Cardiovascular-Protective Properties Of Plant Polyphenols

A thesis submitted for the degree of

MSc

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Abstract

Epidemiological evidence suggests strongly that consumption of fruit and vegetables is associated with a reduced risk of cancer and cardiovascular disease. There is evidence that the polyphenol content of fruit and vegetables contribute to these protective effects. This study examines the antioxidant properties and effects of a range of plant extract polyphenols (PEP) on endothelial function and platelet aggregation, examining the PEP ability to scavenge superoxide and increase nitric oxide bioavailability.

A crude raspberry extract (RE) containing a mixture of polyphenols, a raspberry extract that had been processed through an artificial digestive system (GR), 2 purified polyphenols- quercetin-3'-sulphate (QS) and quercetin-3-glucuronide (QG) were examined together with ascorbic acid (AA) as a positive control.

The scavenging capacity of PEP for superoxide (O_2^-) generated from xanthine/xanthine oxidase, and for the elevated levels of O_2^- from arteries of female stroke prone spontaneously hypertensive rats (SHRSP), was measured by lucigenin chemiluminescence. Nitric oxide bioavailability was measured mainly in carotid artery rings from SHRSP but some studies were carried out using its normotensive control the Wister Kyoto Rat (WKY). It was expressed as the increase in contractile responses to phenylephrine in the presence of the nitric oxide synthase inhibitor L-NAME. ADP dependent platelet aggregation was measured in an agrogometer in plasma from healthy human volunteers in the presence and absence of PEP. Differences between groups were compared by t-test with Bonferoni correction, n = 4-10/ group for all studies.

EC_{50} values for scavenging O_2^- by PEP when O_2^- was generated by xanthine/ xanthine oxidase were as follows with 95% confidence intervals in brackets (ranked in order of potency highest to least potent); QG 0.012μg/ml (0.006-0.022), GR 0.012μg/ml (0.0055-0.024), RE 0.026μg/ml (0.012-0.059), QS 0.047μg/ml (0.020-0.103) and AA 0.192 (0.089-0.415). In contrast to scavenging O_2^- generated by xanthine/ xanthine oxidase, the PEP showed no scavenging effect on the elevated O_2^-

levels in arteries of SHRSP. All PEP showed the ability to increase NO bioavailability in SHRSP; no effect was seen in the control WKY. RE was able to increase NO bioavailability in SHRSP (over the concentration range 90-0.36μg/ml). QG and QS improved NO bioavailability at 50μg/ml (100μmol/l) and 12μg/ml (31μmol/l) respectively. QG and QS were found to increase NO bioavailability at even lower concentrations when given in combination; QG 0.3μg/ml (6 μmol/l) and QS 0.5μg/ml (1μmol/l) when combined. RE at 100μg/ml was the only PEP to show a significant inhibitory effect on platelet aggregation.

In conclusion the PEP have been found to scavenge O_2^- and improve endothelium function but results suggest they have less effect on platelet aggregation. RE appeared to be the most potent but the active ingredients still have to be fully identified and their in vivo bioavailability confirmed. In contrast QS and QG have been identified in plasma and are strong candidates to contribute to the cardiovascular protective effects of fruit and vegetables. Discrepancies between the PEP's ability to scavenge O_2^- and improve NO bioavailability suggest that mechanisms in addition to O_2^- scavenging may contribute to cardiovascular protective effects of PEP.

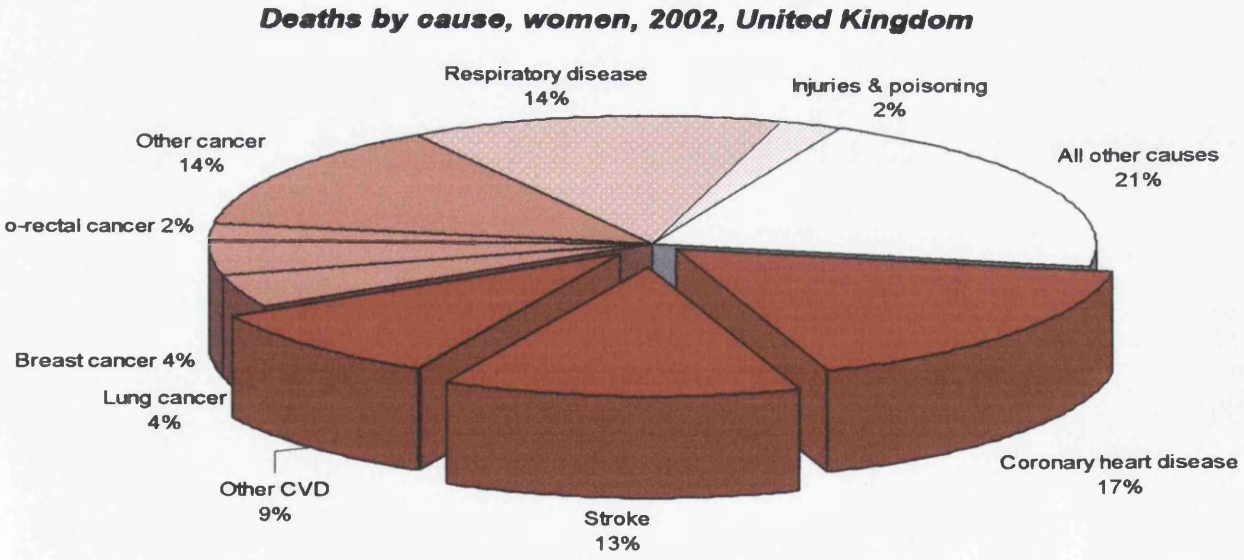
1. Introduction

1.0 Introduction

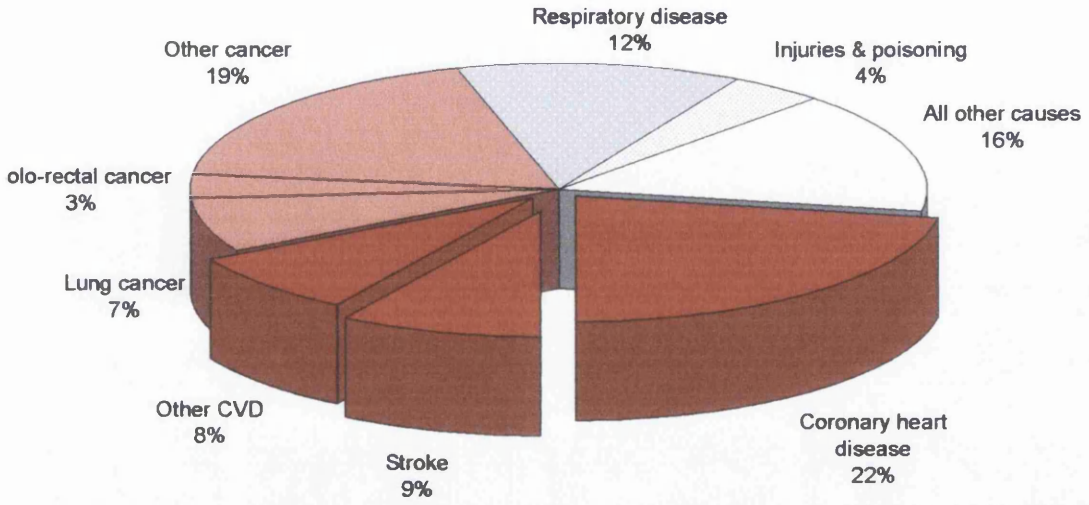
1.1Cardiovascular Disease (CVD) and endothelium function

Cardiovascular disease (CVD) is a major problem worldwide and there is a great need for further research into both causes and treatments. Despite the best efforts of modern medicine CVD claimed the lives of more than 16 million people last year or caused 29.2% of total global deaths in 2003 (World Health Organisation (WHO) 2004). More than 50% of deaths are from heart disease and strokes in industrialized countries (Outside East Asia). CVDs are the major causes of death in the UK, accounting for just under 238,000 deaths in 2002 (BHF-Statistics Database). More than one in five men and one in six women die from coronary heart disease (CHD) (BHF-Statistics Database).

Figure 1 a) Deaths by cause, women 2002, UK, b) Deaths by cause, men 2002, UK (BHF-Statistics Database)



Deaths by cause, men, 2002, United Kingdom



By 2010 CVD is estimated to be the leading cause of death in developing countries. Approximately 20 million people survive heart attacks and strokes every year, but this requires costly clinical care (WHO, 2004).

The rise in CVDs reflects a significant change in dietary habits, physical activity levels, and tobacco consumption worldwide as a result of industrialization, urbanization, economic development and market globalisation (WHO, 2004). Heart disease has no geographic, gender or socio-economic boundaries. Many of the 16.7 million deaths could have been prevented by life style changes: such as having a healthy diet, taking regular physical exercise and not smoking (WHO 2004).

Some of the most important contributing factors in CVD are hypertension, atherosclerosis and diabetes. Hypertension is believed to affect one in four American adults (American Heart Association 1999) and only 47% Americans have optimal blood pressure (BP;<120mmHg systolic/<80mmHg diastolic) (Burt et al 1995). In England hypertension is estimated to effect 41% of men and 33% of women (this is using the definition of hypertension as being systolic >140mmHg or diastolic >90mmHg) (Ramsay et al 1999). Atherosclerosis is virtually ubiquitous in the developed world and begins in childhood.

Endothelium dysfunction is a characteristic feature of hypertension, atherosclerosis and diabetes. Endothelial function is dynamically regulated and the endothelium's vasodilator, anti-inflammatory and antithrombotic properties are markedly diminished by injuries such as hypertension, atherosclerosis, diabetes and inflammation and aging (Verma et al 2003). Endothelial dysfunction in both coronary and peripheral arteries may precede the development of the clinical consequences of atherothombosis (Celermajor et al 1992). Endothelial dysfunction is believed to be one of the earliest stages of atherosclerosis and can be observed in apparently healthy people with risk factors for heart disease (Celermajor et al 1992). Endothelial dysfunction is correlated with sub-clinical measures of CVD and is prospectively associated with an increased risk for clinical CVD events (Schachinger et al 2002).

Endothelial cells play an important role in maintaining the structural and functional integrity of the vasculature.

The primary vasodilator released by the endothelium is nitric oxide (NO) (Furchgott et al 1980; Palmer et at 1987). Other relaxing factors released by endothelium include endoelium-derived hyperpolarizing factor, prostacyclin, C-type natriuretic factor, 5-hydroxytryptamine (5-HT), adenosine triphosphate (ATP), substance P, and acetylcholine (Ach) (Harris et al 2004). Basal blood flow maintains a continual release of endothelium derived relaxing factors, and an increase in blood flow increases the release of relaxing factors in healthy blood vessels. NO is released from the endothelium in response to changes in the shear stress on the vascular wall (Harris et al 2004)

The contracting factors that endothelium releases include endothelin-1, thromboxane A_2, prostaglandin H_2, superoxide anions, and ATP (Harris et al 2004). Normally endothelin levels in plasma are very low. Higher levels have been reported in some disease states such as hypertension. NO can eliminate endothelin-induced arterial constriction and inhibit further release of endothelin from the endothelium (Harris et al 2004).

