction of DBP is delayed and it is statistically significant from day-21 onwards (-18 and -21mmHg on day-21 and day-28). The reasons on the delayed in DBP reduction found in this study is uncertain, it seems a longer EGCG treatment period is required to restore the abnormalities that lead to elevated DBP. On the other hand, it is worthful to further investigate the potential therapeutic role of EGCG in treating isolated systolic hypertension.

Blood pressure lowering effect of EGCG was evidence in genetically-hypertensive and salt-induced hypertensive rodent models (Potenza et al., 2007, Zhang et al., 2018, Luo et al., 2020). It has been proposed that EGCG exerts its antihypertensive effects via suppression in systemic RAS pathway, suppression in sympathetic system and improvement in endothelial functions (Potenza et al., 2007, Yi et al., 2016), in addition via its direct antioxidative property and free radical scavenging activities (Lambert and Elias, 2010). Previous *in silico* and *in vitro* studies have demonstrated that EGCG exhibits its inhibitory effect on renin and ACE (Li et al., 2013, Ke et al., 2017). Meanwhile *in-vivo* studies show that EGCG attenuates the increase of Ang II, ACE, AT1R in SHRSP.Z-Lepr(fa)/IzmDmcr rats (Kochi et al., 2014). In addition, it is well-established that EGCG influences DNA transcription, cellular responses and protein interactions which subsequently modulates enzymatic activities, receptor functions and signal transduction (Krupkova et al., 2016, Ouyang et al., 2020). Along with this evidence, this study further proposes that EGCG exerts its antihypertensive via its modulatory activities on transcriptional levels of intrarenal RAS mediators.

5.3.2 Effects of EGCG on Transcriptional Levels of the Intrarenal RAS-Related Genes and Association with Its Antihypertensive Effect

Intrarenal RAS mediators which include AGT, ACE, Ang II receptors and Ang II are found predominantly in the proximal tubule (Kobori et al., 2003, Ye et al., 2006, Satou et al., 2015, Carey, 2017). The functional data of these local RAS mediators are well documented. However, the biosynthesis and cellular uptake of its components and their cellular processing remains mainly

unelucidated. As tissue-based RAS acts as a paracrine, it may travel and act on the other neighboring tissues, discussion on its functional property would be more meaningful than to debate on the origin of components.

As the secreted substance may subject to transcytosis and cellular uptake by the neighboring tissues, the following discussion focus on the functional property of each upregulated/downregulated gene expressions, rather than focusing on its origin of gene expression in EGCG treated SHR. It is understandable that changes cortical and/or medullary RAS components can lead to paracrine effects and intrarenal RAS responses.

To recap, this present study revealed that EGCG significantly upregulated intrarenal mRNA expressions of receptors (AT2R and MasR) and catalytic enzymes (ACE and ACE2) while significantly downregulated renin expression. Further correlation statistical test showed that the blood pressure reduction is associated with the changes in AT2R, ACE and renin expression levels while upregulated ACE2 and MasR showed a non-statistically association with blood pressure reduction in EGCG treated SHR.

Production of Ang II mainly calatalysed by renin and ACE through a series of biochemical reactions. Nevertheless, extensive research has revealed that Ang II can be produced through non-renin pathway (Nehme et al., 2019). It has been demonstrated tonin and cathepsin G catalyzes the conversion of AGT directly to Ang II (Nehme et al., 2019) and we further hypothesize that the

reduced renin synthesis found in this study may not entirely affect the levels of Ang II in EGCG treated SHR.

5.3.3 The Roles of the Upregulated *Ace* and *Agtr2* in Blood Pressure Reduction in EGCG Treated SHR

The findings of this study suggest that upregulated AT2R may compete Ang II subtract binding with AT1R and further triggers Ang II/AT2R activation and/or forms heterodimerization with AT1R and results losses of Ang II/AT1R cellular response in EGCG treated SHR.

It has been demonstrated EGCG exhibits significant inhibitory activity on circulating/pulmonary ACE. Molecular docking studies show that EGCG inactivate ACE via its binding to the ACE at the position Gln 281, Lys 511 and Tyr 520 through formation of three hydrogen bonds (Ke et al., 2017). *In vitro* ACE inhibitory assay identify that EGCG causes 82% inhibition on ACE activity (Ke et al., 2017). Interestingly, this present study found that EGCG treatment upregulated intrarenal ACE expression in SHR. The underlying mechanism of this occurrence is unclear, but it is believed that augmentation of ACE level is associated with activation of Ang II/AT2R which collectively leads to blood pressure reduction in EGCG treated SHR.

This study postulates that elevated ACE increases Ang II subtracts level and triggers the Ang II/AT2R signaling pathway rather than Ang II/AT1R. In the physiological conditions, AT1R is highly competitive to AT2R and formation rate of Ang II/AT1R complex is higher than Ang II/AT2R (Carey, 2017). Nevertheless, along with the evidence of upregulated AT2R but insignificant changes of the AT1R abundance, this study suggests that binding effects of Ang II/AT2R overtaking those triggered by Ang II/AT1R complex and ultimately results in blood pressure reduction in EGCG treated SHR.

The interaction in between AT1R and AT2R has been suggested to participate in homeostasis of blood pressure regulation (Patel and Hussain, 2018). Ang II/AT1R binding leads to Ca^{2+} influx resulting in vasoconstriction. On the other hand, Ang II/AT2R bindings induces vasorelaxation via activation of the kinin/NO/cGMP system. It has been suggested that AT2R-mediated vasorelaxation may counteract AT1R-mediated vasoconstriction (Patel and Hussain, 2018). In addition, it has been reported that Ang II/AT2R binding in the kidneys triggers production of vasodilators *i.e* nitric oxide, bradykinin and prostaglandins which subsequently leads to blood pressure reduction (Siragy, 2010). This study postulates that elevated AT2R competes Ang II substrates with AT1R and therefore diminishes Ang II/AT1R biological effects in EGCG treated SHR.

