EVALUATION OF BENEFICIAL EFFECTS OF VITAMIN. C AND GREEN TEA POLYPHENOL ANTIOXIDANT IN NORMOTENSIVE AND HYPERTENSIVE RATS WITH OR WITHOUT RENAL FAILURE

MOHAMED IBRAHIM A. ALI LAZHARI

UNIVERSITI SAINS MALAYSIA

2016

EVALUATION OF BENEFICIAL EFFECTS OF VITAMIN C AND GREEN TEA POLYPHENOL ANTIOXIDANT IN NORMOTENSIVE AND HYPERTENSIVE RATS WITH OR WITHOUT RENAL FAILURE

by

MOHAMED IBRAHIM A. ALI LAZHARI

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

December 2016

ACKNOWLEDGEMENT

First of all I would like to prostrate and praise ALLAH for providing me immense strength and courage to accomplish this study.

It is my great pleasure to thank my supervisor Professor Dr. Munavvar Zubaid bin Abdul Sattar for the encouragement and support throughout my study. Indeed, it would have been impossible to complete my research without his constant support and help. His knowledge and logical way of thinking have been of great value for me. I deeply thank him for tremendous amount of patience towards me. I will never forget his kind and humble behavior in all difficult situations in my personal life. He is like a big brother to me and I have immense respect for him.

I am also very thankful to my field supervisor Professor Nor Azizan Abdullah and Co-supervisor Dr. Hassaan Anwer Rathore, for their valuable advice throughout this study.

I would like to extend my gratitude to Professor Emeritus Dr. Edward James Johns, Head of Department of Physiology, University College Cork, Ireland for his great advice and comments.

My special thank go to Dr. Md. Abdul Hye Khan, an academic staff in Medical College of Wisconsin, United State of America for his massive encouragement and cooperation.

Great acknowledgment are also extended to all my wonderful friends and Colleagues in cardiovascular and renal Physiology as well as hypertension and cardiovascular research Lab, Ashfaq Ahmed, Safia Akhtar, Yen Pei, Tan Yong Chia,

Oh Hui Jin, Joo li Khoo, Zaid Ibrahim, Dr. Sheryar Afzal, Dr. Fiaz Ud din Ahmed and Dr. Mohamed Al Hadi for being there to offer me help and support throughout my study in a very nice way.

I highly appreciate and thank all the technical and laboratory staff of School of Pharmaceutical Sciences, including Dr. Isma, Ms. Melawati and Mr. Yusuf from animal research and service centre (ARASC) for their kind advice and valued assistance. Also, I would like to thank lab assistant Mr. Rusli, Mr. Farid, Mr. Nizam and all the administrative staff for providing the facilities.

Last but not the least, thousands of thanks to my wife and kids who always pray for my success and supported me. My highest gratitude goes to my beloved parents and family in Libya for their exceptional love and support which contributed a huge role in my success.

TABLE OF CONTENTS

CHAPTER 3 RESULTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

AKI	Acute kidney injury
Ang II	Angiotensin II
ARF	Acute renal failure
ATN	Acute tubular necrosis
CKD	Chronic kidney disease
COX	Cyclooxygenase
CrCl	Creatinine Clearance
CVD	Cardiovascular diseases
DBP	Diastolic Blood Pressure
EC	Epicatechin
ECG	Epicatechin-3-gallate
EDRF	Endothelium-derived relaxing factor
EGC	Epigallocatechin
EGCG	Epigallocatechin-3-gallate
GC	Gallocatechin
GFR	Glomerular filtration rate
GSH	Glutathione
GT	Green tea
GTPP	Green tea polyphenol
H & E	Hematoxylin and eosin
HD	High dose
HR	Heart rate
LD	Low dose
FE_{Na}	Fractional sodium

FE_K	Fractional potassium
LDL	Low-density lipoprotein
MAP	Mean Artery Blood Pressure
MDA	Malondialdehyde
NADPH	Nicotinamide adenine dinucleotide
NIBP	Non-Invasive Blood Pressure
NO	Nitric oxide
OS	Oxidative stress
PAS	Periodic-Schiff (PAS)
PCT	Proximal convoluted tubules
PGI_2	Prostacyclin
K^+	Potassium
PP	Pulse Pressure
PWV	Pulse wave velocity
RAAS	Renin angiotensin aldosterone system
RCBP	Renal Cortical Blood Perfusion
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Na^+	Sodium
SBP	Systolic Blood Pressure
SHR	Spontaneously hypertensive rat
SOD	Superoxide dismutase
TNF	Tumor necrosis factor
UFR	Urine Flow Rate
VIT. C	Vitamin C

VSMC	Vascular smooth muscle cell
VPR	Volume pressure recorded
WKY	Wistar Kyoto rats

PENILAIAN KESAN MANFAAT VITAMIN C DAN TEH HIJAU POLIFENOL ANTIOKSIDAN DALAM TIKUS NORMOTENSIF DAN HIPERTENSI DENGAN ATAU TANPA KEGAGALAN GINJAL

ABSTRAK

Tekanan oksidatif ialah salah satu daripada factor-faktor di dalam patogenesis hipertensif dengan atau tanpa kegagalan ginjal (RF). Vitamin C (VIT. C) dan teh hijau polifenol merupakan vitamin C dan GTPP agen dietik yang mempunyai tindakan-tindakan berfaedah yang berlainan di dalam badan manusia. Kajian ini bertujuan untuk menilai faedah VIT. C dan GTPP di dalam tikus normotensif dan hipertensif dengan atau tanpa kegagalan ginjal. Kegagalan ginjal telah dicetuskan di dalam tikus normotensive dan hipertensif dengan suntikan cisplatin dos tunggal secara intraperitoneal pada hari pertama di dalam kajian ini (n=6 jantan). Rawatan dengan Vitamin C telah dilakukan pada dua dos iaitu dos rendah (VIT. C-LD; 500mg/day, secara oral) dan dos tinggi VIT. C (VIT. C-HD 1000mg/day, secara oral). Tatkala, dos rendah GTPP (GTPP-LD; 500mg/kg, secara oral) dan dos tinggi GTPP (GTPP-HD; 1000mg/kg, secara oral) telah dimulakan dari hari ke-7 sehingga hari ke-28 eksperimen. Tekanan darah bukan invasif (NIBP) telah diukur hanya pada hari ke-0 dan kajian metabolik telah dijalankan pada hari ke-0, 7, dan 28. Haiwan telah telah dianestetik untuk mengukur tekanan darah, hemodinamik ginjal, penanda oksidatif dan antioksidan di dalam plasma pada hari ke-29. Induksi kegagalan ginjal telah disahkan melalui asai biokimia untuk parameter fungi ginjal dan telah disahkan lagi dengan pemeriksaan histopatologi dengan menggunakan asai hematoxylin dan eosin (H & E). Asai kas protein telah dijalankan dengan menggunakan reagen asid berkala kerja giliran (PAS). Satu nilai kebarangkalian kurang daripada 5 % telah