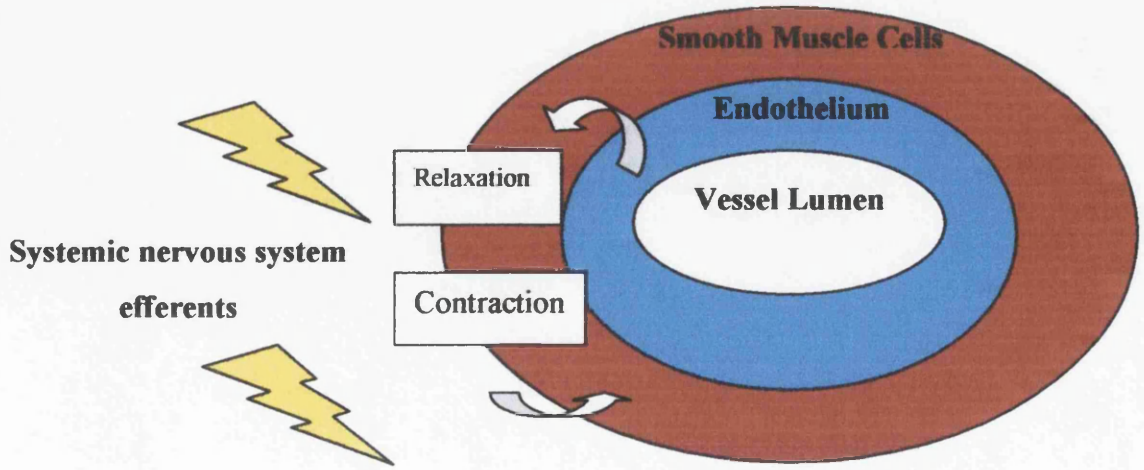
In addition to regulating vessel tone, endothelial cells help prevent the build up of lipids and platelets that initiate the atherosclerotic process. Both NO and prostacyclin

inhibit platelet aggregation, whereas the release of tissue plasminogen activator enhances fibrinolysis (Wu et al 1996). Other anticoagulant factors, such as thrombomodulin, are present on endothelial cell surfaces to prevent cellular adhesion of the vascular wall (Wu et al 1996). All of these mechanisms combine to help prevent the build up of atherosclerotic plaques along arterial walls.

Inflammation is widely thought to play a role in CVD pathogenesis. Damage to the endothelium can enhance inflammatory responses through the release of chemoattractants that promote cellular adhesion and uptake of lipids and macrophages into the abluminal space. Damage to the endothelium results in the release of growth factors such as platelet-derived growth factor, which can promote connective tissue growth and enhance smooth muscle cell proliferation, resulting in vascular wall thickening (Luscher et al 1997).

In a healthy state, the autonomic nervous system (ANS) and the endothelium work together to maintain vascular tone. There is tonic balance between the release of the vasodilating factors from the endothelium and vasoconstricting factors from the sympathetic nerve terminals, Fig 2. The balance between these opposing forces acts on the vascular smooth muscle cells to maintain the appropriate vessel tone (Harris et al 1990).

Figure 2 Interactions autonomic nervous system and endothelium on smooth muscle tone of blood vessels (Adapted from Harris et al 2004)



When the endothelium is damaged NO release may be diminished and the balance between vasorelaxation and vasoconstriction perturbed. NO released from the endothelium decreases the sensitivity of smooth muscle cells to the vasoconstrictor effects of the sympathetic nervous system (SNS) and inhibits central and peripheral SNS activity (Harris et al 2004). In contrast, NO may increase central and peripheral parasympathetic nervous system (PSNS) activity while enhancing sensitivity to PSNS sites of action. This suggests that NO released from endothelial cells may play a modulating role in the balance between the SNS and PSNS branches of the ANS (Harris et al 2004).

Evidence exists for a negative influence of oxidative stress on both ANS and endothelial function (Harris et al 2004). Oxidative stress is said to be present when the radical-scavenging mechanisms are overcome by the level of ROS (Reactive Oxidative Species, e.g. superoxide, hydroxyl radical) (Halliwell et al 1989). Oxygen radicals can attenuate NO mediated responses by combining with it to form peroxynitrite (Beckman et al 1996). Oxidative stress promotes the release of endothelin from endothelium enhancing its activity (Landmesser et al 2001). Exposure to oxidants can damage the endothelium, decreasing the ability of the endothelium to provide a barrier between the blood and sub-endothelial matrix (Lum et al 2001). Oxidative stress promotes leukocyte adhesion to endothelial cells (Lum et al 2001). Oxidative stress also contributes to impairment in ANS function. Oxidation induces neuronal cell death, including apoptosis in SNS neurons (Murphy et al 1998). Peroxynitrite, formed from the interaction of superoxide with NO, is a principal oxidizing agent of the ANS (Harris et al 2004).

1.2 Nitric Oxide in prevention of CVD

Before the 1980s nitric oxide (NO), was believed to be just another toxic molecule, one of a lengthy list of environmental pollutants found in smoke and smog, a free radical gas that is formed in the atmosphere during lightning storms (Koshland 1992). It is now known that NO is also formed in an enzyme catalysed reaction between molecular oxygen and L-arginine in mammals and more primitive species (Rang et al 1999). NO has been found to be involved in areas as diverse as fertilisation (Kuo et al 2000), neurotransmission, immune function, secretion, haemostasis, vascular tone,

peristalsis, arthritis, septic shock, carcinogenesis, erectile function, several degenerative neuronal diseases and pyloric stenosis (Fennell 2002). NO acts as a key signalling mechanism in the cardiovascular system (Furchgott et al 1980; Palmer et al 1987) and nervous system (Snyder et al 1998) and has a role in host defence (Marletta et al 1988).

A physiological function of NO was first realized in the vasculture when it was discovered that the endothelium derived relaxing factor (EDRF) described by Furchgott & Zawadzki in 1980 was NO (Ignarro et al 1987; Plamer et al 1987). As research in the field grew the importance of NO gained recognition, being named "Molecule of the year" by Science in 1992 (Koshland et al 1992) and the 1998 Nobel prize for medicine was awarded to Furchgott, Murad and Ignarro for their work demonstrating the importance of NO as a signalling molecule. In 1980, NO was the subject of only 12 academic papers. In the next 20 years, over 40,000 papers were published on NO and NO research exploded.

Furchgott (1980) observed that despite very potent vasodilating action in vivo, acetylcholine (Ach) does not always produce relaxation of isolated preparations of blood vessels in vitro. The relaxation effect was lost when the intimal surface of the vessel was rubbed off. The possibility that the rubbing of the intimal surface had removed endothelial cells was put forward by Furchgott in 1979, and it was demonstrated that relaxation of isolated preparations of rabbit thoracic aorta and other blood vessels by Ach requires the presence of endothelial cells, and that Ach, acting on muscarinic receptors, stimulates the release of a substance termed EDRF that causes relaxation of the vascular smooth muscle. It was suggested by Furchgott that EDRF could be NO, as nitrovasodilatiors which release NO mimic the effect of EDRF. The biological activity of EDRF and NO was measured by bioassay (Gryglewski et al 1986). In the bioassay the tissue-induced relaxation of EDRF was indistinguishable from that induced by NO. Both substances were equally unstable. Bradykinin caused concentration-dependent release of NO from the cells in amounts sufficient to account for the biological activity of EDRF. The relaxations induced by EDRF and NO were inhibited by haemoglobin and enhanced by superoxide dismutase to a similar degree. Thus it was concluded that EDRF is NO.

It was discovered independently that NO is the endogenous activator of soluble guanylate cyclase, leading to the formation of cyclic GMP (cGMP) a second messenger in many cells including nerves, smooth muscle, monocytes and platelets. In the late 1970s, Murad and collegues had shown that nitroglycerine, nitroprusside and other smooth muscle relaxants increased cyclic guanosine monophosphate cGMP levels in various smooth muscle preparations (Katsuki et al 1977). It was proposed that NO formed from some endogenous precursor activated guanylate cyclase causing the increase in cGMP in intact cells and tissues and vasodilation (Murad et al 1999). Unknowingly, doctors had been using NO to treat angina and hypertension since Victorain times by giving patients drugs based on amylnitrite. Brunton published the first comprehensive report on the potential use of amyl nitrite as a treatment for angina in 1869 (Lincoln et al 1997).

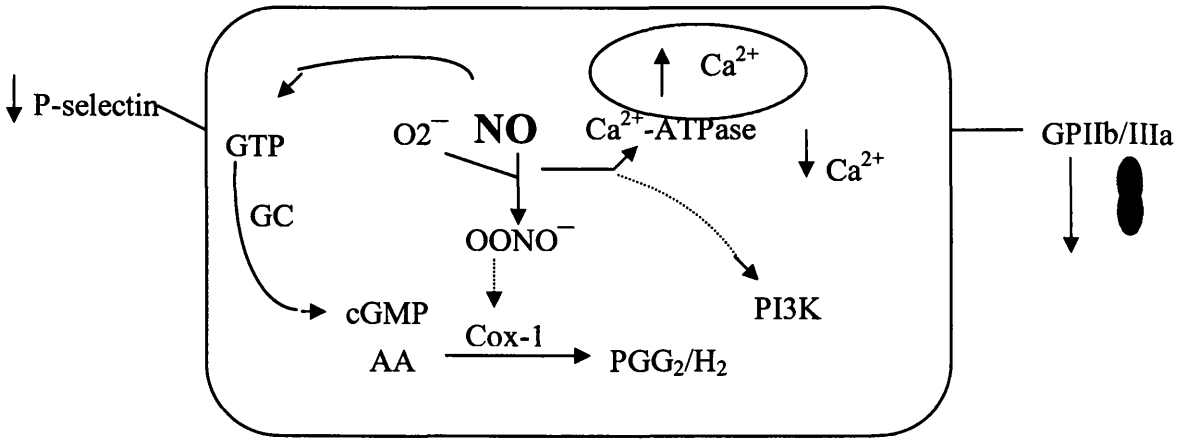
Reduced NO bioavailability is a common finding in many forms of vascular disease. A reduction in either NO or NO-mediated vasodilation characterized as endothelium dysfunction has been reported in vascular diseases as diverse as hypertension (Cifuentes et al 2000), hypercholesterolaemia (Creager et al 1990), diabetes (Calver et al 1992) and chronic renal failure (Wever et al 1999). It has been demonstrated that patients with essential hypertension have impaired endothelium-dependent vasodilation (Panza et al 1993). Panza illustrated this by investigating the effect of the arginine analogue N^G-monomethyl-L-arginine (L-NMMA, inhibitor of nitric oxide synthase) on forearm blood flow. L-NMMA was infused under baseline conditions, during infusion of Ach (an endothelium-dependent dilator), and sodium nitroprusside (direct smooth muscle dilator) to 11 hypertensive patients and 10 control subjects. L-NMMA produced a significantly greater decrease in blood flow in control subjects than in hypertensive patients. The vasodilator response to Ach was reduced in patients compared with control subjects. L-NMMA did not modify the response to sodium nitroprusside in either control subjects or patients. The findings strongly indicated that a defect in the endothelium derived NO system partly accounted for both the increase in vascular resistance under basal conditions and impaired response to endothelium-dependent vasodilators.