It has been showed that AT1R functionality is influenced by the interaction with the presence of AT2R which may lead to different cellular responses. Administration of AT2R agonist inhibits AT1R-mediated activation of sodium transporters, NKA and NHE3 through the formation of the AT1R-AT2R heterodimer and results in attenuation of the functionality of AT1R (Yang et al., 2012). Receptor heterodimerization affects the expression of receptors, sensitization/desensitization and the effect of agonists on signal transduction which resulting in various functional consequences (Rukavina Mikusic et al., 2020). These discoveries support our research findings in suggesting upregulated AT2R may decreases functionality of AT1R, either via Ang II-dependent and/or -independent mechanisms as proposed above.

5.3.4 The Roles of the Upregulated *Ace2* and *MasR* in Blood Pressure Reduction in EGCG Treated SHR

This present study shows that EGCG stimulates ACE2/Ang-(1-7)/MasR axis through upregulation of *Ace2* and *Mas1* expression. Elevated ACE2 increases production of Ang (1-7) and this phenomenon may further trigger Ang (1-7)/MasR signaling pathway and results in a series of reactions (vasorelaxation, increased renal blood flow, urinary sodium excretion and reduced renal vascular resistance) that leads to blood pressure reduction in EGCG treated SHR, albeit the correlation of gene expression changes with the blood pressure reduction is non-statistically significant. In addition, elevated MasR may also play a role in forming heterodimer with AT1R and AT2R that results in different biological events.

The elevated MasR expressions found in EGCG treated SHR may play a crucial role in determining the functionality of AT1R and AT2R via the process of receptor heterodimerization. It has been found that AT1R-MasR heterodimer attenuates functionality of AT1R while AT2R-MasR heterodimer enhances both AT2R and MasR-mediated signalling pathways that associated with blood pressure reduction (Patel and Hussain, 2018, Rukavina Mikusic et al., 2020). Therefore, this present study further proposes that upregulated AT2R and MasR may collectively promote heterodimerization of (i) AT1R-AT2R (ii) AT1R-MasR (iii) AT2R-MasR that subsequently leads to blood pressure reduction in EGCG treated SHR.

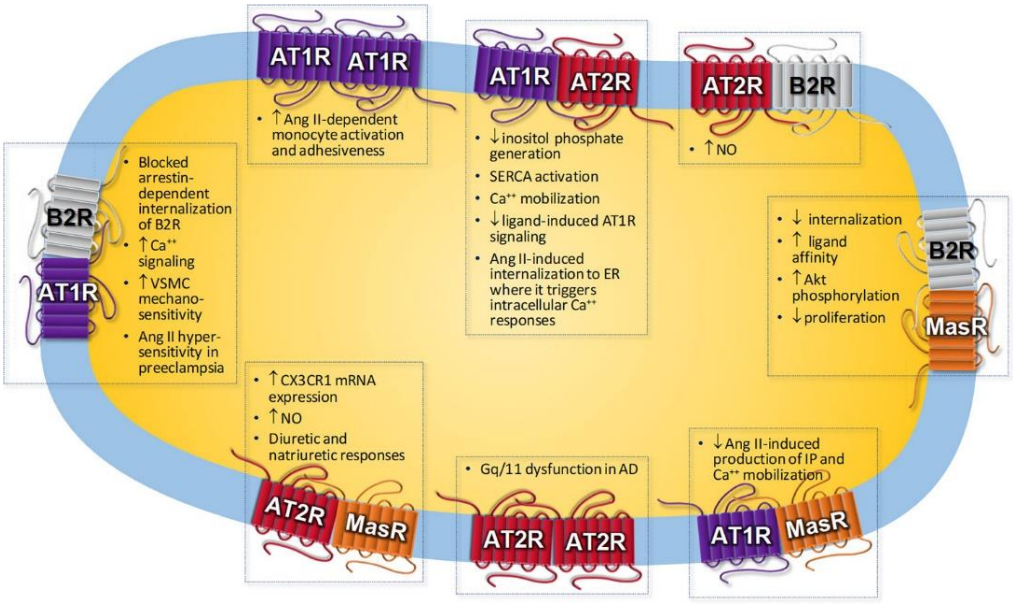

Figure 5.2: The heterodimerization between the RAS receptors [Adapted from Rukavina Mikusic et al. (2020)].

The existing data related to effect of EGCG/catechins on counter-regulator RAS components are very limited and perhaps the findings of this present study could serve as the fundamental information for further studies in

elucidating the underlying mechanism of EGCG in modulating Ang II/AT2R and Ang (1-7)/MasR signaling pathways.

5.3.5 The Roles of the Downregulated *Ren* in Blood Pressure Reduction in EGCG Treated SHR

Reduced renal renin synthesis found in this present study could be due to (i) the direct effect of EGCG on renin transcriptional levels (ii) the negative feedback loop of AT2R activation in EGCG treated SHR (iii) negative feedback loop of elevated ACE and non-renin-dependent-Ang II. Certainly, reduced renin synthesis is significantly associated with the blood pressure lowering effect found in the EGCG treated SHR regardless via its endocrine or paracrine effects on systemic or intrarenal RAS.

Blood pressure lowering effect of EGCG has been associated with its inhibitory property on circulating renin. *In vitro* study suggested that EGCG inhibit renin activity in a non-competitive and dose-dependent manner (Li et al., 2013). It has been hypothesized that EGCG binds to renin active site and results in renin dysfunction (Li et al., 2013). On the other hand, it has been demonstrated that GTE supplementation reduced renin expression in chronic cyclosporin-treated rats (Ryu et al., 2011).