diset untuk mempertimbangkan perbezaan signifikan parameter sistemik hemodinamik seperti tekanan darah sistolik (SBP), tekanan darah diastolic (DBP) dan min tekanan arteri (MAP) yang telah menunjukkan bahawa SHR+RF+VIT. C-HD, SHR+RF+GTPP-LD dan SHR+RF+GTPP-HD telah berkurang secara signifikan (semua $P<0.05$) apabila dibandingkan dengan SHR. Min tekanan arteri VIT. C-HD-RF telah berkurang secara signifikan (semua $P<0.05$) apabila dibandingkan dengan WKY-NRF dan VIT. C-HD-NRF. Data hemodinamik sistemik telah menunjukkan bahawa VIT. C-HD, GTPP-LD dan GTPP-HD mempunyai kesan mengurangkan tekanan darah yang signifikan di dalam kumpulan kegagalan ginjal SHR. Tambahan pula, aruhan kegagalan ginjal oleh cisplatin telah menunjukkan peningkatan tekanan oksidatif dan kekejangan arteri serta pengurangan perfusi darah kortikal ginjal yang jelas. Rawatan dengan VIT.C dan GTPP telah meningkatkan status antioksidan di dalam plasma, mengurangkan kekejangan arteri dan meningkatkan perfusi darah kortikal ginjal secara signifikan yang mungkin dengan meningkatkan kepekatan nitrik oksida dalam plasma di dalam kedua-dua modal normotensif dan hipertensif yang mengalami kegagalan ginjal apabila dibandingkan dengan kumpulan WKY-RF dan SHR-RF. Fungsi perkumuhan ginjal dan kreatinin di dalam plasma modal-modal kegagalan ginjal normotensif dan hipertensif telah meningkat secara signifikan (semua $P<0.05$) apabila dibandingkan dengan kumpulan-kumpulan tersebut yang tidak mengalami kegagalan ginjal. Rawatan dengan dos VIT. C and GTPP yang berlainan dalam modal-modal normotensif dan hipertensif yang mengalami kegagalan ginjal telah terbalikkan kerosakan yang disebabkan oleh cisplatin dan pulihkan keupayaan penyerapan semula ginjal yang telah dipamerkan melalui pengurangan fungsi perkumuhan ginjal, pengurangan tahap kreatinin dalam plasma dan peningkatan nitrik oksida. Pemulihan fungsi perkumuhan dan penyerapan ginjal dengan rawatan VIT. C dan GTPP telah disahkan melalui

histopatologi ginjal dengan menggunakan reagen hematoxylin dan eosin (H&E) dan asid berkala kerja giliran (PAS). Pendapatan ini mencadangkan secara kolektif bahawa potensi antioksidan oleh VIT. C and GTPP merupakan pengantara vascular dan kesan perlindungan ginjal melalui peningkatan tahap NO di dalam modal normotensive dan hipertensif yang mengalami kegagalan ginjal.

EVALUATION OF BENEFICIAL EFFECTS OF VITAMIN C AND GREEN TEA POLYPHENOL ANTIOXIDANT IN NORMOTENSIVE AND HYPERTENSIVE RATS WITH OR WITHOUT RENAL FAILURE

ABSTRACT

Oxidative stress is one of the factors in the pathogenesis of hypertension with or without renal failure (RF). Vitamin C (VIT. C) and green tea polyphenol (GTPP) are the VIT. C and GTPP dietary agents which have different beneficial actions in human body. The present study aimed to evaluate the beneficial of VIT. C and GTPP in normotensive rats and hypertensive rats with or without renal failure. Renal failure was induced in normotensive and hypertensive rats by a single dose of intraperitoneal injection of cisplatin on day 1 in this study (n=6 males). Treatment with VIT. C was done at both low doses VIT. C-LD; 500mg/day, orally), and VIT. C high dose (VIT. C-HD 1000mg/day, orally). While GTPP low dose (GTPP-LD; 500mg/kg, orally) and GTPP high dose (GTPP-HD; 1000mg/kg, orally) were started from day 7 until day 28 of the experiment. Non-invasive blood pressure (NIBP) was measured only on day 0 and metabolic studies were performed on days 0, 7, and 28. Animals were anaesthetized to measure blood pressure, renal hemodynamics, plasma oxidative and antioxidant marker on day 29. Induction of renal failure in both models was confirmed by biochemical assays for renal functional parameters and further confirmed by histopathological examinations using hematoxylin and eosin assay (H&E). Protein cast assays were carried out by using periodic acid-Schiff (PAS) reagents. A probability value of less than 5% was set to consider significant differences systemic hemodynamic parameters like systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial pressure (MAP) have shown that

SHR+RF+VIT. C-HD, SHR+RF+GTPP-LD and SHR+RF+GTPP-HD are significantly reduced (all $P<0.05$) when compared to SHR. VIT. C-HD-RF significantly reduced (all $P<0.05$) mean arterial pressure (MAP) when compared to that of WKY-NRF and (VIT. C-HD-NRF. Systemic hemodynamic data showed that VIT. C-HD, GTPP-LD and GTPP-HD have significant blood pressure lowering effects in renal failure groups of SHRs. Moreover, cisplatin-induced renal failure rats exhibited marked increase in oxidative stress, increased arterial stiffness and reduced renal cortical blood perfusion. Treatment with vitamin C (VIT. C) and green tea polyphenol (GTPP) significantly increased (all $P<0.05$) the antioxidant status in plasma, reduced the arterial stiffness and increased the renal cortical blood perfusion possibly by increasing plasma nitric oxide (NO) concentration of both normotensive and hypertensive models of renal failure when compared to WKY-RF and SHR-RF group. Renal excretory function and creatinine in the plasma of normotensive and hypertensive models of renal failure were significantly increased (all $P<0.05$) compared to their non-renal failure groups. Treatment of normotensive and hypertensive models of renal failure with different doses of VIT. C and GTPP reversed the damage induced by cisplatin and restored the reabsorption ability of the kidney as exhibited by a reduction in the excretory function of the kidney, reduction in of creatinine levels in the plasma and elevation of nitric oxide The amelioration of renal excretory function and reabsorption due to VIT. C and GTPP treatment were confirmed by histopathology of the kidney using hematoxylin and exotoxin (H&E) and Periodic acid-Schiff (PAS) staining. These findings collectively suggest that antioxidant potential of VIT. C and GTPP mediates the vascular and renoprotective effects through increased NO level in normotensive and hypertensive model of renal failure.