NO not only acts as a vasodilator it has important anti-platelet properties. This was first shown for NO vasodilators. This inhibitory effect can be potentiated by

maintaining the intracellular thiol redox state (Loscalzo et al 2001). NO has been shown to have important antiplatelet actions activating guanylate cyclase, inhibiting phosphoinositide 3-kinase, impairing capacitance calcium influx, and inhibiting cyclooxygenase-1. Endothelial NO also limits platelet activation, adhesion, and aggregation (Loscalzo et al 2001), fig 3.

Figure 3 Effects of NO on platelet signalling and function (Adapted from Loscalzo et al 2001)

NO derived from endothelial cells or from platelets, suppresses platelet activation by activating guanylyl cyclase (GC), leading to an increase in the conversion of GTP to cGMP, enhancing calcium ATPase-dependent refilling of intracellular Ca^{2+} stores, inhibiting the activation of P13K. As a result of second-order effects mediated by the first two of these signalling systems, intracellular Ca^{2+} flux is suppressed, leading to suppression of P-selectin expression and of the active confirmation of glycoprotein IIb/IIIa (GPIIb/IIIa) required for the binding fibrinogen. NO also reacts with O_2^- to from peroxynitrite ($OONO^-$), which can react with protein tyrosin residues on cyclooxygenase-1 to inhibit enzyme conversion of arachidonic acid (AA) to prostaglandins G_2 and H_2 (PGG_2/H_2), with a resulting reduction in thromboxane A2 synthesis. Solid arrows indicate activation; dashed arrows inhibition.



Platelets are an important source of NO, and this platelet-derived NO pool limits recruitment of platelets to the platelet-rich thrombus (Loscalzo et al 2001). Under normal conditions of blood flow and shear stress the source of NO on platelets is endothelial derived NO (Loscalzo et al 2001). However, under conditions of endothelial dysfunction or denudation, especially in the setting of an acute coronary syndrome, other sources of NO become important in regulating platelet responses. A constitutive NOS has been found in human platelets and megakaryocytic cells (Loscalzo et al 2001). Using an NO-selective microelectrode, adapted to a platelet

aggregometer, Freedman (1997) showed that this platelet derived NO not only modestly modulates platelet activation to strong and weak agonists but also, more importantly, markedly inhibits platelet recruitment to the growing platelet thrombus. These in vitro findings were confirmed in an animal model of deficient platelet-derived NO, the NOS3-Null mouse. In this model, Freedman et al (1999) found that there is no detectable NOS3 gene in marrow cells, that platelets generate no detectable NO on activation, and that the bleeding times of NOS3-null mice are correspondingly shorter than those in wild type mice. Thus, endothelial and platelet derived NO pools both contribute to normal haemostatic function, and a deficiency of either pool enhances haemostatic responses to acute vascular injury (Loscalzo et al 2001).

Loss of NO results in the loss of many of the antiatherogenic effects of NO such as the prevention of LDL oxidation, reduction of expression of monocyte chemoattractant protein-1, CD11/CD18, P-selectin, and the adhesion molecules VCAM-1 and ICAM-1, all of which prevent leukocyte adhesion and migration (Loscalzo et al 2001).

Nitric oxide is an uncharged diatomic molecule composed of seven electrons from nitrogen and eight electrons from oxygen (Beckman 1996), fig 4.

Figure 4 Structure of NO (Beckman et al 1996)

Nitric Oxide is a free radical, with an unpaired electron

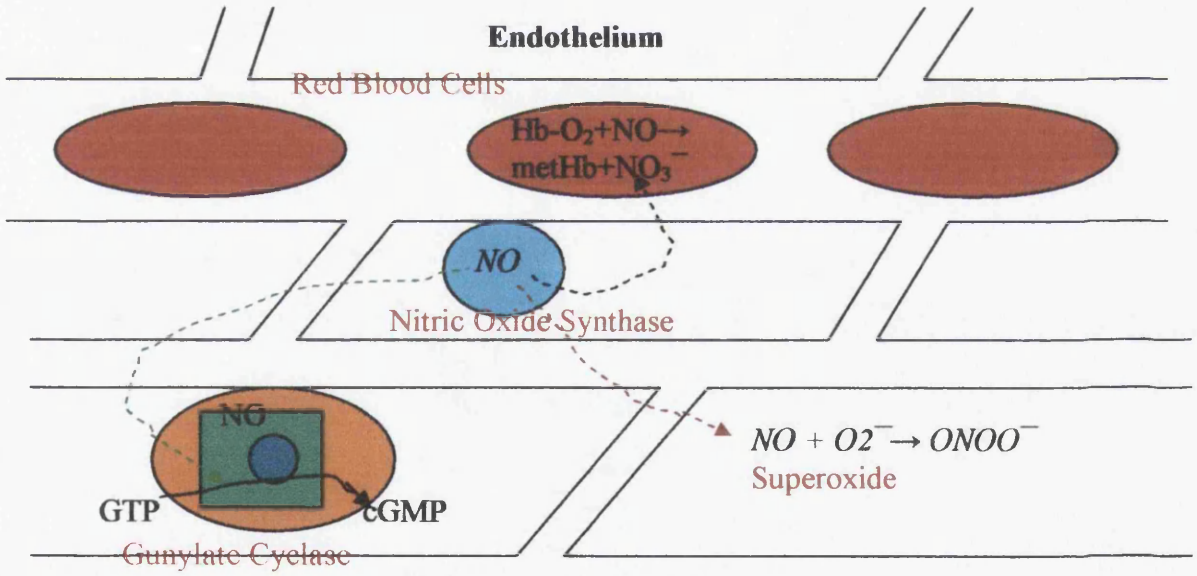
$$\bullet NO$$

Nitric Oxide

NO only reacts rapidly with a select range of free radicals, and with transition metals like heme iron. NO has a half-life of 1.6-16 seconds in vivo (Beckman et al 1996). With the reaction rates as a guide the biological chemistry of NO can be simplified in a reasonable approximation to just three reactions; its activation of guanylate cyclase, responsible for signal transduction; its destruction by reaction with oxyhemaglobin; and its transformation to peroxynitrite ($ONOO^-$) by the reaction with superoxide (O_2^-) (Beckman et al 1996) fig 5.

Figure 5 Three principal reactions of NO simplified by reaction rates (Adapted from Beckman et al 1996)

Nitric oxide produced in the endothelial cell will diffuse out far faster than it will react with most intracellular components. Nitric oxide binds to and activates guanylate cyclase (green dashed arrow). If it diffuses into a red blood cell, it will be eliminated by oxyhemoglobin ($Hb\text{-}O_2$) to form nitrate (black dashed arrow). NO reacts with oxygen to give nitrogen oxides (NO_2 and N_2O_3), which react with other molecules e.g. thiols, or amines, or they simply hydroylase to form nitrate (NO_3^-) (Goldstein et al 1995). The reaction with superoxide to form peroxynitrite is normally limited by the micromolar concentration of superoxide dismutase in cells (dark red dashed arrow). metHb = methemoglobin.



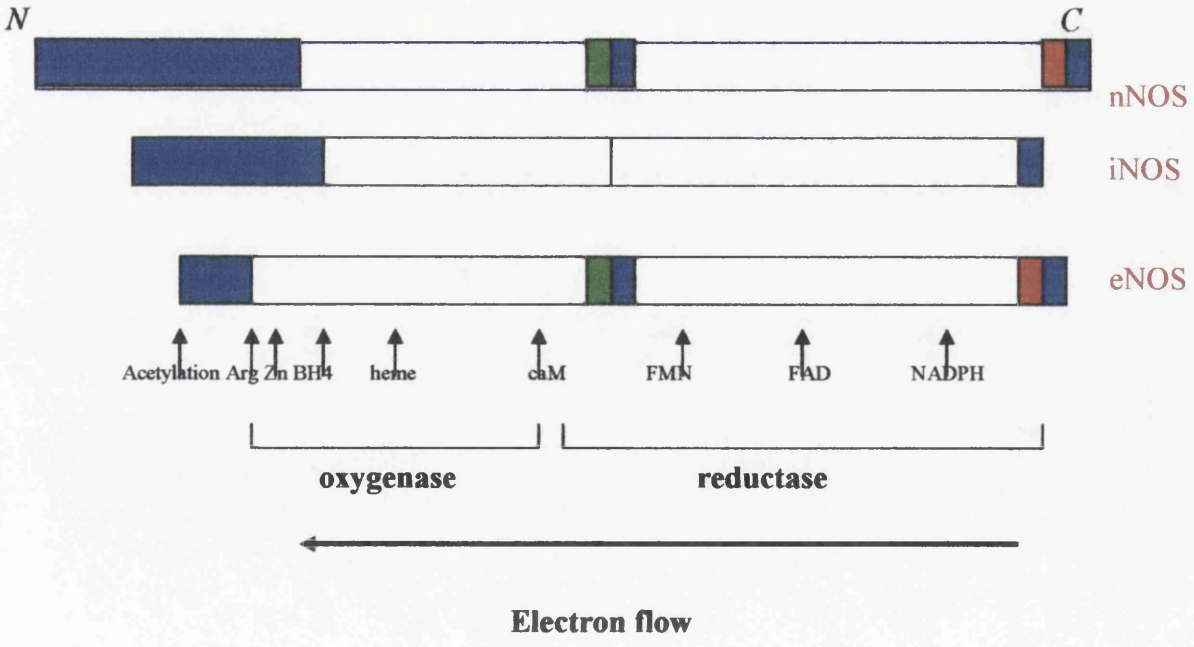
NO can diffuse through most cell diameters (on the order of 100-200μm), with little consumption or direct reaction (Beckman et al 1990). The site of NO producing cells is unimportant, because of its extremely high diffusion coefficient, the action of NO depends more on the number of NO producing cells than the location (Beckman et al 1990). In vivo the effects of NO are greatly limited by the reaction of NO with

haemaglobin which, drains a large proportion of NO from the vessel wall. Direct measurement of NO using a porphyrinic microsensor showed that the endothelium can produce 10 to 40 times the amount of NO required to activate guanylate cyclase (Beckman et al 1996).

NO synthase (NOS) enzymes are central to the control of NO biosynthesis. There are three known isoforms of NOS; an inducible form (iNOS or NOS-II), expressed in macrophages, kupffer cells, neutrophils, fibroblasts, vascular smooth muscle and endothelial cells in response to pathological stimuli such as invading microorganisms and two so called constitutive forms that are present under physiological conditions in endothelium (eNOS or NOS- III) and neurons (nNOS or NOS- I) (Rang et al 1990), fig 6. All three NOS isoforms are dimeric enzymes, bearing similarities to the cytochrome P450 enzymes.