It is uncertain that if reduced renin synthesis is due to the upregulation of AT2R in EGCG treated SHR. Previous studies have demonstrated that activation of AT2R suppressed renin synthesis via negative feedback

mechanism (Siragy et al., 2005). Siragy et al. (2005) has demonstrated administration of AT2R blocker, PD123319 significantly upregulated renin mRNA expression and this research team concluded that AT2R regulates RAS activity via its inhibitory action on renin synthesis. This discovery supports our postulation that upregulated AT2R might play a crucial role in suppressing renin expression. Nevertheless, it is premature to conclude the causal relationship of AT2R and renin expressions and certainly more studies are required to establish the chronological events.

In addition to its action in generation of Ang II, studies have demonstrated that renin itself may directly trigger cellular effects via activation of p38 mitogen-activated protein kinase that subsequently may lead to vascular damage and raised blood pressure (Potthoff et al., 2016). Reduced renin undeniably can diminish RAS overactivation and inhibit further blood pressure elevation (Simko et al., 2021).

The renin system is also regulated by the levels of Ang II levels through short loop negative feedback mechanism. The subcutaneously infusion of Ang II was found to suppress the renin production in the renal proximal tubule cells (Neubauer et al., 2018).

5.4 Limitations and Further Studies

A positive control drug is not included in this study as there is a lack of suitable candidate drug that is able to modulate the gene expression of multiple intrarenal RAS components simultaneously. The current antihypertensive drugs including renin inhibitor, ACE inhibitor, and ARB focus on inhibition of the enzyme activities or receptor functions rather than acts as molecular medicines to alternate the gene expression of the key proteins.

Our study had confirmed the dual effects of the EGCG on RAS components at gene expression. Further study can be conducted through co-administration of the EGCG with AT2 blocker to confirm the role of AT2 in the antihypertensive effects of EGCG.

CHAPTER 6

CONCLUSION

The NOAEL of 28-day oral EGCG is 250 mg/kg *b.w.* in SHR. Administration of EGCG beyond this NOAEL causes elevated hepatic lipid peroxidation and liver damage indicated by the elevated ALT and AST levels. EGCG at its NOAEL dose exhibits blood pressure lowering effects and it is statistically associated with the activation of the intrarenal counter-regulatory axis (ACE/Ang II/AT2R) and suppression of the classical axis (REN) gene expressions.

In future clinical studies, liver functions should be closely monitored to further establish the safety of EGCG during long-term usage. Meanwhile, the findings on the effect of EGCG on intrarenal RAS transcriptional levels in models of hypertension could serves as the fundamental evidence to support further research questions and research gaps.

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APPENDICES

Appendix A

Table A: List of chemicals, consumables, software, equipment, and assay kits.

Items	Manufacturer	Country
Chemicals and reagents		
2,7-Dichlorofluoroscin Diacetate, DCFDA (CA093)	Canvax	Córdoba, Spain
50X TAE buffer (B49)	Thermo Fisher Scientific	Massachusetts, USA
6X gel loading dye (B7025)	New England Biolabs	Massachusetts, USA
Agarose	Thermo Fisher Scientific	Massachusetts, USA
Bleach (Clorox®)	Clorox	California, USA
Di-sodium hydrogen phosphate, Na_2HPO_4	Chemiz	Selangor, Malaysia
Epigallocatechin gallate (Taevigo®)	Taiyo	Gevelsberg, Germany
Food pellet (702P)	Gold Coin	Selangor, Malaysia
Hydrochloric acid, HCl	Chemiz	Selangor, Malaysia
Nuclease free water (AM9938)	Thermo Fisher Scientific	Massachusetts, USA
Potassium chloride, KCl	Chemiz	Selangor, Malaysia
Potassium dihydrogen phosphate, KH_2PO_4	Chemiz	Selangor, Malaysia
Primers	Integrated DNA Technologies	Iowa, USA
RedSafe™ nucleic acid staining solution (21141)	iNtRON,	Seongnam-si, South Korea

RNA stabilizing solution (RNAlater^{TM}, AM7021)	Thermo Fisher Scientific	Massachusetts, USA
Sodium chloride, NaCl	Chemiz	Selangor, Malaysia
Tris base, $(CH_2OH)_3CNH_2$	Fisher Scientific	Massachusetts, USA
Plasticwares and Consumables		
0.5 mL microcentrifuge tubes	Corning	New York, USA
1.5 mL microcentrifuge tubes	Nest	Jiangsu, China
10 µL micropipette filter tips	Labcon	California, USA
10 µL micropipette tips	Labcon	California, USA
10 mL syringe	Terumo	Tokyo, Japan
100 µL micropipette filter tips	Labcon	California, USA
100 µL micropipette tips	Labcon	California, USA
1000 µL micropipette filter tips	Labcon	California, USA
1000 µL micropipette tips	Labcon	California, USA
15 mL centrifuge tubes	SPL Life Sciences	Gyeonggi-do, South Korea
2 mL microcentrifuge tubes	Nest	Jiangsu, China
21 G needles	Terumo	Tokyo, Japan
3 mL syringe	Terumo	Tokyo, Japan
50 mL centrifuge tubes	Nest	Jiangsu, China
Blade	B.Braun	Melsungen, Germany
PCR cooling blocks	Labcon	California, USA
qPCR strip tubes with caps	Paradigm	Wisconsin, USA
Laboratories Equipment		
–20°C freezer (DW-40L262)	Haier	Qingdao, China
4°C chiller	hi-TEN	Selangor, Malaysia