CHAPTER 1

INTRODUCTION

1.1 The kidneys

Kidneys are one of the major organs of primary importance in the urinary as well as the cardiovascular system. They not only excrete metabolic wastes from the body but also maintain homeostasis. Kidneys selectively excrete these waste products from the plasma and sequentially reabsorb some important metabolic products during various steps of reabsorption, tubular secretion and urinary excretion. Kidneys act as endocrine glands by releasing renin, which ultimately regulates blood pressure. It is reported that persistent elevated blood pressure is due to improper function of the kidneys (Sattar & Johns, 1994; Applegate, 2000; Schrier et al., 2004).

A pair of kidneys is present on each side of the vertebral column on the 12^{th} thoracic and 3^{rd} lumbar vertebrae. The appearance of the kidney is bean shaped with an indent on the middle part, called the hilum. A cross section of the kidney shows that it is made up of cortex and medulla. Both are considered as functional tissues of the kidney (Applegate, 2000).

The nephron is known as the functional unit of kidney. Each kidney contains about 12 million nephrons. A nephron comprises of two major parts, the renal corpuscle which is a rich network of capillaries called the glomerulus and renal tubules which extend into renal corpuscle. Renal tubules are in connection with

glomerulus that carries fluid and some waste product through proximal convoluted tubules, loop of Henle, and distal convoluted tubules to bladder (Applegate, 2000).

1.1.1 The glomerulus

The glomerulus comprises of a rich network of capillaries and a balloon like structure known as Bowman's capsule. Each glomerulus carries afferent arterioles that carry the blood towards glomerulus and efferent arterioles which carry blood away from glomerulus. Glomerulus maintains a pressure gradient which is responsible for filtration of different substances like creatinine and albumin. Amount of fluid which is filtered through glomerulus in one minute is known as glomerular filtration rate (GFR) and its normal value is 125ml/min. This is mostly 20 % of renal plasma flow (Applegate, 2000a).

1.1.2 Proximal convoluted tubules

Proximal convoluted tubule (PCT) is the first part of renal tubules and is responsible for reabsorption of 60-70 % of electrolytes like sodium and potassium. PCT consists of a convoluted and a straight part (Abdel-Raheem et al., 2010). From the PCT, fluid flows to the loop of Henle which descends down to the medulla. Each loop of Henle consists of an ascending and a descending part. The descending loop of Henle and lower part of the ascending region has thin walls and is therefore called the thin segment of loop of Henle, while the upper part of the ascending loop of Henle has thick walls and is therefore named as thick segment of the ascending limb (Figure 1). At the end of this ascending limb is a plaque like structure called macula densa which controls the function of the nephrons. Beyond macula densa, the fluid enters into the distal tubules which lie in the renal cortex like the PCT. The distal

tubules lead to the collecting tubules, which collectively lead to the formation of the collecting duct (Guyton, 1996).

1.2 Renal hemodynamics

The total blood flow to the kidneys in an adult of 70 kg is approximately 1100 mL/min. Although the weight of both kidneys is less than 1% of the total body weight, the kidneys receive 20-25% of total cardiac output (5 L/min) (Guyton, 1996; Applegate, 2000). The micro vessels in the kidneys have the ability to auto-regulate the renal plasma flow. Altered distribution of plasma in kidney may cause changes in renal function. Therefore, for normal kidney function, the uniform distribution of renal plasma flow is very important (Regan et al., 1995).

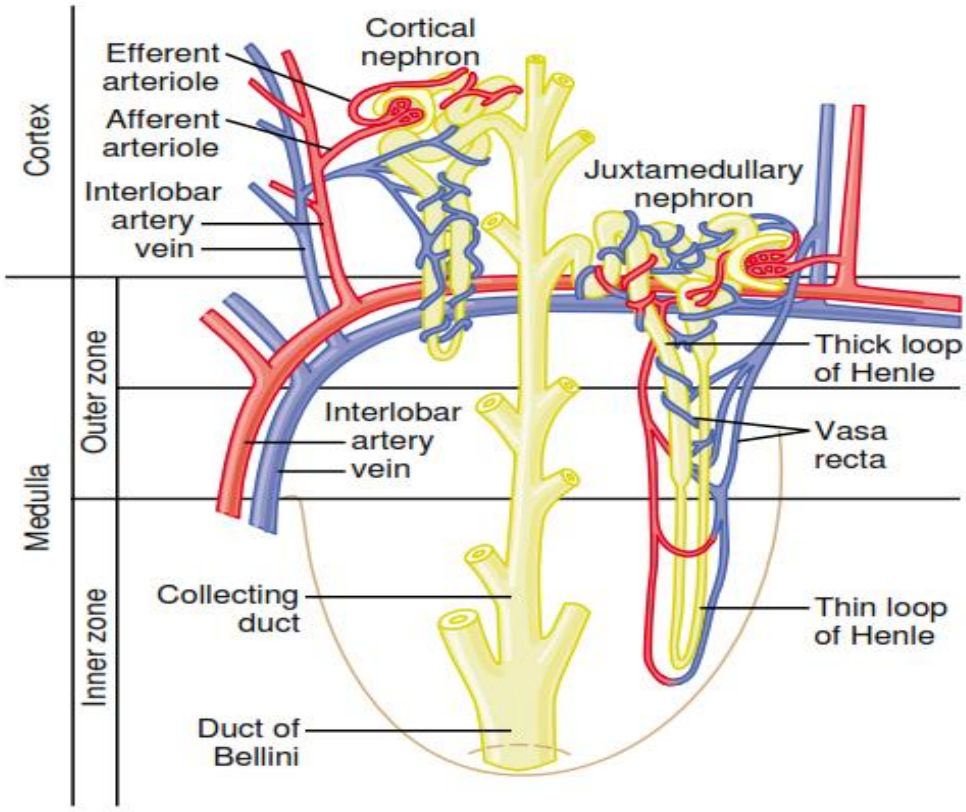

Figure 1.1 Schematic diagrams showing the relationship between blood vessels renal tubules (adapted from (Hall, 2010)