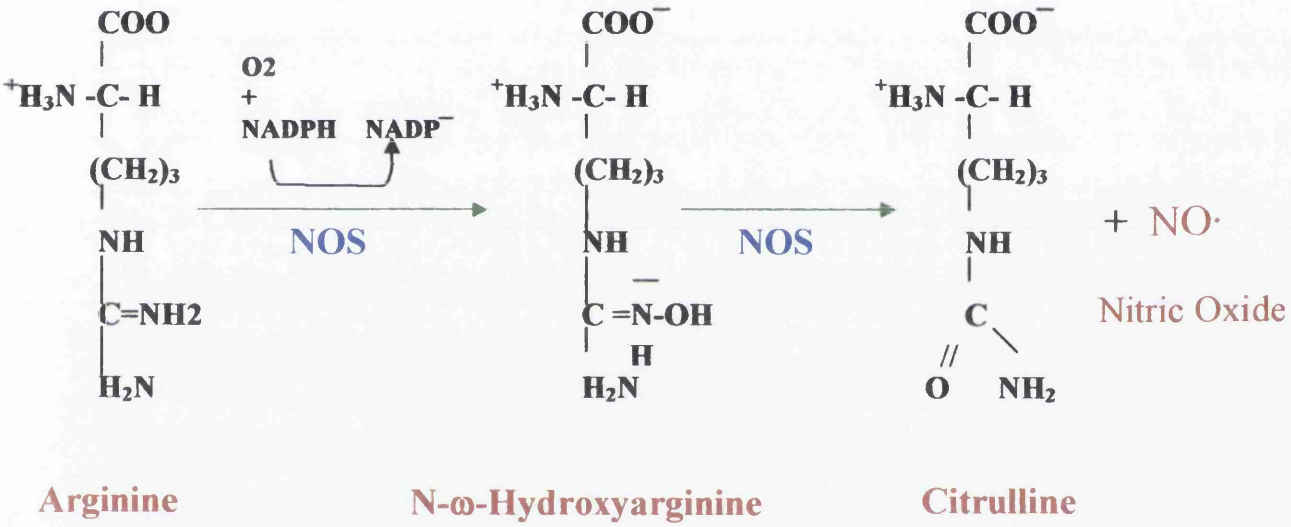
Figure 6 Structure of nitric oxide synthase (NOS) isoforms (Adapted from Goovers 2001).

Homology in amino acid sequences between the 3 NOS isoforms (white boxes), homologous in nNOS and eNOS (green boxes), isoform specific sequences (red boxes). For eNOS, regions of acetylation and in the binding of cofactors are indicated as well as the oxygenase and reductase domain and the direction of the intramolecular electron flow.



NO is synthesised from L-arginine through a five-electron oxidation step that produces NO and citrulline through the production of an N^G-hydroxy-L-arginine intermediate fig 7. Several cofactors are required for NO generation; these include flavin mononucleotide (FMN), flavin adeninedinucleotide (FAD) and tetrahydrobiopterin (BH_4). Futhermore, the enzyme contains binding sites for haem and the allosteric activator calmodulin, (Gover et al 2001). The constitutive enzymes generate small amounts of NO (picomolar) whereas the activity of iNOS is approximately a thousand times greater (Rang et al 1999).

Figure 7 Production of NO (Stryer 1995)



The regulation of eNOS is complicated and occurs at every level from transcription to translation and protein-to-protein interactions. Several factors up-regulate eNOS transcription including shear stress, protein kinase C inhibitors, hydrogen peroxide (H_2O_2), oestrogen, vascular endothelial growth factor (VEGF), insulin, basic fibroblast growth factor and low concentrations of oxidised LDL (Gover et al 2001). Transcription of eNOS can be down regulated by factors such as hypoxia, erythropoietin and high concentrations of oxidised LDL (Gover et al 2001).

Phosphorylation of eNOS also plays a role in eNOS regulation but this depends on the kinase and the residue involved and is not completely understood. The protein kinase

Akt phosphorylates a specific eNOS serine leading to eNOS activation (Dimmeler et al 2003). This renders eNOS more sensitive to calcium/ calmodulin. VEGF, oestrogen, insulin and shear stress can also cause calcium independent activation of eNOS (Gover et al 2001).

eNOS plays a central role in the regulation of basal vascular tone and thus hypertension. This is illustrated by the eNOS knock out (KO) mouse. The KO mouse has no endothelial-dependent vasodilation to Ach unlike wild type mice, and is hypertensive having both elevated systemic and pulmonary blood pressure. Blood pressure is about 30mmHg greater in KO mice than wild type mice (Huang et al 1995).

1.3 Superoxide (O_2^-) and pathogenesis of cardiovascular disease (CVD)

Numerous studies suggest that levels of O_2^- are increased in CVD. Enhanced NO biodegradation by vascular O_2^- occurs in most forms of CVD (Münzel et al 1999). NO is scavenged by O_2^-, which produces peroxynitrite ($ONOO^-$). The latter is a strong oxidant with only minimal vasodilator activity (Murphy et al 1998). O_2^- may be produced by xanthine oxidase (XO), cyclooxygenase and mitochondrial oxidases (Münzel et al 1999; Rajagopalan et al 1996) but vascular NAD(P)H oxidases are the principal source of O_2^-in most forms of CVD (Münzel et al 1999).

Recent clinical and experimental studies provide indirect evidence that in chronic heart failure (CHF) the production of oxygen derived free radicals is increased (Münzel et al 1999). Polymorphonuclear leukocyte production of oxygen-derived free radicals has been reported to be increased 4-fold in patients with heart failure compared to controls (Prascal et al 1992). Dahalla et al (1994) showed that the production of O_2^- in cardiac tissue is increased as a consequence of reduced antioxidant reserve in heart failure. In patients with CHF, levels of malondialdehyde are increased, compatible with increased lipid peroxidation by oxygen-derived free radicals (Diaz-Velez et al 1996).

Baursachs (1999) added to the understanding of the interactions between O_2^- and NO in heart failure. These authors induced heart failure in rats by producing myocardial

infarction. The animals had a marked degree of endothelial dysfunction, despite increased expression of both eNOS and soluble guanyl cyclase. Incubation of aortas from these animals with radical scavengers normalized cGMP responses to sodium nitroprusside and improved vascular relaxations. These investigators also identified vascular NADPH oxidase as the likely source of vascular O_2^- in this model (Buarsachs et al 1999).

There is also evidence for excess O_2^- production in patients with coronary artery disease (CAD). In isolated rings of internal thoracic artery (ITA) and saphenous vein (SV) from patients that had undergone coronary artery by pass graft surgery (CABG), treatment with superoxide dismutase and tiron to scavenge O_2^- potentiated carbachol-stimulated relaxation in ITA and SV. In addition tiron treatment resulted in a significant increase in basal NO in arteries (Hamilton et al 1997).

Spiekermann et al (2003) characterized the vascular activities of XO and NAD(P)H oxidase, and their relationship with flow-dependent, endothelium-mediated vasodilation in patients with CAD. The authors presented the first electron spin resonance measurements of XO and NAD(P)H oxidase activity in human coronary arteries. The results support the concept that increased activities of both enzymes contribute to increased vascular oxidant stress in patients with CAD. Furthermore, the results suggested that increased XO activity contributes to endothelial dysfunction in patients with CAD and may thereby promote the atherosclerotic process (Spiekermann et al 2003).

In vessels from rats with either genetic (Grunfield et al 1995) or angiotensin II-induced (Rajagopalan et al 1996) hypertension, enhanced endothelial O_2^- production has been reported. Lucigenin chemiluminescence has shown increased levels of O_2^- in several models of hypertension (Grunfeld et al 1995; Rajagopalan et al 1996). Direct measurement of NO using a porphyrinic microsensor showed a reduction in endothelial NO generation in both spontaneously hypertensive rats (SHR) (Broukouych et al 1999) and stroke-prone spontaneously hypertensive rats (SHRSP) (Grunfeld et al 1995), which was associated with increased O_2^- levels.

Loss of NO mediated-vasodilation (related to increased release of O_2^-) also occurs in diabetes (Landmesser et al 2001). Diabetes has been associated with increased NAD(P)H oxidase activity (Landmesser et al 2001). Human endothelial cells treated with a high glucose concentration have been shown to increase both O_2^- levels and DNA synthesis (Zanetti et al 2001).

O_2^- may contribute to the pathogenesis of atherosclerosis, in addition to being an effective scavenger of NO, by mediating LDL oxidation. The altered bioactivity of oxidized compared with unoxidized LDL was first reported by Chrisolm and colleagues (1983). It was shown that monocyetes/ macrophages, the major inflammatory cell component of atherosclerotic lesions, could cause LDL oxidation (Cathcart et al 2004). Macrophage-mediated LDL oxidation has been shown to be entirely dependent on the production of O_2^- by the NADPH oxidase enzyme complex (Cathcart et al 2004). Myeloperoxidase and ceruloplasmin have also been implicated in mediating lipid and LDL oxidation in atherosclerotic lesions (Chisolm et al 1999). O_2^- is a required substrate or cofactor in the oxidation reactions catalysed by these enzymes, particularly myeloperoxidase, ceruloplasmin, and lipoxygenases (Cathcart et al 2004).

In non-vascular tissues, XO, mitochondrial oxidases and arachidonic acid are the major sources of oxidative molecules, whereas NADPH oxidase appears to be the most important source of O_2^- in vascular cells (Griendling et al 1994). The NAD(P)H enzyme comprises five subunits: three cytosolic subunits, namely, $p22^{phox}$, $p47^{phox}$, and a low weight molecular weight G protein (Rac 1 or Rac 2), and two membrane associated subunits $p22^{phox}$ and $gp91^{phox}$ (Ying et al 2004). The assembling of cytosolic subunits of NAD(P)H oxidase to the membrane facilitates electron transfer from NAD(P)H to molecular oxygen and leads to the production of O_2^-. Relative mRNA expression of NAD(P)H subunits $p67^{phox}$ (2.5%) and $gp91^{phox}$ (1.1%) are much lower in endothelial cells than in leukocytes (Ying et al 2004). It is suggested that the expression level of these two subunits may be the rate limiting factors for O_2^- production. NAD(P)H oxidase is expressed in endothelial cells, smooth muscle cells and adventitial fibroblasts. It is upregulated by angiotensin II (Ying et al 2004), sheer stress and cytochromes. In cultured vascular smooth muscle angiotensin II, platelet derived growth factor (PDGF-BB) and thrombin potently activate NAD(P)H oxidase

whereas, in cultured endothelial cells, angiotensin II, vascular endothelial cell growth factor, tumour necrosis factor-alpha and lysophosphatidylcholine were found to activate NAD(P)H oxidase (Cai et al 2000).

In humans treatment with AT_1 receptor antagonists not only reduces blood pressure, but also attenuates O_2^- production, presumably by inhibiting this oxidase (Bobik et al 2003). In spontaneously hypertensive rats AT_1 receptor antagonism lowers vascular O_2^- levels by in part down regulating $p22^{phox}$. The importance of NAD(P)H enzymes in blood pressure regulation has been demonstrated in $p47^{phox}$ -/- mice, in which the rise in blood pressure in response to angiotensin II is severely blunted compared to wild type $p47^{phox}$ +/+ mice (Bobik et al 2003).