Analytical balance (BSA224S-CW)	Sartorius	Göttingen, Germany
Autoclave (SX-500)	Tomy Digital Biology	Tokyo, Japan
Automatic ice flake maker (ZBS-100)	Remi	Selangor, Malaysia
Benchtop centrifuge (Sorvall™ ST 16R)	Thermo Fisher Scientific	Massachusetts, USA
Benchtop pH meter (6175)	Jenco	Penang, Malaysia
Drying oven (ED 53)	Binder	Tuttlingen, Germany
Gel electrophoresis system (Mini-Sub Cell GT Cell)	Bio-Rad	California, USA
Imaging system (UVP BioSpectrum® 410)	Analytik Jena	California, USA
Laminar flow cabinet (AHC-4D1)	Esco Lifesciences Group	Tampines, Singapore
Microcentrifuge (5415R)	Eppendorf	Hamburg, Germany
Multimode microplate reader (Spark®)	Tecan	Zürich, Switzerland
Non-invasive blood pressure system (CODA® Monitor)	Kent Scientific	Connecticut, USA
Real-time PCR system (StepOnePlus™)	Applied Biosystems	Massachusetts, USA
Thermal cycler (Veriti™)	Applied Biosystems	Massachusetts, USA
Tissue homogenizer (TH)	Omni International	Georgia, USA
Ultra low temperature freezer (MDF-U74V)	PHCbi	Tokyo, Japan
Water purification system (Milli-Q® Integral 5)	Millipore	Massachusetts, USA

Waterbath (WNB 22)	Memmert	Schwabach, Germany
Weighting balance (ELB2000)	Shimadzu	Kyoto, Japan
	Software	
GraphPad Prism version 8.3	GraphPad Software	California, USA
Microsoft Excel	Microsoft	Washington, USA
Primer BLAST	National Center for Biotechnology Information	Maryland, USA
SPSS Statistics for Windows, version 26	IBM	New York, USA
StepOne Software version 2.3	Applied Biosystems	California, USA
VisionWorks®	Analytik Jena	California, USA
	Assay kits	
Alanine Transaminase Colorimetric Activity Assay Kit (700260)	Cayman Chemical	Michigan, USA
Aspartate Aminotransferase Colorimetric Activity Assay Kit (701640)	Cayman Chemical	Michigan, USA
Caspase 3 Activity Colorimetric Assay Kit (E-CK-A311)	Elabscience®	Wuhan, China
High-Capacity cDNA Reverse Transcription Kit (4368814)	Applied Biosystems	Massachusetts, USA
Luna® Universal qPCR Master Mix (M3003E)	New England Biolabs	Massachusetts, USA
Monarch® Total RNA Miniprep Kit (T2010S)	New England Biolabs	Massachusetts, USA

Thiobarbituric Acid Reactants (TBARS) Colorimetric Assay Kit (E-BC-K298-M)	Elabscience®	Wuhan. China

APPENDIX B

Table B: The weight of tissue sample being extracted, the concentration and purity of extracted RNA sample.

ID	Weight (mg)	Concentration (ng/µL)	260/280 ratio	260/230 ratio
Renal cortex				
Control				
1	27.4	1092.6	2.07	2.18
2	29.0	1586.3	2.04	2.07
3	28.1	1048.0	2.06	2.16
4	27.6	1157.1	2.05	2.16
5	28.7	968.1	2.06	2.14
6	28.2	1082.8	2.04	1.92
7	26.0	901.4	2.04	2.16
EGCG 250 mg/kg b.w.				
1	27.4	1117.2	2.05	2.19
2	26.8	961.8	2.05	2.04
3	28.8	868.7	2.05	2.15
4	29.0	1171.4	2.06	2.19
5	28.8	796.5	2.04	2.15
6	29.1	1253.2	2.06	2.20
7	28.1	1392.0	2.05	2.21
Renal medulla				
Control				
1	25.1	708.2	2.07	2.21
2	26.0	796.5	2.07	2.20
3	29.4	1188.5	2.06	2.17
4	29.4	847.8	2.06	2.22
5	28.9	1017.8	2.06	2.20
6	26.0	977.2	2.05	2.18
7	26.2	865.5	2.06	2.22
EGCG 250 mg/kg b.w.				
1	29.7	883.6	2.05	2.15
2	25.3	805.0	2.06	2.22
3	29.9	893.9	2.06	2.21
4	29.6	1048.3	2.05	2.11
5	28.8	856.8	2.05	2.23
6	28.3	944.3	2.05	2.22
7	27.0	766.0	2.06	2.21