1.2.1 Factors influencing renal hemodynamics

Factors that influence renal hemodynamic are divided into intrinsic and extrinsic factors. Intrinsic factors include auto regulation mechanism, renal renin angiotensin system, eicosanoid and kidney. Extrinsic pathway includes sympathetic nervous system, angiotensin II, vasopressin, dopamine and histamine. Besides these intrinsic and extrinsic factors, there are also roles for endothelial nitric oxide (NO) and atrial natriuretic peptides (Unwin, 2000).

1.2.2 Regulation of vascular tone: Role of endothelium

Endothelium is a very thin, monolayer of endothelial cells; it is the innermost lining of blood and lymphatic vessels. Endothelial cells play a major role in the control of vascular tone and vascular homeostasis and thus control blood flow by releasing its potent relaxing and contracting factors. Important vascular functions that involve endothelial cells include: vasculogenesis, angiogenesis and arteriogenesis, inflammation, regulation of blood pressure and apoptosis. Due to its strategic position at the blood/tissue interface, it lies in direct contact with various circulating factors such as antioxidants, oxidized low-density lipoprotein (LDL), and also pro-inflammatory cytokines like the tumour necrosis factor (TNF) or interleukins (IL) (Cines et al., 1998; Brown & Hu, 2001). Therefore, endothelial dysfunction may be a very important hallmark in the pathogenesis of thrombosis and cardiovascular diseases (CVD) such as atherosclerosis and hypertension and may thereby lead to many damaging processes to vascular cells and the surrounding tissue (Matz & Andriantsitohaina, 2003).

Endothelial cells also play a major role in the regulation of vascular tone by releasing vasoconstrictor agents such as endothelin-1 (ET-1), angiotensin II (Ang II) and thromboxane A_2 (TXA_2) (Moncada & Vane, 1978; Furchgott & Zawadzki, 1980; Ignarro et al., 1987; Feletou & Vanhoutte, 1988; Luescher & Barton, 1997) and a very potent vasodilator agent, nitric oxide (NO) which also acts as a free radical scavenger and prostacyclin (PGI_2). Both NO and PGI_2 exert vasodilator effect on blood vessels and inhibit platelet activation. Since NO is released from the endothelium, it is also known as endothelium-derived relaxing factor (EDRF). NO is biosynthesized from L-arginine by endothelial NO synthase (eNOS) enzyme. It can diffuse freely across vascular smooth muscle cells. It exerts its vasorelaxation

properties via various pathways including activation of guanylate cyclase enzyme, which further leads to the production of cyclic guanosine monophosphate (cGMP).. Furthermore, prostacyclin exerts its vasodilator action via (cyclooxygenase (COX), also known as prostaglandin H synthase (PGHS) or prostaglandin endoperoxide synthase pathway. It is mainly mediated by prostacyclin receptors and intracellular peroxisome proliferator-activated receptors (PPAR) beta (Lüscher & Vanhoutte, 1986).

1.3 Indices for arterial stiffness

Cardiovascular diseases have been considered as the leading cause of morbidity and mortality in developing countries and cannot be predicted by classical predictors alone (Laurent et al., 2001). An increase in arterial stiffness may elevate systolic blood pressure (SBP) which ultimately raises the after load. This raised after load results in a reduction in the diastolic blood pressure which will modify the coronary perfusion (Koen et al., 2011; Safar, 1989).

Many indices have been introduced to estimate the stiffness of the arteries. In 1994, the International Society of Hypertension in its meeting decided to postulate an interim measurement. None of the method was found to be superior over the other and some challenges in measurement and interpretation were faced (O'Rourke et al., 2002). In this study pulse wave velocity (PWV) was used as an index for estimation of arterial stiffness. Measurement of oxidative stress parameters can give an idea about endothelial dysfunction.

1.3.1 Pulse wave velocity

PWV is taken as a marker of arterial stiffness (Sutton-Tyrrell et al., 2005). PWV can be defined as the distance travelled (Δx) by the pressure wave divided by the time (Δt) taken for the wave to travel that distance.

$$PWV = \Delta x / \Delta t$$

PWV can be measured between two sites of an artery, at a known distance apart using "foot to foot" technique of the waveform to calculate the transit time. As shown in Figure 1.2 PWV measurement requires only two pressure wave forms recorded simultaneously with invasive catheters, or mechanical tonometers applied non-invasively to the pulse across the skin. It is considered as a marker of arterial stiffness (Wilkinson, et al., 1998; Nichols, 2005). There is a strong correlation between PWV and cardiovascular events and both cause mortality (Blacher et al., 1999; Cruickshank et al., 2002; Laurent et al., 2001).