XO is capable of generating O_2^- and hydrogen peroxide (H_2O_2). Recently it has been shown that vascular endothelial cells possess glycosaminoglycan (GAG) receptors, which reversibly bind XO via saturable, high affinity binding sites (White et al 1996). This serves to concentrate the enzyme at the cell surface, which when supplied by its substrates xanthine or hypoxanthine generates O_2^- and H_2O_2 by reducing O_2. This association may be relevant to atherosclerosis, since it has been argued that an early response to hypercholesterolaemia is a localized hypoxia in the vessel wall, which would lead to ATP catabolism and the formation of XO substrate (White et al 1996). These substrates accumulate under ischaemic or hypoxic conditions. A XO inhibitor, oxypurinol was used to reduce both O_2^- and blood pressure in the spontaneously hypertensive rat (SHR) model of hypertension (Nakazono et al 1991). Similarly, XO inhibition with allopurinol in a rabbit model of hypercholesterolaemia resulted in increased relaxations to Ach and reduced O_2^- (White et al 1996). Human saphenous veins and internal mammary arteries of patients undergoing CABG treated with a specific XO inhibitor (allopurinol) showed a reduction in O_2^- production of approximately 32% and 42% respectively (Raha et al 2000). Thus XO is an important source of O_2^- present in endothelial dysfunction.

In cardiovascular diseases it has been demonstrated that eNOS, when uncoupled from the essential cofactor tetrahydrobiopterin (BH_4), can act as a source O_2^- (Gover et al 2001). Under normal conditions eNOS produces NO and low levels of $ONOO^-$. (For normal function, eNOS requires substrates NADPH, L-arginine and O_2 and the

cofactors FMN, FAD, BH4, Calmodulin, and Ca^{2+}). When the cofactor BH_4 is not present in sufficient concentration oxidation of arginine and the reductions of molecular oxygen by eNOS is 'uncoupled' resulting in the incomplete reduction of molecular oxygen to O_2^- (Gover et al 2001). NOS has been shown to be an important source of O_2^- in animal models of hypertension (Bobik et al 2003). In patients BH_4 has been shown to improve endothelium-dependent vasodilation in hypercholesterolaemia (Stroes et al 1997), coronary artery disease (Tiefenbacher et al 2000) and diabetes (Heitzet et al 2000). The availability of L-arginine also plays an important role in the generation of O_2^- by eNOS (Gover et al 2001). N^G-nitric-L - Arginine methyl ester (L-NAME) results in increased O_2^- production. In controls O_2^- detection increased by 63% in the pressence of L-NAME, this is the result when eNOS is functioning normally. In CCF patients, incorporation of L-NAME reduced O_2^- production by 39%, indicating O_2^- production by eNOS uncoupling and the importance of L-arginine (Dixon et al 2003).

O_2^- is produced by the reduction of molecular oxygen by one electron, which has two paired electrons (Beckman et al 1996).

$$\cdot O\text{-}O\cdot + 1e^- \rightarrow {}^-{:}O\text{-}O\cdot \ (O_2^-)$$

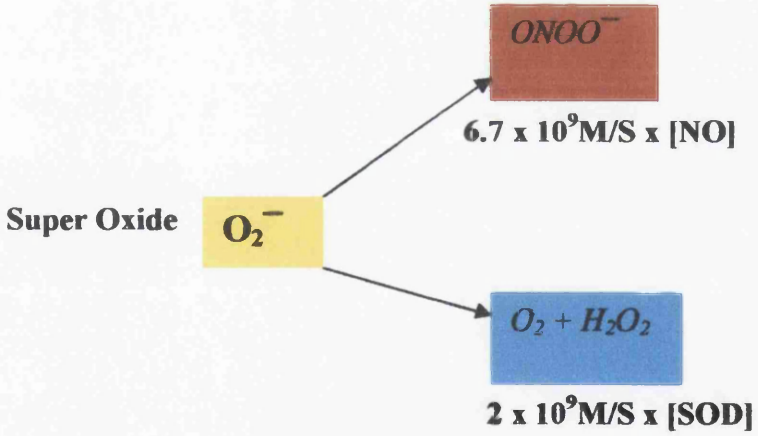
Consequently, superoxide still has one unpaired electron that rapidly combines with NO, with the release of 22 kcal/mol energy (Beckman et al 1996).

$$^-{:}O\text{-}O\cdot + \cdot N\text{-}O \rightarrow {}^-{:}O\text{-}O\text{-}N\text{-}O$$

The large Gibbs energy makes the formation of peroxynitrite ($ONOO^-$) essentially irreversible. The reaction rate for $ONOO^-$ was determined to be $6.7\pm 0.9 \times 10^9$M/S, which is approximately 6 times faster than the competing reaction of SOD for O_2^-, which is itself the fastest known enzyme-catalysed reaction (2×10^9M/S), fig 8 (Beckman et al 1996).

Figure 8 The reaction rates of O_2^- with NO and SOD (Adapted from Beckman et al 1996)

Superoxide (O_2^-) concentrations are controlled by reaction rates with possible targets multiplied by the target concentration. Superoxide dimutase (SOD) is present in micromolar concentrations and is generally the major drain of removing O_2^-. Concentrations of nitric oxide (NO) that cause vasorelaxation are on the order of 5-10nM and will not effectively compete with SOD for O_2^-. When the concentration of nitric oxide rises to micromolar concentrations, it can effectively compete with SOD because of its rapid rate constant.



The interaction between O_2^- and NO leads to the formation of $ONOO^-$; $ONOO^-$ is more reactive and damaging than its precursors, therefore more toxic to cells than H_2O_2, O_2^- and NO (Murphy et al 1998). $ONOO^-$ has been associated with atherosclerosis, hypertension, ischaemia-reperfusion injury, rheumatoid arthritis, and neurodegenerative diseases. The major limitations of $ONOO^-$ production are the amount of NO and O_2^- produced and the amount of other reactants (especially oxyhaemoglobin and SOD respectively) available to compete for them. $ONOO^-$ has a relatively long half-life of approximately 1.9s at pH 7.4, this permits diffusion across several cell diameters. $ONOO^-$ decomposes to generate a potent oxidant similar to hydroxyl radical (HO·) in reactivity (Beckman et al 1990), and it may cross cell membranes through anion channels as has been demonstrated for O_2^- (Murphy et al 1998).

$ONOO^-$ is capable of oxidising iron/sulphur centres, lipids, zinc fingers, cytochrome C, and protein thiols in cells. In the main, the majority of $ONOO^-$ formed will react with CO_2 in the blood and with thiols in cells. In vivo $ONOO^-$ reacts with CO_2 to produce nitroperoxocarbonate ($ONO_2CO_2^-$), which is short lived and decomposes to carbon dioxide and nitrate. $ONOO^-$ also reacts with haemoglobin, haem-containing peroxidases, glutathione synthase and proteins containing zinc-thiolate centres, including DNA-binding transcription factors (Fennell 2002). When $ONOO^-$ reacts with glutathione it generates oxidized glutathione and O_2^-, which are scavenged by glutathione reductase and SOD respectively. This seems to be the main cellular system of protection against $ONOO^-$; there are no specific enzymes or antioxidants for $ONOO^-$ (Fennell 2002).

$ONOO^-$ inactivates antioxidants such as glutathione synthase, cysteine and α-topopherol. The reaction with CO_2 results in the generation of more reactive species (Murphy et al 1998). $ONOO^-$ reaction compounds (largely $^{\cdot}NO_2$ and CO_3^-) produce hydroxyl radicals, initiate lipid peroxidation, nitrate tyrosine residues and damage DNA (Murphy et al 1998). This results in disruption of the cell membranes, cell signalling and cell survival (Beckman et al 1996).

$ONOO^-$ inactivates MnSOD, thus increasing the amount of O_2^- available to react with NO and establishing an autocatalytic spiral in increasing mitochondrial peroxynitrite formation (Murphy et al 1998). $ONOO^-$ has also been associated with the uncoupling of NOS by BH_4 oxidation leading to another positive feed back loop of O_2^- production (Milstien et al 1999).

Large amounts of $ONOO^-$ rapidly lead to cell death due to overbearing oxidative stress. This can occur in vivo under conditions of ischaemia-reperfusion or inflammation. Lower levels of $ONOO^-$ formation can still result in cell death by induction of apoptosis (Murphy et al 1998).

In vivo $ONOO^-$ oxidation of proteins, nitration of protein tyrosine residues to 3-nitrotyrosine is widespread in human atherosclerotic lesions. Because nitrotyrosine is negatively charged, its incorporation disrupts protein shape and function (Beckman et al 1996). Tyrosine nitration also blocks tyrosine phosphorylation thereby disrupting tyrosine kinase signalling pathways. Tyrosine nitration is also believed to modify cytochrome C, an important component of both the mitochondrial electron transport chain and apoptosis (Murphy et al 1998).

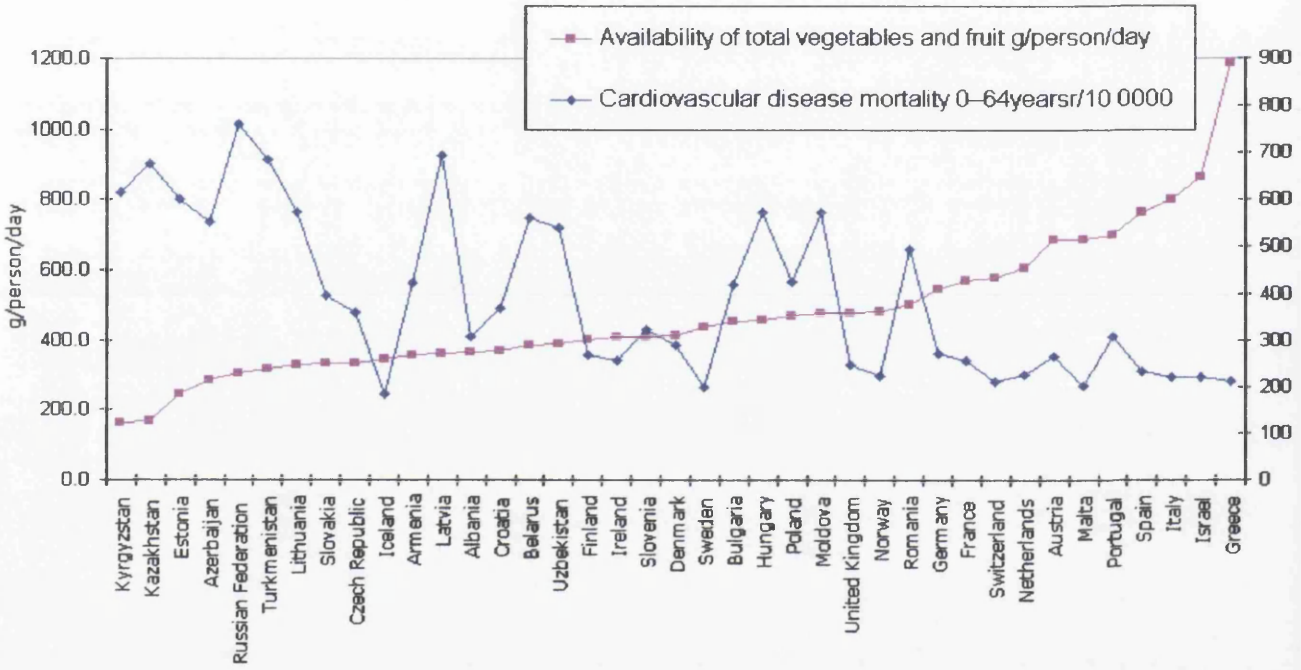
$ONOO^-$ causes generalized oxidative damage, reacting with DNA to form 8-hydroxydeoxyguanosine and single –strand DNA breaks; it causes protein oxidative damage and the formation of protein carbonyls; induces lipid peroxidation; oxidises thiols; and hydroxylates phenolics (Murphy et al 1998). $ONOO^-$ can damage DNA by reacting directly with DNA, inhibit DNA repair processes and can damage it by increasing production of H_2O_2 and alkylating agents (e.g. nitrosamines) both of which are genotoxic (Fennell 2002).