APPENDIX C

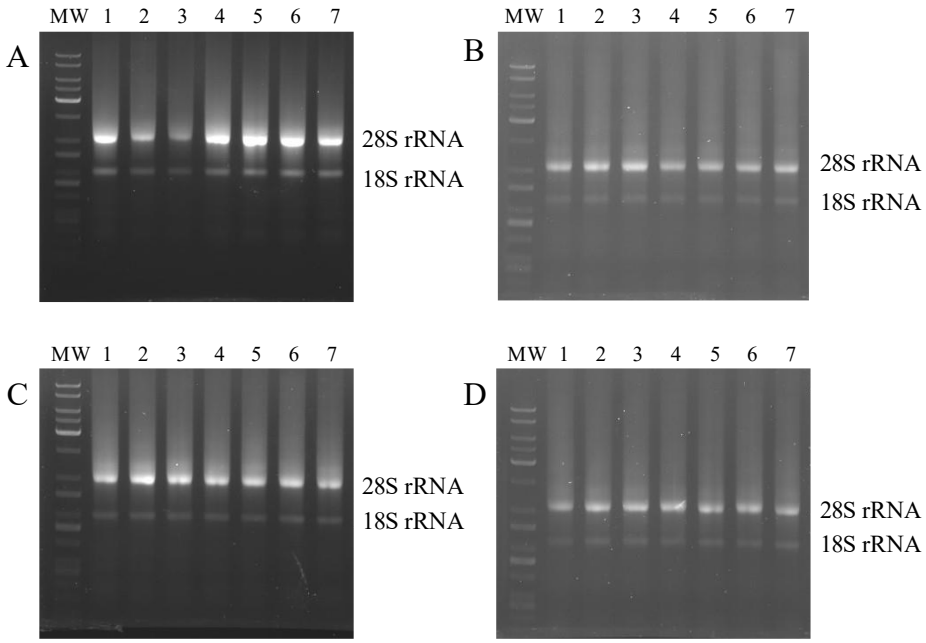
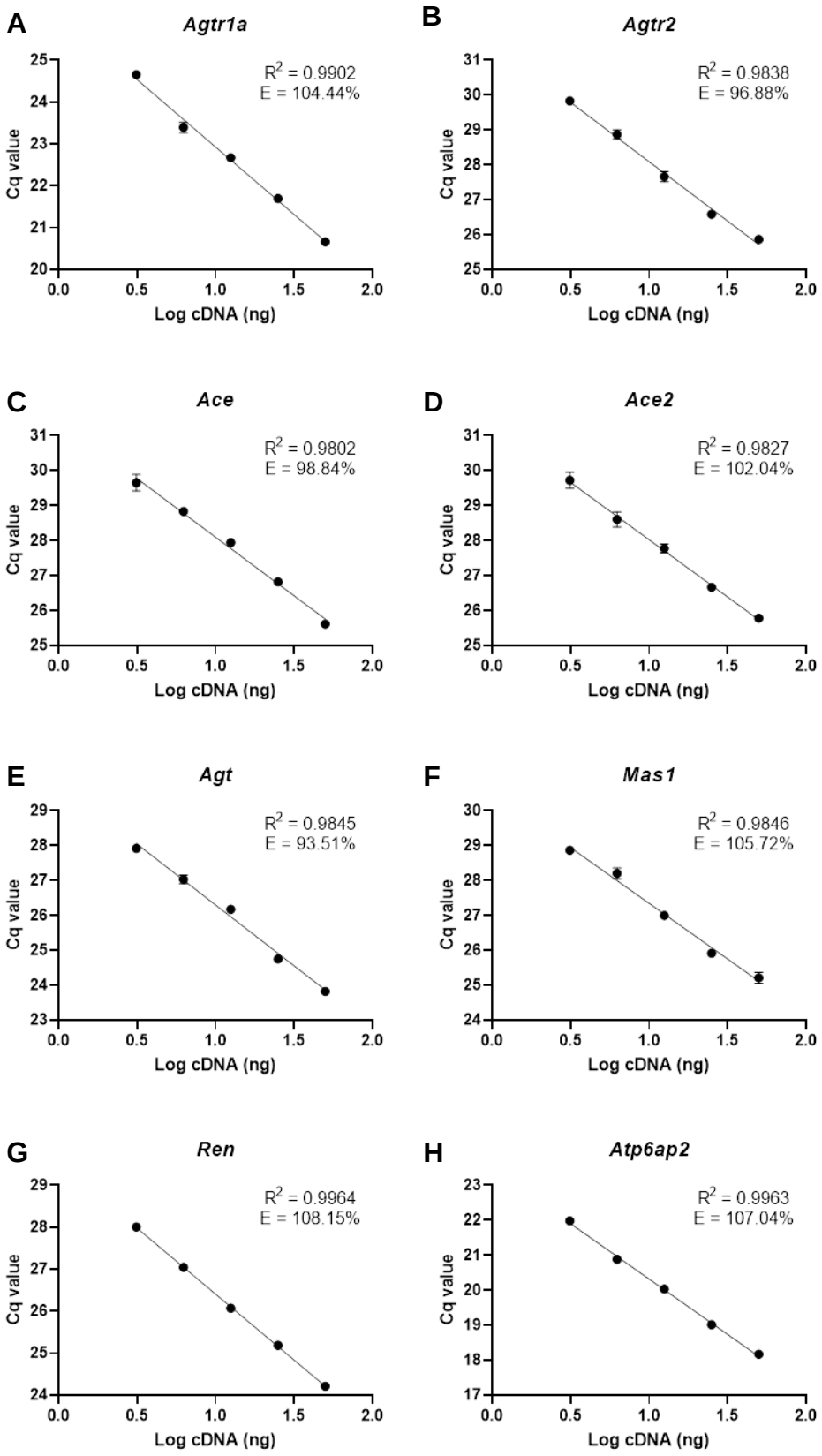


Figure C: The bleach gel image for renal cortex (A) control and (B) 250 mg/kg *b.w.* EGCG groups and renal medulla (C) control and 250 mg/kg *b.w.* EGCG groups. (Lane MW: molecular weight marker; Lane 1 to 7: samples)

APPENDIX D


A
Agtr1a
R2 = 0.9902
E = 104.44%
B
Agtr2
R2 = 0.9838
E = 96.88%
C
Ace
R2 = 0.9802
E = 98.84%
D
Ace2
R2 = 0.9827
E = 102.04%
E
Agt
R2 = 0.9845
E = 93.51%
F
Mas1
R2 = 0.9846
E = 105.72%
G
Ren
R2 = 0.9964
E = 108.15%
H
Atp6ap2
R2 = 0.9963
E = 107.04%
Cq value
Log cDNA (ng)

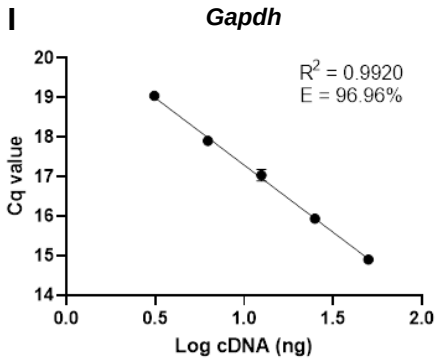


Figure D: Amplification efficiency of (A) *Agtr1a*, (B) *Agtr2*, (C) *Ace*, (D) *Ace2*, (E) *Agt*, (F) *Mas1*, (G) *Ren*, (H) *Atp6ap2*, and (I) *Gapdh* using the Luna® Universal qPCR Master Mix.

Table D: Primer efficiency and R2 value of reference and RAS-related genes.