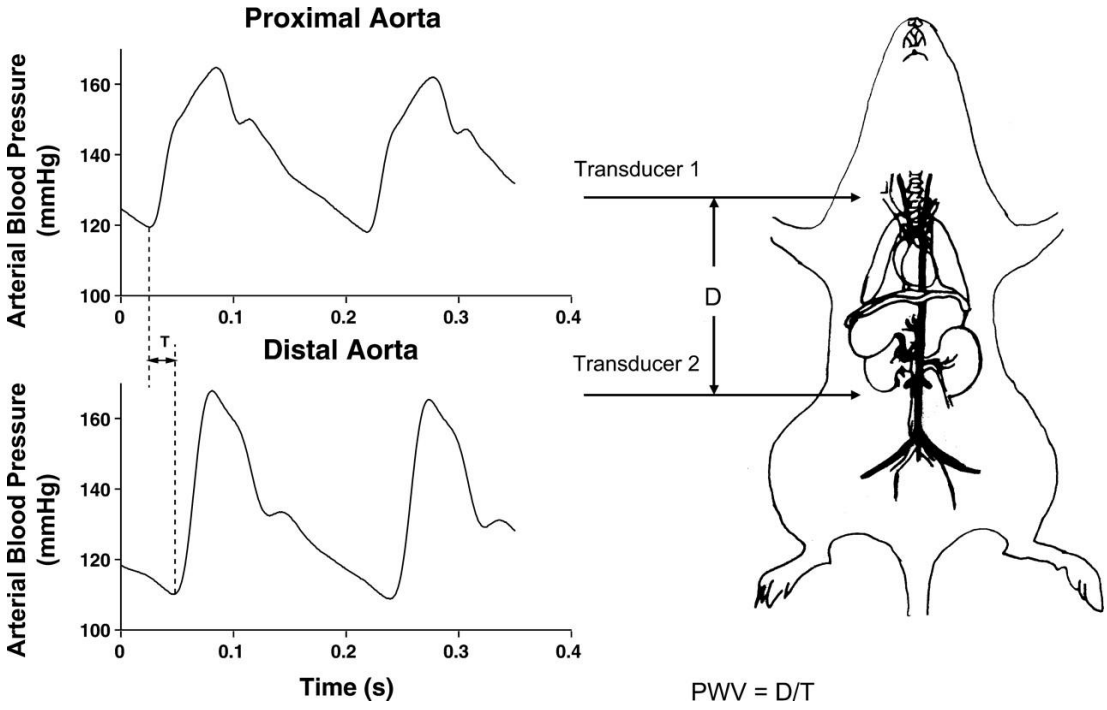

Figure 1.2: Schematic illustration of the measurement of pulse wave velocity (PWV) in the rat. PWV is the ratio of the distance (*D*) between the tips of the 2 catheters and the difference between the time at the minimal values of proximal and distal blood pressure. Adapted from (Cosson et al., 2007)

1.3.2 Mechanisms of arterial stiffness

Arterial stiffening or hardening in renal disease involves several mechanisms. It occurs as a result of many independent and inter-dependent factors. One of the factors which influence greatly in the stiffness of vascular wall is the imbalance between collagen and elastin (Zieman et al., 2005).

Elastin is an important protein responsible for vascular flexibility, however in state of inflammation within the body that leads to increased degradation of collagen and elastin (Park & Lakatta, 2012).

Renal failure model is a well-established model of reactive oxygen species, and it has been reported that increased oxidative stress also plays a role in arterial

elasticity. Oxidative injury may lead to vascular inflammation and increase cell cellular proliferation, which may subsequently lead to impaired arterial elasticity (Csiszar et al., 2002; Park & Lakatta, 2012).

Arterial stiffening in renal disease is also driven by accumulation of calcium crystals (arterial calcification) within the internal wall of artery (Bazan et al., 2007).

It has been also reported that several genes and molecules are associated with vascular wall stiffening (Laurent et al., 2005). Structural and cellular components take part in development of arterial stiffness as shown in Figure 1.3

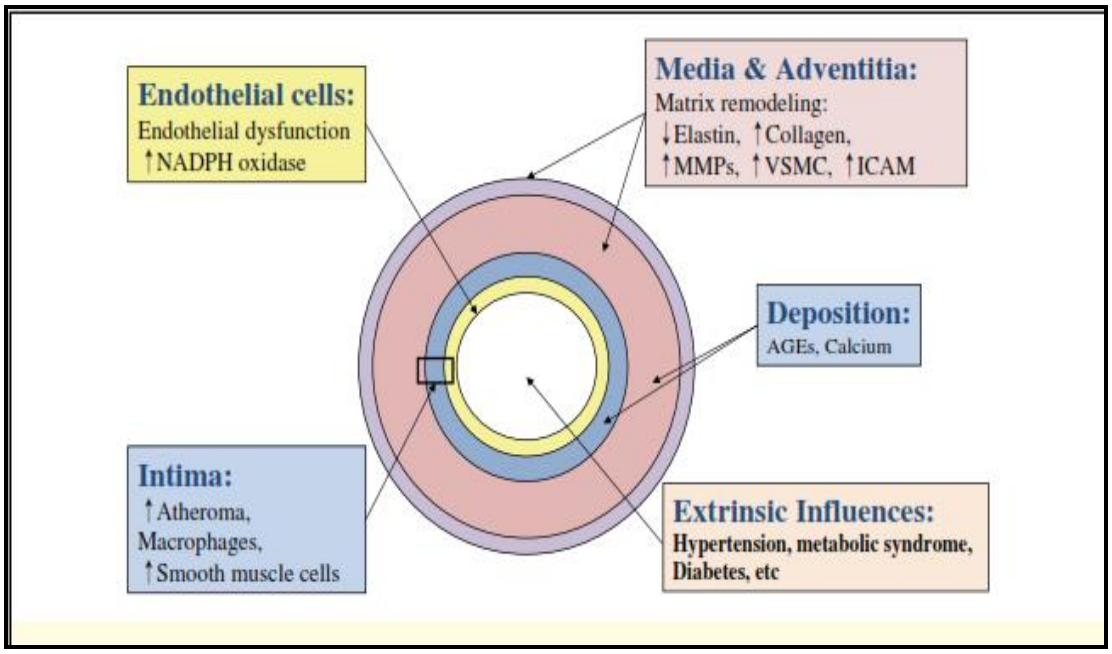

Figure 1.3: Summary of the causes of arterial aging adapted from (Lee & Oh, 2010)

Vascular changes have been affected by hemodynamic forces (Wolinsky & Glagov, 1969) and some extrinsic factors like hormones, salts and glucose metabolism and regulations. Some pathological states like hypertension, diabetes, metabolic disorders or simply aging can cause vascular changes which increase the arterial stiffness by different mechanisms.

The stability and compliance of blood vessels depends upon the functional contribution and stability of its two components, elastin and collagen. The concentration of both components determines the functional capacity of blood vessels. Any turnover in the concentration of both may shift blood vessel and lead to not only poor conduction of blood, but also many pathological states. Deregulation of this balance due to involvement of inflammatory mediators leads to over production of collagen and reduces the production of elastin leading to increased arterial stiffness (Johnson et al., 2001).

Hypertension also increases the collagen production (Xu et al., 2000) which will increase the stiffness. The intima of stiffened vessels show disoriented endothelial cells. Elastin is broken due to collagen deposition and infiltration of vascular smooth muscle cells (VSMC), mononuclear cells and macrophages. Cytokine levels, tumor growth factor (TGF)-β, intracellular cell adhesion molecules and matrix metalloproteinase are increased (Lakatta & Levy, 2003). Therefore, inflammatory mediators are also responsible for increased arterial stiffness. Therapeutic agents which maintain the balance between elastin and collagen and inhibit these inflammatory mediators can be used in the treatment of arterial stiffness.