$ONOO^-$ has some vasorelaxant properties but is less potent than NO, being very short-lived (Beckman et al 1996). $ONOO^-$ like NO causes vessel relaxation increases cGMP in smooth muscle and inhibits platelet aggregation and leukocyte adhesion (Beckman et al 1996). These properties may be the result of the reaction of $ONOO^-$ with thiols producing S-nitrothiols, which release NO (Murphy et al 1998). However these effects are very short lived.

1.4 Fruit and vegetable consumption, vitamin C and E trials, and Cardiovascular Protection

Numerous ecological and epidemiological studies strongly indicate that a high consumption of fruit and vegetables is associated with a reduced risk of cancer (American Institute for Cancer research, 1997) and cardiovascular disease (Joshipura 2001; Liu et al 2000), Fig 9.

Figure 9; Consumption of fruit and vegetables and incidence of CVD mortality (BHF-statistic data base, 2002)



Despite cultural differences in cuisines worldwide that are associated with different macronutrient profiles, there are some shared characteristics of healthy dietary patterns. Most notably they include fruit and vegetables, legumes, whole grains, and fish, and because of this, all are high in fibre, relatively high in ω-3 fatty acids, and low in saturated fat, trans fat, and dietary cholesterol (Hu et al 2002). There have been a few prospective cohort studies examining the cardio-protective effects of fruit and vegetables that have reported negative findings (Ness et al 1997). However most studies included in the meta-analysis carried out by Ness were consistent with a strong protective effect of fruit and vegetables for stroke and a weaker protective effect for coronary heart disease. The Mediterranean diet, which is rich in fruit and

vegetables that have been minimally processed, has been reported to reduce the recurrence of myocardial infarction in the Lyons Heart Study (de Lorgeril 1999).

Other studies in which highly selected populations underwent short-term intensive dietary interventions increasing fruit and vegetable intake substantially, a rise in plasma antioxidant concentrations and lowering of blood pressure was observed (Appel et al 1997).

A randomised, controlled trial investigating the effects of a 6-month intervention to increase fruit and vegetable consumption to an extra five portions a day, also produced favourable results. The study was carried out in 690 participants from a healthy general population. It was found that plasma concentrations of α-carotene, β-carotene, luetin, and ascorbic acid increased by more in the invention group than in the control group. Groups did not differ for changes in lycopene, retinal, α-tocopherol, γ-tocopherol, or total cholesterol concentrations. Systolic blood pressure fell more in the intervention group than in controls (difference=4.0 mmHg, 2.0-6.0; $p<0.0001$), as did diastolic blood pressure (1.5mm Hg, 0.2-2.7; $p=0.02$). It was concluded that the intervention on fruit and vegetable consumption leading to a rise in plasma antioxidants and lowering of blood pressure would be expected to reduce cardiovascular disease in the general population (John et al 2002). The results were in accordance of the DASH (Dietary Approaches to Stop Hypertension trial) (Appel et al 1997).

A much larger study conducted by Bazzano et al (2002), took advantage of the large participant size and prolonged follow up experience of participants in the National Health and Nutrition Examination Survey Epidemiolgic Follow-up Study (NHEFS) to examine the association between fruit and vegetable intake and the risk of subsequent cardiovascular disease. 9608 adults took part in the study, aged 25-74 years, who were free of cardiovascular disease at the time of their baseline examination (between 1971 and 1975). Fruit and vegetable intake at baseline was measured with a food-frequency questionnaire. The incidence of and mortality from cardiovascular disease was obtained from medical records and death certificates. Over approximately the next 19 years, 888 strokes (218 fatal), 1786 ischaemic heart disease events (639 fatal), 1145 cardiovascular disease deaths, and 2530 all-cause deaths were documented.

Consuming fruit and vegetable more than three times a day compared with once a day was associated with a 27% lower stroke incidence {relative risk}, 42% lower stroke mortality, 24% lower ischaemic heart disease mortality, and 15% lower all-cause mortality after adjustment for the established cardiovascular disease risk factors. The results showed a strong inverse association of fruit and vegetable intake with the risk of cardiovascular disease and all-cause mortality in the general US population. As a result an increase in consumption of fruit and vegetables to 400g or five portions a day has been advocated by national and international bodies (James et al 1988, Report of WHO study group 1990) on the assumption that such a change would reduce the incidence both of cancer and cardiovascular disease.

The mounting evidence that consumption of fruit and vegetables benefits our health and has cardio-protective properties has lead to research into what bioactive compounds, the extra nutritional constituents that naturally occur in small quantities in plant products and lipid rich foods (Kitts et al 1994) are contributing most to this apparent cardio-protective effect. Observational epidemiologic studies suggest that dietary nutrients such as potassium, antioxidants, and folic acid abundant in fruit and vegetables are associated with a lower incidence of mortality from cardiovascular disease (Khaw et al 1987; Tribble et al 1999). In the Nurses Health Study and the Health Professionals follow up study, a 4% reduction in coronary heart disease for each increase in serving of fruit and vegetable intake per day was noted (Joshipura et al 2001). Green leafy vegetables and vitamin C rich fruit and vegetables were reported to contribute the most to this apparent protective effect. Vitamin C has been found to improve vascular endothelium function in vitro, by increasing nitric oxide bioavailability (Ulker et al 2003).

Vitamin C and E have been reported as the most important antioxidants (Frei et al 1989). Vitamin C alone or in combination with vitamin E has been shown to enhance NO generation and reduce blood pressure in hypertensive animals (Xu et al 2000). Vitamin C is an important water-soluble antioxidant in human plasma and is capable of scavenging oxygen derived free radicals such as superoxide anion (Frei et al 1989). Studies of administration of intra-arterial vitamin C in the forearm in subjects with cardiovascular risk factors, other than age, have reported increased endothelium-dependent relaxation in forearm resistance vessels (Landmesser et al 2001; Taddei et

al 1995). In one study Taddei et al (1995), observed that impaired endothelium-dependent relaxation in response to acetylcholine was reversible by vitamin C in healthy sedentary older subjects.

Vitamin E supplementation improves cell-mediated immunity in mice and in humans. In addition to modulating the oxidation of low-density lipoproteins, vitamin E can modulate immune/ endothelial cells interactions, thus reducing the risk of CVD (Meydani et al 1999). Vitamin E is reported to be a major antioxidant in LDL particles; it exerts potent antioxidant effect against free-radical-mediated oxidation of LDL, but is not capable of inhibiting LDL oxidation mediated by non-radical mechanisms (Diaz et al 1997). However, when vitamin E works as an antioxidant it is oxidized to harmful radicals which need to be reduced back to a α-tocopherol, e.g., by vitamin C (Packer et al 1979).

A study investigating the antioxidant effects of both vitamin C and E found that both had protective properties in vitro in a model of genetic hypertension associated with enhanced oxidative stress. The ability of vitamin C (10 to 100μol/L) or E (100μol/L) to modulate vascular function by regulating enzymatic activities of endothelial nitric oxide synthase and NAD(P)H oxidase, in the thoracic arteries of male spontaneously hypertensive rats and their normotensive counterparts Wistar-Kyoto rats was examined. The results showed that both vitamins improved endothelial function, reduced O_2^- production as well as NAD(P)H oxidase activity, and increased eNOS activity and NO generation in SHR aortas to the levels observed in the aortas of the WKY vitamin treated rats. It was concluded that endothelial NAD(P)H oxidase is the major source of vascular O_2^- in SHR and that vitamin C and E are critical in normalizing genetic endothelial dysfunction through the regulation of eNOS and NAD(P)H oxidase activities (Ülker et al 2003).

In a prospective study; the Antioxidant Supplementation in Atherosclerosis Prevention (ASAP), a 6-year study examining the combinational effect of 136 IU of vitamin E plus 250mg of slow-release vitamin C given twice daily, it was concluded that supplementation with this combination slowed down atherosclerotic progression in hypercholesterolemic patients (Salonen et al 2003). 440 adults aged 45 to 69 years with serum cholesterol ≥ 5.0mmol/L completed the study. The common carotid artery

intima-media thickness was measured. In both sexes combined, the average annual increase of the mean was 0.014mm in the un-supplemented and only 0.010mm in the supplemented group. The effect was larger in subjects with either low baseline plasma vitamin C levels or CCA plaques. Vitamin E had no effect on HDL cholesterol (Salonen et al 2003).

However, in prospective placebo controlled clinical trials the protective effects of oral vitamin supplements is not a constent finding. The MRC/ BHF Heart Protection Study on antioxidant vitamin supplementation had 20, 536 UK high-risk patients, aged 40-80 years with coronary heart disease, or other occlusive arterial disease including diabetic participants. The subjects were allocated randomly to receive antioxidant vitamin supplementation (600mg Vitamin E, 250 mg Vitamin C, and 20 mg β-carotene daily) or matching placebo. An average of 83% of participants in each treatment group remained compliant during the scheduled 5-year treatment period. The results showed plasma concentrations of α-tocopherol had doubled, vitamin C had gone up by a third and β- carotene had quadrupled. Despite the increase in blood vitamin levels there was no significant differences in the 5 year mortality from, or incidence of, any type of vascular disease, cancer, or other major outcome (Heart protection study group 2002).

In the Primary Prevention Project (PPP- 2001) a randomised controlled open 2x2 factorial trail in 4495 people, mean age 64.4 years with one or more of the following: hypertension, hypercholesterolaemia, diabetes, obesity, family history of premature myocardial infarction, the effects of low-dose aspirin (100mg/day) and vitamin E (300mg/day) were investigated. It was concluded that low-dose aspirin given in addition to treatment of specific risk factors contributed an additional effect, but that vitamin E showed no additional protective effect. Other intervention studies which do not support the hypothesis, that vitamins are responsible for the CVD and cancer protective effects, observed when intake of fruit and vegetables is high include the following trials; Alpha-Tocopherol 1994; Omenn et al 1996; Hennekenset et al 1996; The Heart Outcomes Prevention Evaluation Study 2000. This lead to the conclusion that mechanisms other than antioxidant vitamin content must contribute to the health benefits of eating fruit and vegetables (Appel et al 1997).

Indeed one study showed treatment with the Mediterranean diet but not oral vitamin C improved endothelium dependent and endothelium independent dilation (Singh et al 2002). Vitamins do not explain the "French paradox", that is why, despite a high saturated fat diet short in vitamins, the French have a lower incidence of coronary heart disease (Ulbricht et al Southgate 1991; Renaud et al 1992). Epidemiological studies have suggested that the antioxidants in red wine may be responsible for this finding. Red wine is a rich concentrated source of polyphenolic substances (Kris-Etherton et al 2002).