Genes	Efficiency (%)	R^2
Agtr1a	104.44	0.9902
Agtr2	96.88	0.9838
Ace	93.51	0.9845
Ace2	98.84	0.9802
Agt	102.04	0.9827
Mas1	105.72	0.9846
Ren	108.15	0.9964
Atp6ap2	107.04	0.9936
Gapdh	96.96	0.9920

APPENDIX E

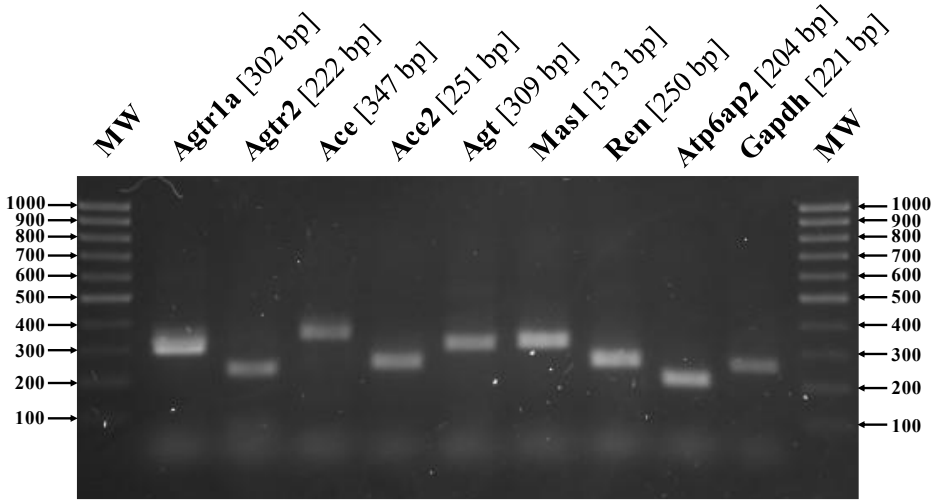
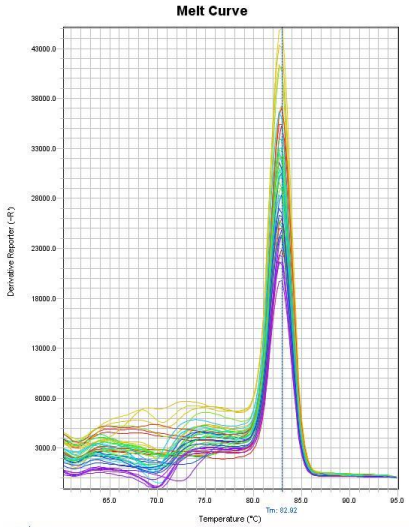
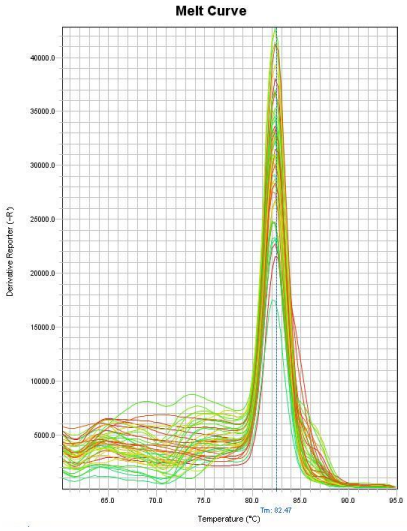


Figure E: Agarose gel image of PCR product for reference and RAS-related genes. The 2% (w/v) agarose gel was electrophoresis at 80 V for 40 min. The actual amplicon size was retrieved from previous publications and validated using Primer BLAST software.

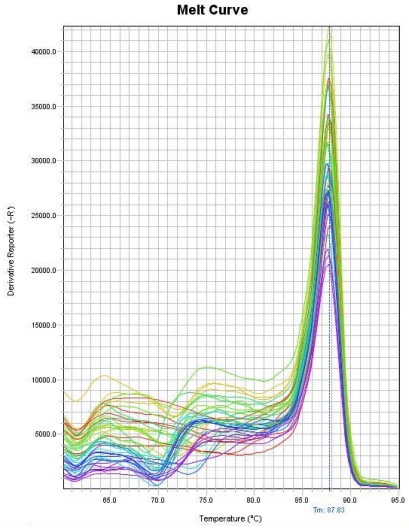
APPENDIX F



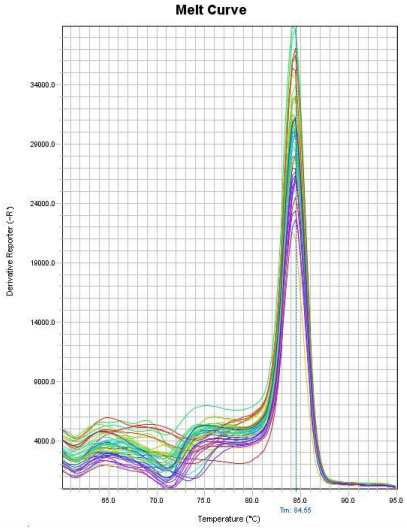
A. *Agtr1a* (Tm = 82.92°C) [302 bp]



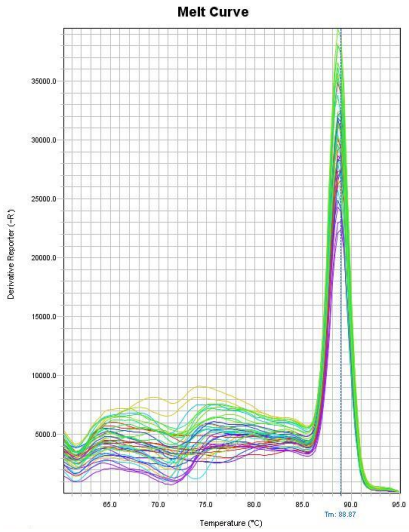
B. *Agtr2* (Tm = 86.16°C) [222 bp]