1.3.3 Factors influencing arterial stiffness in renal insufficiency

Arterial stiffness is increased in chronic cases of renal insufficiency. Increased arterial stiffness in renal insufficiency is due to involvement of several mechanisms, mainly extra pressure on intima wall, hypertension, and thickening of intima wall of blood vessels. Due to the activation of local and systemic renin angiotensin aldosterone system (RAAS), there is increased production of extracellular matrix metalloproteinase and VSMC proliferation. Arterial stiffness

in renal diseases is also initiated by diffused calcification without the involvement of inflammation and this histological picture is different from calcification in atherosclerotic plaque (Abedin et al., 2004; Goldsmith et al., 2004).

1.4 Oxidative stress (OS)

In the human body both oxidants and antioxidants are present and they mostly control the activities of each other. Compared to antioxidants, oxidative stress exists when oxidants are increased in our body. The net result is the increase in free radicals which ultimately cause damage to different organs of the body. This damage may play a significant role in the pathogenesis of different diseases. Oxidative stress plays a role in a number of diseases, like atherosclerosis, diabetes, inflammation and ischemia reperfusion injury (Griendling & Fitz Gerald, 2003; LeRoith et al., 2004).

1.4.1 Reactive oxygen species

Reactive oxygen species (ROS) are generally produced during normal cellular function. ROS includes hydroxyl radicals, superoxide anion, hydrogen peroxide and nitric oxide. Due to their high chemical reactivity, they lead to lipid peroxidation and oxidation of DNA and proteins of the cell. Under normal conditions, cellular antioxidant systems of the cell minimize the damage caused by ROS, however oxidative stress starts to develop when the level of ROS generation exceeds the level of cellular antioxidants (Valko et al., 2005).

1.4.2 Reactive nitrogen species

Reactive nitrogen species (RNS) are reactive metabolites of nitrogen (radical and non-radical). The most biologically reactive and important RNS are nitric oxide ($NO^{\bullet}$), peroxynitrite ($ONOO^{-}$) and nitrogen dioxide ($NOO^{\bullet}$). $NO^{\bullet}$ is a small molecule,

an important oxidative biological signalling molecule with one unpaired electron generated in biological tissues *via* nitric oxide synthases (NOSs). It has many vital physiological properties including neurotransmission, blood pressure regulation, defence mechanisms, smooth muscle relaxation and immune regulation (Bergendi et al., 1999). When NO reacts with superoxide anion, it produces a potent oxidizing agent known as peroxynitrite anion, which plays a role in lipid oxidation and DNA fragmentation (Carr et al., 2000).

1.5 Antioxidants

Antioxidants prevent cell damage by inhibiting the oxidation of other molecules. They scavenge the free radicals which are highly reactive species responsible for cell damage and possessing major chance of various cardiovascular diseases. Multiple mechanisms are involved in the defence system of antioxidants. For example, superoxide dismutase scavenges superoxide and converts it to less reactive species. In patients with amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, a defective gene for superoxide dismutase is found. This throws light on the existence of superoxide dismutase in biological systems, as well as other antioxidant enzymes like catalase and glutathione peroxidase, which suggests that oxidative damage can lead to cellular damage. Furthermore, apart from major antioxidant enzymes, several other small-molecule antioxidants (basically lipid-soluble and water-soluble antioxidants) also play important roles in antioxidant defence systems. The lipid-soluble antioxidants are localized to cellular membranes and lipoproteins, whereas the water-soluble antioxidants are present in aqueous fluids, such as blood and the fluids within cells and surrounding them. They are particularly important in blood and the fluids present in the extracellular space and generally become active in times when there is less availability of antioxidant

enzymes or when there is a total absence. A potential antioxidant should have the following capabilities: (i) Must be able to react with biologically active oxygen species (ii) the resultant pro-oxidant oxidant should not be more toxic than the metabolites removed from the body (iii) must be present in the body in sufficient quantity (iv) half of the antioxidant should be long enough to react with oxidants (Marklund, 1986).

1.5.1 Enzymatic antioxidants

Superoxide dismutase (SOD) is one of the important enzymatic antioxidants which play a significant role in the intracellular space. It helps in catalysis of superoxide radical to non-radical molecules O_2 and H_2O_2. In humans there are three SOD isoforms (Cu/Zn SOD, Fe/Mn SOD and Ni SOD) (Richardson et al., 1975; Borgstahl et al., 1992 Barondeau et al., 2004) are known to be present. All the three isoforms of SOD catalyze dismutation of the superoxide. Depending on the site or tissue of origin, concentration of these enzymes differ (Marklund, 1986).

Majority of aerobic organisms (except some bacteria and algae) commonly have catalase enzyme inside the body. It mainly catalyzes the decomposition of H_2O_2 to water and oxygen. In humans, it is present in every organ but is abundantly present in the liver and erythrocytes.

$$2\ H_2O_2 \rightarrow 2H_2O + O_2$$

The enzyme glutathione peroxidase plays a major role in the reduction of lipid hydro peroxides into their corresponding alcohols and free hydrogen peroxide to water. For the optimum physiological activity of GPx, sufficient concentration of GSH is required. Depending on the number of subunits in the selenium bond at the active centre, it is mainly found in two forms namely, selenium-dependent and

selenium-independent GPx. Selenium-dependent GPx is further composed of four subunits whereas selenium-independent GPx (glutathione-S-transferase, GST) catalysis detoxication of various xenobiotics (Holovska et al., 1998).