Polyphenol compounds are present in virtually all plant foods; green tea, red wine, apple, orange juices, cocoa and legumes are plant foods that are particularly high in polyphenols. Flavonoids (a major class of polyphenols) and other phenolic compounds appear to contribute highly to the antioxidant capacity observed in certain fruit and vegetables (Gao et al 2001). The antioxidant capacities, measured as oxygen radical absorbance (ORAC) of some flavonoids, have been reported to be several times stronger on a molar basis than vitamins C and E (Gao et al 1999, Wang et al 1997). Population studies have reported an inverse association between flavanoid intake and the risk of coronary disease (Hertog et al 1996). The phenolic compounds identified in red wine include phenolic acids, flavonols, monomeric catechins, and polymeric anthocyanidins. Catechin, a flavan-3-ol compound is the most abundant polyphenol in red wine and is present at concentrations up to 300mg/ml (Singleton et al 1988). Flavanols (quercetin and kaempferol) are present in smaller concentration approximately 30mg/ml, and phenolic acids at 140mg/l. All of these phenolic compounds have been shown to have antioxidant properties in vitro (Frankel et al 1993). Studies have shown that red wine inhibits oxidation of LDL in vitro (Frankel et al 1993) and increases the antioxidant capacity in plasma. There is evidence that phenolic compounds have anti-thrombotic properties that appear to be the result of reduced susceptibility of platelet aggregation, reduced synthesis of prothrombotic and proinflammatory mediators, decreased expression of adhesion molecules, and tissue factor activity (Rotondo et al 2000). There is also evidence that polyphenols can improve endothelium function by modulating the production of nitric oxide, resulting in vasorelaxation (Rotondo et al 2000).

The effects of polyphenols appear to be independent of whether the source is wine or grape. In one study it was found that juice from purple grapes (7ml/kg body weight per day for 14 days) decreased platelet aggregation (58% vs. 39% control), increased platelet derived nitric oxide release (6.0pmol/10^8 platelets vs. 3.5 pmol/10^8 platelets), and reduced O_2^- production (30 vs. 19 Arbitury units). Thus it was concluded that these protective effects were independent from alcohol consumption. A number of studies have found that moderate alcohol consumption (1 to 3 drinks/day) attenuates the risk of CHD (Rotondo et al 2000). However other studies have shown that only red wine has protective effects, beer and sprits being with out effect (Truelssen et al 1998). Therapeutic properties of polyphenols have been reported from food sources other than grapes, such as semisweet chocolate (Rein et al 2000) and green tea (Negishi et al 2004, Ying et al 2004).

1.5 Polyphenols: Chemistry metabolism and structure activity relationship

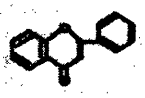
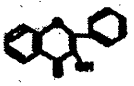
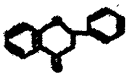
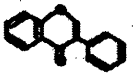
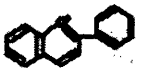
Polyphenols are a class of phytochemicals found in high concentrations in wine, tea and grapes (Harbory et al 1997). Phenolic compounds are ubiquitous in the plant kingdom and the term refers to substances that possess an aromatic ring bearing one or more hydroxyl substituients (Rice-Evans et al 1996).

Over 8000 phenolic compounds have been identified in a dozen chemical sub-categories. The major dietary families of polyphenols are the flavan-3-ols, flavonols, flavanones and anthocyanins. Over 4,000 flavonoids have been identified. These are one of the most nutritionally important classes of polyphenols and are widley distributed in plant food, Table 1.1.

Natural polyphenols can range from simple molecules such as phenolic acid to large highly polymerised compounds such as tannins. Conjugated forms of polyphenols are the most common, where various sugar molecules, organic acids and lipids are linked with the phenolic ring structure. Differences in this conjugated chemical structure account for different chemical classification and variation in the modes of action and health giving properties of the various compounds.

Table 1.1; General structure and Trolox equivalent antioxidant activities (TEAC) of some dietary flavonoids (Adapted from Rice-Evans et al 1996)

The assay for the total antioxidant activity (TAA), or the Trolox equivalent antioxidant activity (TEAC), measures the concentration of Trolox solution with an equivalent antioxidant potential to a standard concentration of the compound under investigation. The TEAC is defined as the concentration of Trolox solution with equivalent antioxidant potential to a 1mM concentration of the compound under investigation.

Class	Structure	Flavonoid	Dietary Source	TEAC(mM)
Flavanol		(+)-catechin	Tea	2.4
		(-)-epicatechin	Tea	2.5
		Epigallocatechin gallate	Tea	4.75
			Fruit skins	1.43
Flavone		chrysin	Parsley, celery	1.45
		apigenin	Red wine, buckwheat citrus, tomato skin	2.4
		rutin		
		luteolin	Red pepper	2.1
		luteolin glucosides		1.74-0.79
Flavonol		kaempferol	Broccoli, black tea, grapefruit, leek	1.34
		quercetin	Apple skin, broccoli, onion, olive oil, lettuce, red wine, tomato, tea	4.7
		myricetin	Cranberry grapes, red wine	3.1
		tamarixetin		
Flavanone		naringin	Citrus, grapefruit	0.24
		naringenin	Citrus fruits	1.53
		taxifolin	Citrus fruits	1.9
		eriodictyol	Lemons	1.8
		hesperidin	Oranges	1.08
Isoflavone		genistin	Soybean	1.24
		genistein	Soybean	2.9
		daidzin	Soybean	1.15
		daidzein	Soybean	1.25
Anthocynidin		apigenidin	Colured fruits	2.35
		cynidin	Cherry, raspberry, strawberry	4.42

Flavonoids have the diphenylpropane (C6C3C6) skeleton (Rice-Evans et al 1996), are benzo-γ-prone derivatives consisting of phenolic and pyrane rings and are classified accordingly to substitutions, fig 10. Dietary flavonoids differ in the arrangements of hydroxyl, methoxy, and glycosidic side groups, and in the conjunction between the A- and B- rings, fig 11. During metabolism, hydroxyl groups are added, methylated, sulphated or glucuronidated. The most commonly occurring flavones and flavonols are those with dihydroxylation in the 3′ and 4′ positions of the B ring, and to a lesser extent, those with a lone B ring-hydroxyl group in the 4′ position. Most dietary flavonoids occur in food as O-glycosides and polymers (Rechner et al 2002). The

most common glycosidic unit is glucose, but other examples include glucorhamnose, galactose, arabinose, and rhamnose (Rechner et al 2002).

Figure 10 Nuclear Structure of flavonoid (Heim et al 2002)

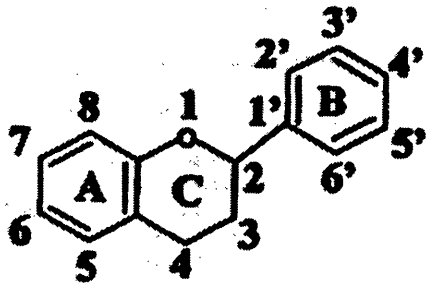
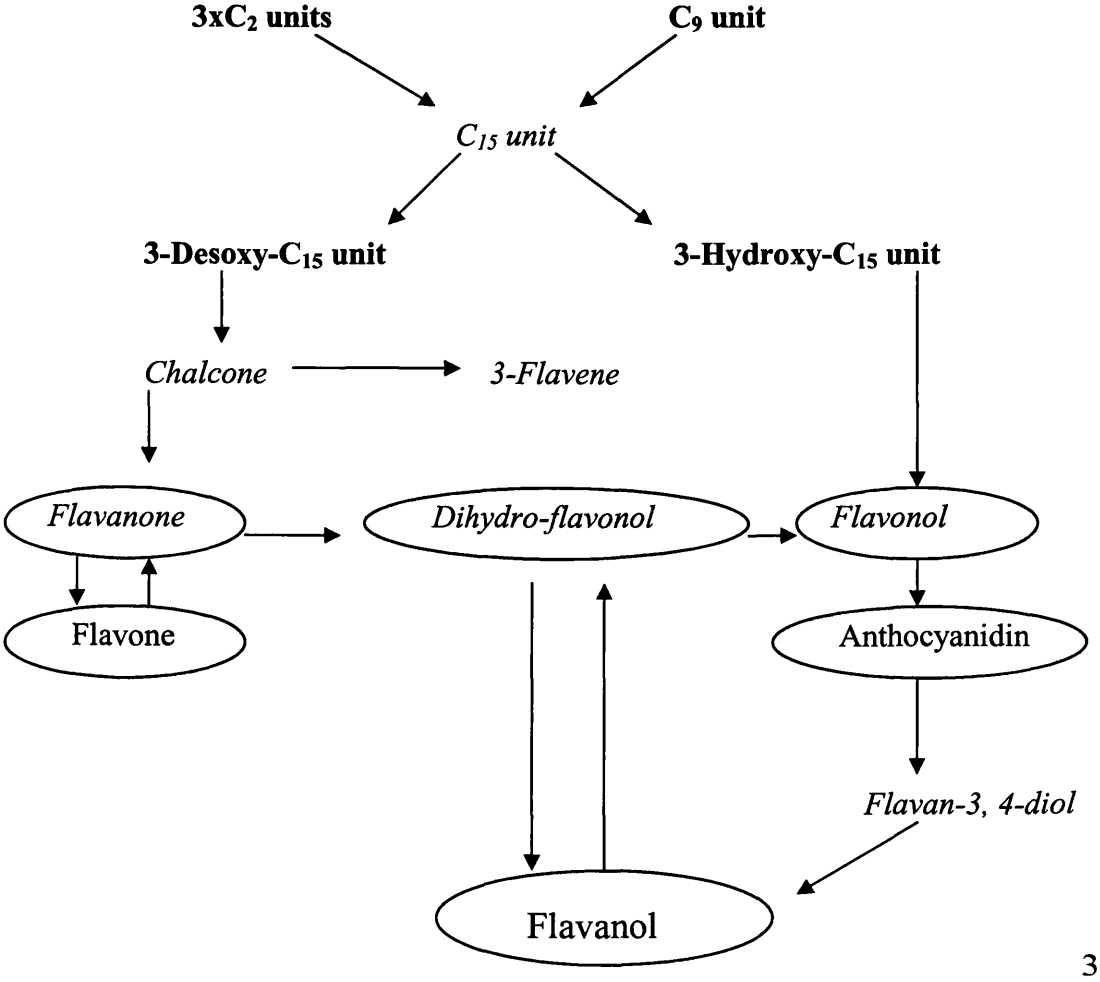


Figure 11 Interconnections of flavonoid subgroups (Adapted from Rice-Evans et al 1996)



Polyphenols particularly flavonoids are among the most potent plant antioxidants (Rechner et al 2002). Flavonoids and phenolic acids can act as anti-oxidants by a number of pathways, but perhaps the most significant is by free radical scavenging in which the phenolic compounds can break the free radical chain reaction. Polyphenols form complexes with reactive metals such as iron, zinc and copper reducing their absorption. This may seem a negative side effect (reducing nutrient absorption), but excess levels of such elements (metal cations) in the body can promote the generation of free radicals and contribute to the oxidative damage of cell membranes and cellular DNA. In general, polyphenols (flavonoids) are thought to deliver health benefits by four main mechanisms;

- Direct free radical scavenging (scavenging of hydroxyl, peroxyl, or synthetic radicals).
- Termination of the chain reaction in the lipid phase, involving peroxyl radicals and hydroperoxides.
- Protection and regeneration of other dietary antioxidants (such as ascorbate, which may reduce and recycle the flavonoid radical or vice-versa).
- Cheletion of metal ions (diavalent cations used to initiate oxidative events).