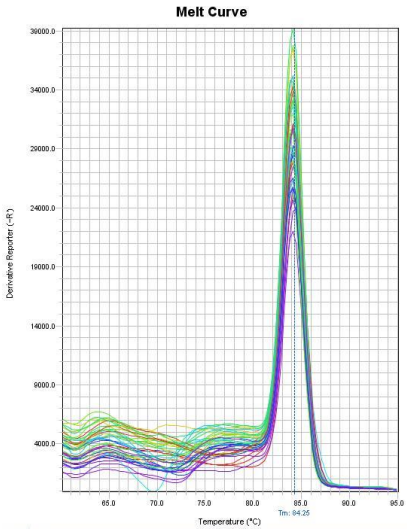
C. *Ace* (Tm = 87.83°C) [347 bp]



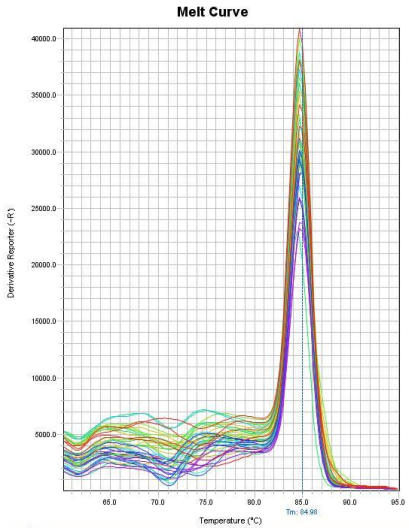
D. *Ace2* (Tm = 84.55°C) [251 bp]



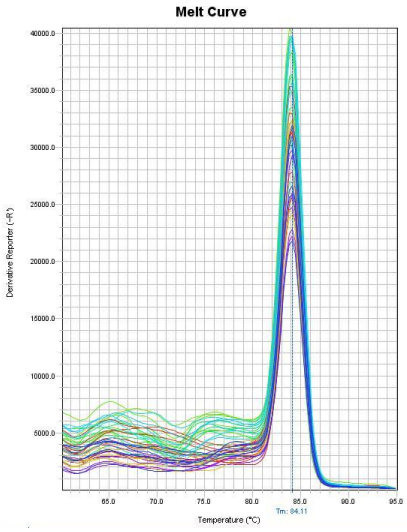
E. *Agt* (Tm = 88.87°C) [309 bp]



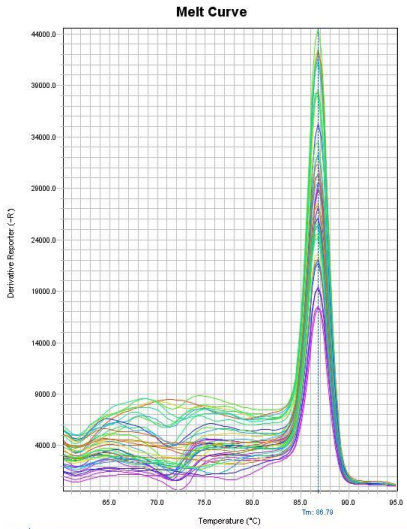
F. *Mas1* (Tm = 84.25°C) [313 bp]



G. *Ren* (Tm = 84.98°C) [250 bp]



H. *Atp6ap2* (Tm = 84.11°C) [204 bp]



I. *Gapdh* (Tm = 86.79°C) [221 bp]

Figure F: Melt curve analysis of (A) *Agtr1a*, (B) *Agtr2*, (C) *Ace*, (D) *Ace2*, (E) *Agt*, (F) *Mas1*, (G) *Ren*, (H) *Atp6ap2*, and (I) *Gapdh* amplicon.

APPENDIX G

Table G: Effects of EGCG on relative organ weights.

Organ index	**EGCG (mg/kg *b.w.*)**				
(g/100 g *b.w.*)	**0**	**50**	**250**	**500**	**1000**
Heart	0.41 ± 0.02	0.41 ± 0.03	0.45 ± 0.07	0.44 ± 0.03	0.43 ± 0.01
Lungs	0.46 ± 0.04	0.49 ± 0.04	0.46 ± 0.04	0.50 ± 0.04	0.52 ± 0.05
Liver	3.39 ± 0.35	3.56 ± 0.18	3.54 ± 0.17	3.511 ± 0.26	3.61 ± 0.08
Stomach	0.19 ± 0.01	0.18 ± 0.01	0.20 ± 0.01	0.20 ± 0.02	0.20 ± 0.01
Spleen	0.52 ± 0.10	0.49 ± 0.06	0.44 ± 0.03	0.49 ± 0.07	0.50 ± 0.08
Small intestine	1.74 ± 0.39	1.65 ± 0.26	1.40 ± 0.22	1.83 ± 0.14	1.62 ± 0.15
Large intestine	0.94 ± 0.12	0.88 ± 0.06	0.92 ± 0.16	0.99 ± 0.13	1.00 ± 0.14
Kidneys	0.78 ± 0.02	0.81 ± 0.04	0.78 ± 0.04	0.79 ± 0.03	0.81 ± 0.02

Relative organ weights were calculated through the normalization of organ weight to body weight. Data were expressed as mean ± SD.

APPENDIX H

Table H: Effects of EGCG on plasma ALT, AST, creatinine, urea and hepatic ROS, TBARS and caspsase-3 levels.