1.5.2 Non-enzymatic antioxidants

Glutathione (GSH) plays a very important role within the cells: organism protection from oxidative damage and detoxification processes. In the physiological environment, however, it is mostly present in the reduced form. Although it is a non-enzymatic antioxidant, interestingly, it is also a cofactor for some antioxidant enzymes and hence participates in detoxication mechanisms by transferring amino acids through the cell membrane including glutathione peroxidase, glutathione transferase, and dehydroascorbate. Transferrin participates by inhibiting the formation of $HO^{\bullet}$ radical and lipid peroxidation and hence also acts as an antioxidant. It is a plasma protein binding Fe (III) atom. Lipid peroxidation cannot be catalyzing if transferrin is bound to iron ions (Aruoma & Halliwell, 1987). Another important extracellular antioxidant found in humans is ceruloplasmin, which is a Cu (II) transporting protein. Its specialty is that it can bind with all the plasma copper atoms. It also participates in the Fenton reaction and releases Fe (II) by changing its oxidative number. In the human extracellular space, antioxidant activity of ceruloplasmin is greatly contributed by albumin. An important function of serum albumin is to protect the hypochlorous acid (HOCl), produced by myeloperoxidase mediated oxidative damage (Ďuračková, 2008). This enzyme scavenges OH^{-} radicals and oxygen (Ďuračková, 2008). GSH reverts Vitamin C and E into their active forms for their antioxidant actions. The ratio between GSH and its oxidized form is indicative of the redox state of the cell (Schafer & Buettner, 2001).

Vitamin E consists of a set of related compounds like α, β, γ and δ tocopherol. Among all these members, δ tocopherol being a fat soluble vitamin has the highest availability and antioxidant capability. Vitamin E scavenges or traps oxygen free radicals and inhibits the formation of chain reactions. Among these chain reactions tocopherol redical is propagated and can be reduced by ascorbate and glutathione to an active α tocopherol (Bast et al., 1991).

Flavonoids being phenolic compounds are widely distributed in the plant kingdom. There are more than 4000 different derivatives isolated and the number of new isolated derivatives is increasing constantly. Quercetin and catechins are among flavonoids and are present in food as free monomers. Flavonoids may be bound to saccharides or glycosides. Several studies have endorsed the usefulness of flavonoid-rich food in various diseases like coronary heart disease, myocardial infarction, cancer, neurodegenerative diseases, psychic diseases and other chronic diseases (Knekt et al., 2002; Trebaticka et al., 2006).

Oxidative stress has been found to play a significant role in disease pathology. Some supplements containing flavonoids have been considered to exert therapeutic role in different diseases by their antioxidant mechanism.

1.6 Vitamin C (Ascorbic acid)

Vitamin C is used as ascorbic acid (reduced form) and dehydroascorbic acid (oxidized form). Vitamin C is one of the important water soluble vitamins that are present in plenty, in fresh fruits like orange, pineapple, raspberries, lemons, grapefruit, watermelon, papaya, strawberries, cantaloupe, mango, and cherries and in vegetables like broccoli ,tomato, cauliflower, cabbage, and green and red peppers. It is also present in feeds of plant origin that are relevant for animal nutrition,

particularly in green feeds and silages (Adikwu & Deo, 2013). Among nutrients, vitamin C which is an essential component in many fruits and vegetables like oranges, lemons and grapes, is widely used and consumed in our daily life. Vitamin C prevents the oxidation of other substrates by donating its electron. During this process, vitamin C is oxidized to a relatively stable radical known as ascorbyl.

The average adult has a body pool of 1.2–2.0 g of ascorbic acid that may be preserved with 75 mg/dl of ascorbic acid. However, humans cannot synthesize ascorbic acid due to the absence of the enzyme L-gulonolactone oxidase. In humans, vitamin C is supplemented through food or as tablets. Vitamin C intake has been found to have a very profound effect in the reduction of cardiovascular disease risk (Mayne, 2003; May & Harrison, 2013). On the contrary, few studies have failed to see any effect of vitamin. C supplementation on the reduction of cardiovascular disease risk (Watanabe et al., 1998; Protogerou et al., 2002). It is a cofactor for several enzymes including the hydroxylation of proline, lysine essential for the synthesis of collagen; radical scavenger activity and NO-sparing function (May & Harrison, 2013). Supplements of vitamin C have achieved tremendous advantages as an antioxidant since it inhibits the genesis of atherosclerosis due to its effect on vascular remodelling, endothelial function, and lipid peroxidation.

Vitamin C is responsible for the synthesis of collagen. It also plays an important role in converting plasma iron into ferritin. It is important for neutrophil function, decreases circulating glucocorticoids and is also important for immune response. It also reduces oxidized tocopherol to its effective form in liver. Vitamin C is a dietary antioxidant which is used to prevent atherosclerosis. In atherosclerosis free radicals are involved at the early phase of endothelial dysfunction and in later phase of oxidation of the low-density lipoproteins (Korish & Arafah, 2008). It reduces the

adverse effects of ROS and RNS that can cause oxidative loss to macromolecules such as lipids, DNA and proteins, which are involved in cardiovascular disease, stroke, cancer, neurodegenerative diseases and cataractogenesis (Imam et al., 2011).

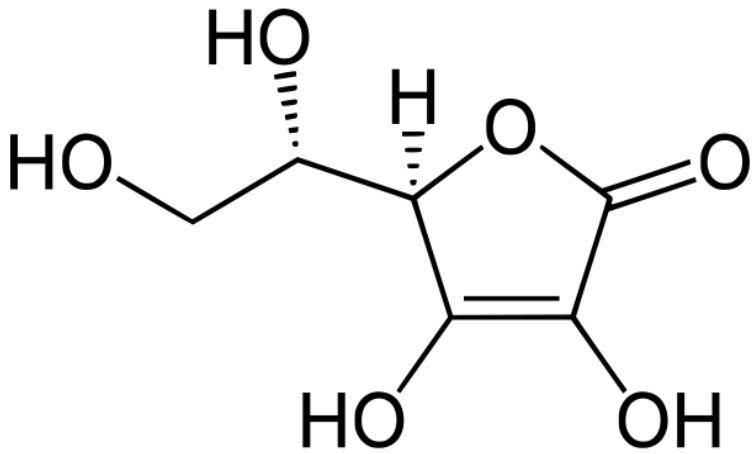

Figure 1.4: Structure of vitamin C (ascorbic acid) adapted from Zümreoglu-Karan, 2006

1.7 Green tea polyphenol (GTPP)

1.7.1 General description

Tea is the most widely used drink in the world, second to water. On the basis of the level of oxidation, tea can be divided into three types; Green tea, Oolong tea and black tea (Velayutham et al., 2008; Chan et al., 2011). All the three types originate from *Camellia sinensis*, but are treated differently to achieve different standards of oxidation.