Parameters	EGCG (mg/kg *b.w.*)				
	0	**50**	**250**	**500**	**1000**
Plasma					
ALT (U/L)	17.80 ± 2.20	15.99 ± 5.53	16.11 ± 4.84	22.11 ± 5.76	25.93 ± 7.40*
AST (U/L)	31.25 ± 5.88	29.81 ± 6.07	34.17 ± 4.86	34.83 ± 6.73	44.50 ± 10.79*
Creatinine (umol/L)	49.62 ± 2.50	48.43 ± 1.69	48.48 ± 2.40	52.78 ± 4.03	49.62 ± 6.04
Urea (mmol/L)	9.24 ± 1.40	9.49 ± 1.24	9.40 ± 1.55	10.75 ± 1.57	8.64 ± 1.48
Liver					
ROS (fold of control)	1.00 ± 0.26	1.69 ± 0.65	1.08 ± 0.27	1.91 ± 1.21	1.24 ± 0.52
TBARS (nmol/mg protein)	6.94 ± 1.21	7.22 ± 0.64	7.28 ± 0.69	8.42 ± 1.34*	8.62 ± 0.77*
Caspase-3 (fold of control)	1.00 ± 0.18	1.00 ± 0.07	0.94 ± 0.11	1.01 ± 0.08	1.02 ± 0.12

ALT: alanine aminotransferase; AST: aspartate aminotransferase; ROS: reactive oxygen species; TBARS: thiobarbituric acid reactive substances. Data were expressed as mean ± SD. *$p < 0.05$ vs vehicle, Dunnett's test

APPENDIX I

Table I: Effects of EGCG on blood pressures.

Blood pressure parameters (mmHg)	EGCG (mg/kg *b.w.*)		*p*-value
	0	250	
Systolic blood pressure (SBP)			
D0	182 ± 11	181 ± 15	0.914
D7	188 ± 8	177 ± 3*	0.012
D14	193 ± 6	180 ± 5**	0.002
D21	196 ± 4$^{\#}$	179 ± 7***	0.000
D28	199 ± 8$^{\#\#}$	176 ± 7***	0.000
Diastolic blood pressure (DBP)			
D0	123 ± 15	124 ± 15	0.924
D7	129 ± 9	123 ± 4	0.158
D14	135 ± 9	124 ± 9	0.051
D21	140 ± 7$^{\#}$	122 ± 13*	0.011
D28	142 ± 7$^{\#\#}$	121 ± 6***	0.000
Mean blood pressure (MBP)			
D0	142 ± 13	143 ± 15	0.968
D7	148 ± 8	141 ± 3*	0.048
D14	154 ± 7	142 ± 7*	0.013
D21	159 ± 6$^{\#}$	140 ± 11**	0.004
D28	161 ± 7$^{\#\#}$	139 ± 7***	0.000

Data were expressed as mean ± SD. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs day 0; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs vehicle, two-way ANOVA followed by Dunnett's test.

APPENDIX J

Table J: Relative mRNA expression of RAS-related genes in renal cortex and medulla.

Relative mRNA expression of RAS-related genes	EGCG (mg/kg *b.w.*)		*p*-value
	0	250	
Renal Cortex			
Agtr1a	1.00 ± 0.15	0.95 ± 0.07	0.569
Agtr2	1.00 ± 0.10	2.42 ± 1.23*	0.010
Ace	1.00 ± 0.19	1.18 ± 0.32	0.297
Ace2	1.00 ± 0.22	1.29 ± 0.19*	0.032
Agt	1.00 ± 0.22	1.07 ± 0.24	0.675
Mas1	1.00 ± 0.30	0.94 ± 0.30	0.687
Ren	1.00 ± 0.20	0.85 ± 0.20	0.173
Atp6ap2	1.00 ± 0.18	1.12 ± 0.12	0.189
Renal Medulla			
Agtr1a	1.00 ± 0.28	0.95 ± 0.10	0.869
Agtr2	1.00 ± 0.54	1.90 ± 0.70*	0.045
Ace	1.00 ± 0.14	1.38 ± 0.20**	0.003
Ace2	1.00 ± 0.07	1.07 ± 0.14	0.272
Agt	1.00 ± 0.17	1.13 ± 0.19	0.254
Mas1	1.00 ± 0.51	2.01 ± 0.60*	0.019
Ren	1.00 ± 0.57	0.37 ± 0.20*	0.023
Atp6ap2	1.00 ± 0.14	0.89 ± 0.14	0.202

Data were normalized to the *Gapdh* gene expression and expressed as mean ± SD, n = 6. *$p < 0.05$, **$p < 0.01$ vs vehicle, independent t-test.

LIST OF PUBLICATIONS

Parn, K.W., Ling, W.C., Chin, J.H. and Lee, S.-K. Safety and Efficacy of Dietary Epigallocatechin Gallate Supplementation in Attenuating Hypertension via Its Modulatory Activities on the Intrarenal Renin-Angiotensin System in Spontaneously Hypertensive Rats. *Nutrients* 2022, 14(21), p.4605. http: doi.org/10.3990/nu14214605

LIST OF CONFRENCE PARTICIPATION AND PUBLICATION IN CONFERENCE PROCEEDING

Parn, K.W., Ling, W.C. and Lee, S., 2021. Subacute oral toxicity study of epigallocatechin-3-gallate in spontaneously hypertensive rats. Malaysian Society of Pharmacology and Physiology 34th Scientific Meeting 2021. 15 – 17 July 2021. [Oral Presentation] Abstract published in *Frontiers in Pharmacology*. https://www.doi.org/10.3389/978-2-88971-008-9.

Parn, K.W., Ling, W.C. and Lee, S., 2021. High-Dose Subacute Oral Epigallocatechin-3-gallate Induces Hepatotoxicity in Spontaneously Hypertensive Rats. The 32nd Great Wall International Congress of Cardiology. 27 – 31 October 2021. [Poster Presentation]

Parn, K.W., Ling, W.C. and Lee, S., 2021. EGCG mediates its antihypertensive effects via upregulation of angiotensin type II receptor in a primary hypertension rodent model. 3rd Biennial Medical and Health Science International Conference. 22 – 26 November 2021. [Poster Presentation] Abstract published in *The Malaysian Journal of Pathology*, 44 (1), pp. 133 – 162.