Green tea contains catechins in greater concentration than black and oolong tea. *In vitro* and *in vivo,* catechins are proven to be potentially strong antioxidants. In Chinese traditional medicines, since ancient times, green tea is considered as a healthy beverage. Green tea has some potential pharmacological properties such as

anti-hypertensive effect, body weight control, and antibacterial and antiviral activity. It is also used for solar ultraviolet protection, to increase bone mineral density and as neuroprotective. Green tea polyphenols also have anti-fibrotic action both on the arteries and skin.

Certificate analysis of GTPP extract 95% are shown in the table 2.3 page 82 which received from Nova Company.

Polyphenols, especially flavanols and flavones, are available abundantly in green tea, which is nearly 30% dry weight of the fresh leaf (Balentine et al., 1997). Catechins are the most common flavones and fundamentally include (-) epicatechin (EC), (-) epicatechin-3-gallate (ECG), (-) epigallocatechin (EGC), (-) epigallocatechin-3-gallate (EGCG), (+) catechins and (+) gallocatechin (GC). EGCG, the most abundant catechin in green tea, accounts for about 65% of the total catechins content (Zaveri, 2006) as shown in the Figure 1.5.

The exogenous administration of extract of green tea in rats was reported to increase the standard of the endogenous antioxidants such as glutathione peroxidase (GPx) and reductase, SOD and catalases. *In vitro* studies have shown that the epigallocatechin in green tea have angiotensin converting enzyme inhibitor properties (Actis-Goretta et al., 2006; Guerrero et al., 2012). Several studies conducted on animal models suggested that green tea lowers the blood pressure by reducing or suppressing the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) ROS in the vascular system.

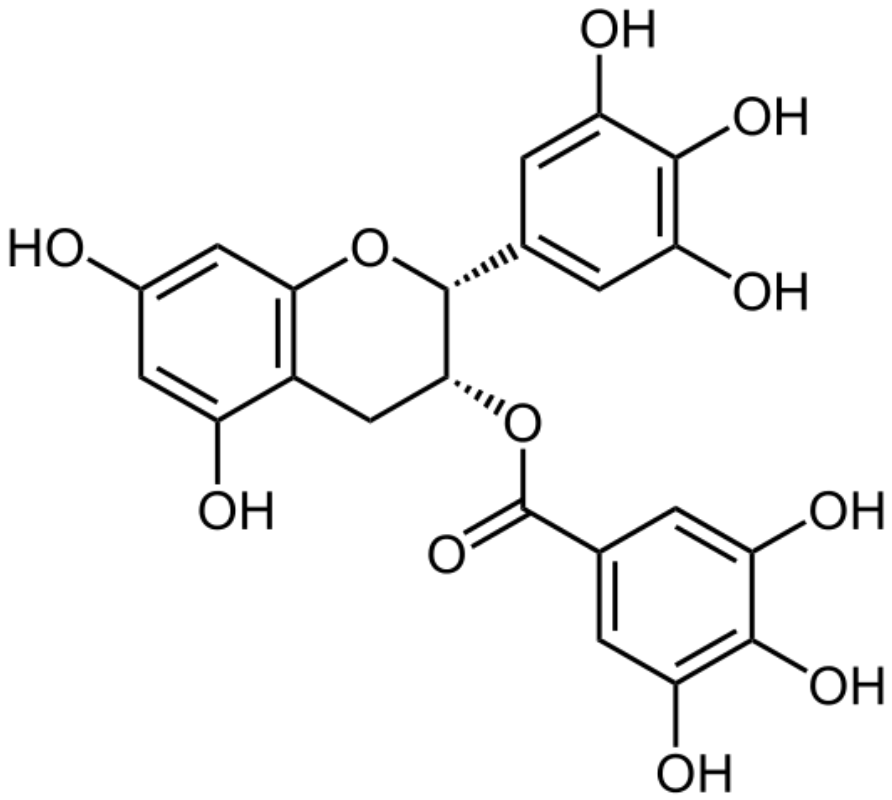

Figure 1.5: Chemical structure of Epigallocatechin gallate in green tea adapted from Zaveri, 2006

1.7.2 Polyphenols and vascular tone: Role of endothelial cells

One of the major reasons for the progression of CVD is hypertension. Long standing high pressure and changes within the vascular walls may affect the heart, which can ultimately lead to cardiac failure. However, antioxidant consumption may have beneficial effect on the vascular tone. It is reported that drop in blood pressure and heart rate in hypertensive patients is markedly associated with increase in nitric oxide (NO) level after 30 minutes of polyphenol consumption (Matsuo et al., 2001). Similarly, red wine polyphenolic compounds (RWPC) were shown to possess hypotensive effect by inducing endothelium-dependent relaxation of the vessels of the aorta and mesenteric artery which are mainly associated with nitric oxide production (Fitzpatrick et al., 1993; Hodgson & Croft, 2006). There is well

documented evidence on the hypotensive activity of polyphenol from different sources like red wine, grape kin or an isolated polyphenol (quercetin). Interestingly, polyphenols fail to show its hypotensive effect when nitric oxide synthesis is chronically inhibited which suggests that its blood pressure lowering activity is mainly due to nitric oxide production. Similarly, another study showed that increased NO synthase activity helps to prevent L-nitro arginine-methyl ester-induced hypertension, cardiovascular remodelling and vascular dysfunction following red wine polyphenols. Studies have demonstrated that polyphenol extract and delphinidin (a major anthocyanin present in red wine), enhances the release of Ca^{2++} which leads to NO release (Stoclet et al., 1999; Martin et al., 2002). This strongly suggests that polyphenol mediated NO production is mainly through an extracellular Ca^{2+} dependent mechanism in endothelial cells (Andriambeloson et al., 1999).

Endothelial dysfunction may play a vital role in the development of CVD. Nitric oxide is a very potent mediator released from the endothelial cells which is an important regulator of vascular tone. When endothelium mediated NO release fails to cause sufficient vasodilatation of the blood vessels, it is known as endothelial dysfunction. It can be deduced that the antioxidant property of plant polyphenols plays a major role in the prevention of hypertension in experimental and clinical studies and can reduce the risk of CVD *via* activation of endothelial nitric oxide synthase (eNOS).

1.7.3 Green tea and human health

1.7.4 Green tea: Nutritional value

Green tea consumption contributes to the overall daily fluid intake, while the calories intake is insignificant if sugar is avoided, and the caffeine intake is lower