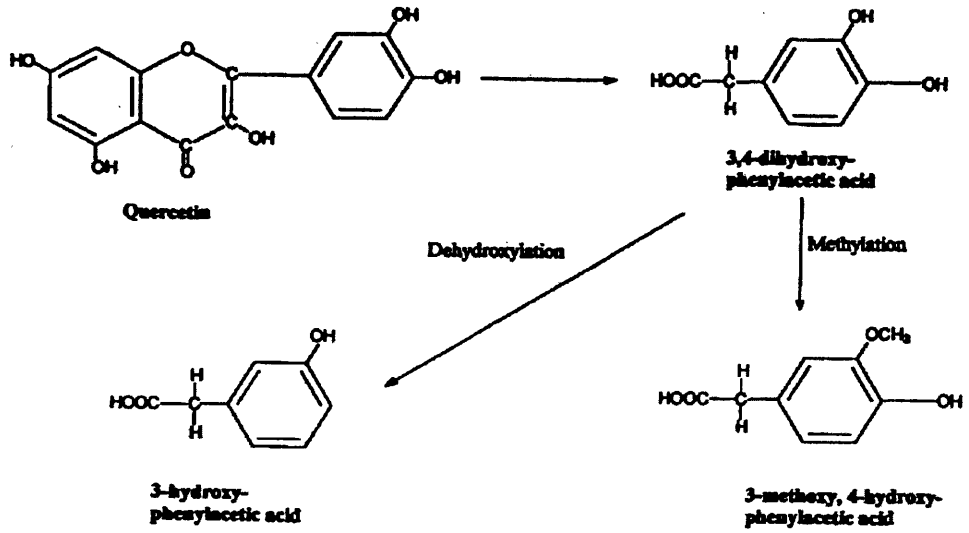
It was suggested in 1959 that flavonoids are rapidly absorbed and converted to a variety of hydroxyaromatic acids, which are rapidly eliminated in the urine (Rechner et al 2002). Due to molecular size, absorbtion of polymeric flavonoids across the intestinal epithelium requires preliminary degradation to smaller, low molecular weight compounds. Polyphenols presence in plasma appears to be largely transient, although a short half-life does not prelude these compounds from playing an important role in disease prevention. Peak concentrations of flavonoids typically occur approximately 2hr after ingestion, although one study reported peak levels at 24hours following an oral dose of epicatechin (Rechner et al 2002). In healthy volunteers, the half-life of quercetin has been reorted to be high ranging from 20-72 hours (Walle et al 2001). Provided that sufficient dietary intake is sustained, this long half-life of quercetin is conducive to accumulation in plasma and concomitant bioactive potential. Indeed several flavonoids and anthocyanins have been detected in

human plasma at levels that allow biological activity although there is less evidence regarding absorption of phenolic acids (Heim et al 2002).

Although quercetin represents but one of hundreds of dietary flavonoids, it is among the most abundant, potent, and widely studied and provides insight into the absorption and metabolism of polyphenols. The average daily intake of flavonoids in the occidental diet is 23mg, of which quercetin represents 60 to 75% (Rice-Evans et al 1996), fig 12.

The absorption kinetics varies considerably among foods, owing to the heterogeneity of sugars and other functional groups of the flavan nucleus (Rice-Evans et al 1996). Absorption may also depend on dosage, vehicle administration, antecedent diet, sex differences, and microbal population of the colon (Pannala et al 2001).

Figure 12 Representation of quercetin metabolism (Rice-Evans et al 1996)



Due to the affinity of flavaonoid hydroxyl groups for proline residues, the antioxidant capacity of catechin gallates in vitro is attenuated by presence of proteins such as β-casein (Arts et al 1998). This suggests proteins in the food itself, and the bloodstream may potentially mask the biological activity of polyhydroxylated flavonoids (Rice-Evans et al 1996). However, addition of milk to black tea had no effect on the

Evans et al 1996). However, addition of milk to black tea had no effect on the absorption of quercetin or kaempferol in a group of healthy individuals (Rice-Evans et al 1996).

During the past 15 years structure activity relationships study has generated several consistent lines of evidence supporting the role of specific structural components as requisites for radical scavenging, chelation and oxidant status, structural components of interest are;

- Hydroxyl groups
- O-methylation
- The 2-3 double bond and 4-oxo function
- Carbohydrate moieties
- Degree of polymerisation

(Heim et al 2002)

The spatial arrangement of substitiuents is perhaps a greater determinant of antioxidant activity than the flavan backbone alone. Both the configuration and total number of hydroxyl groups substantially influence several mechanisms of antioxidant activity. Free radical scavenging capacity is primarily attributed to the high reactivities of hydroxyl substituents that participate in the following reaction (Heim et al 2002):

$$F\text{-}OH + R^{\cdot} \rightarrow F\text{-}O^{\cdot} + RH$$

F-OH= Flavonoid hydroxyl group
R$^{\cdot}$=Reactive oxygen species
F-O$^{\cdot}$ = Stable flavonoid radical
RH=Hydrogen reactant

The B-ring hydroxyl configuration: this is the most significant determinant of scavenging of ROS (Rice-Evans et al 1996). Hydroxyl groups on the B-ring donate hydrogen and an electron to hydroxyl, peroxyl, and peroxynitrite radicals, stabilizing them and giving rise to relatively stable flavonoid radicals. Among structurally

homologous flavones and flavannones, peroxyl and hydroxyl scavenging increases linearly and curvilinearly, respectively, according to the total number of OH groups (Rice-Evans et al 1996).

A 3′4′-catechol stucture in the B-ring strongly enhances lipid peroxidation inhibition (Rice-Evans et al 1996). This arrangement is a salient feature of the most potent scavengers of peroxyl, superoxide, and peroxynitrite radicals (Rice-Evans et al 1996).

O-methylation: Differences in antioxidant activity between polyhydroxylated and polymethoxylated flavonoids are most likely due to differences in both hydrophobicity and molecular planarity. Although the ratio of methoxy to hydroxyl substituents does not necessarily predict the scavenging ability of a flavonoid, the B-ring is particularly sensitive to the position of the methoxy group. Alternating a 6′-OH/4′-Ome configuration to 6′-Ome/4′-OH completely abolishes the scavenging of DPPH by inducing coplanarity (Rice-Evans et al 1996).

The 2-3 unsaturated double bond in conjugation with a 4-oxo function: This is a distinguishing feature among general flavonoid structural classes (Heim et al 2002). Comparison of quercetin with taxifolin suggests that in flavonoids fulfilling other structural criteria, the 4-oxo and double bond distinguishes the better antioxidant. Quecetin is a more potent inhibitor of ferrous sulphate-induced MDA formation than taxifolin; both structures have a 4-oxo group, but taxifolin is saturated between carbons 2 and 3. Flavonoids with a 2-3 double bond in conjugation with a 4-carbonyl group exhibit lower IC_{50} values (indicative of stronger antioxidant activity) in a microsomal system compared to those with saturated heterocycles. The majority of research suggests that flavonoids lacking one or both features are less potent antioxidants than those with both elements (Heim et al 2002).

Carbohydrate moieties: Aglycones are more potent antioxidants than their corresponding glycosides (Gao et al 1999). Daidzein and genestein aglycones exhibit greater TEAC values (1.25 and 2.9) than their 7-glycosides (1.15 and 1.24, respectively) (Heim et al 2002). Aside from the presence and total number, the position and structure of the sugar plays important role. For example luteolin and quercetin aglycones significantly exceeded their 3-,4′- and 7-*O*-glucosides in retarding

the accumulaion of hydroperoxides in membrane bilayers, but a 4′- sugar was more suppressive than 3- or 7-substitution. Whether the sugar moiety is glucose, rhamnose, or rutinose is relevant. For example, compared to rutinose, a rhamnose moiety on quercetin significantly reduces scavenging of radicals generated by stimulated human neutrophils (Heim et al 2002). Aside from occupying free OH groups necessary for hydrogen abstraction and radical scavenging, any sugar substituent is capable of (i) diminishing coplanarity of the B-ring relative to the rest of the flavonoid, and/or (ii) lending hydrophilicity and altering access to lipid peroxyl and alkoxyl radicals during the propagation of lipid peroxidation (LPO) in membranes.

Polymerization of flavonoids: Procyanidin dimmers and trimers are more effective than monomeric flavonids against O_2^-, but the activities of dimmers and trimers differ little (Heim et al 2002). Tetramers exhibit greater activity against peroxynitrite and O_2^-mediated oxidation than trimers, while heptamers and hexamers demonstrate significantly greater O_2^- scavenging properties than trimers and tetramers. It appears that to a point, increasing the degree of polymerisation enhances the effectiveness of procyanidins against a variey of radical species (Heim et al 2002).

1.6 Polyphenols, vasodilation and nitric Oxide bioavailability

Polyphenols have shown many properties suggestive of an NO sparring effect. They enhance many of the important functions carried out by NO.

Polyphenols derived from red wine, grape juice and grape skin have been shown to enhance endothelium-dependent vasorelaxation of the rat-precontracted aorta (Fitzpatrick et al 1993). Wine polyphenols fed to SHRSP for 8 weeks attenuated the elevation in blood pressure and improved aortic biomechanical properties compared with control animals (Mizutani et al 1999). Red wine polyphenols encompass anthocyanins, proanthocyanidins, monomeric flavanols, flavonols, and phenolic acids, as well as stilbene derivatives. Of these resveratol (stilbene) (Orallo et al 2002), delphinindin (anthocyanidin) (Andriambelsan et al 1998), and quercetin (flavanol) (Flesch et al 1998) were the red wine polyphenols to show a vasorelaxation effect.

Andriambelson and collegues (1997) reported that concentrations of red wine extract enriched in polyphenolic compounds (RWPC) from 10^{-5} to 10^{-2} g/l elicited enhanced NO generation, cyclic guanosine 3′, 5-monophosphete accumulation and endothelial-dependent relaxation in aortic rings from Wister Kyoto Rats. The study was expanded to investigate which groups of polyphenols were able to cause endothelium-dependent vasorelaxation. RWPC were chromatographically resolved into 10 fractions in order to separate the two groups of polyphenols that contain polymerised flavanols and anthocyanins. The endothelium-dependent and –independent relaxing effects of these fractions were compared to those of commercially available polyphenols. Fractions enriched into either anthocyanins or oligomeric condensed tannins exhibited endothelium dependent vasorelaxant activity (maximal relaxation in the range of 59%-77%) compared to the original RWPC. However polymeric condensed tannins elicited a weaker vasorelaxant response than the original RWPC (maxiamal relaxation in the range 20-47%). Furthermore representatives of phenolic acid derivatives (benzoic acid, vanillic acid, gallic acid), hydroxycinnamic acid (p-coumaric acid, caffeic acid) or the flavanol [(+)-epicatechin] classes failed to induce vasorelaxation. Among the anthocyanins, delphinidin (maximal relaxation being 89%), but not malvidin or cyanidin, showed endothelium-dependent vasorelaxation (Andrambeloson et al 1998).

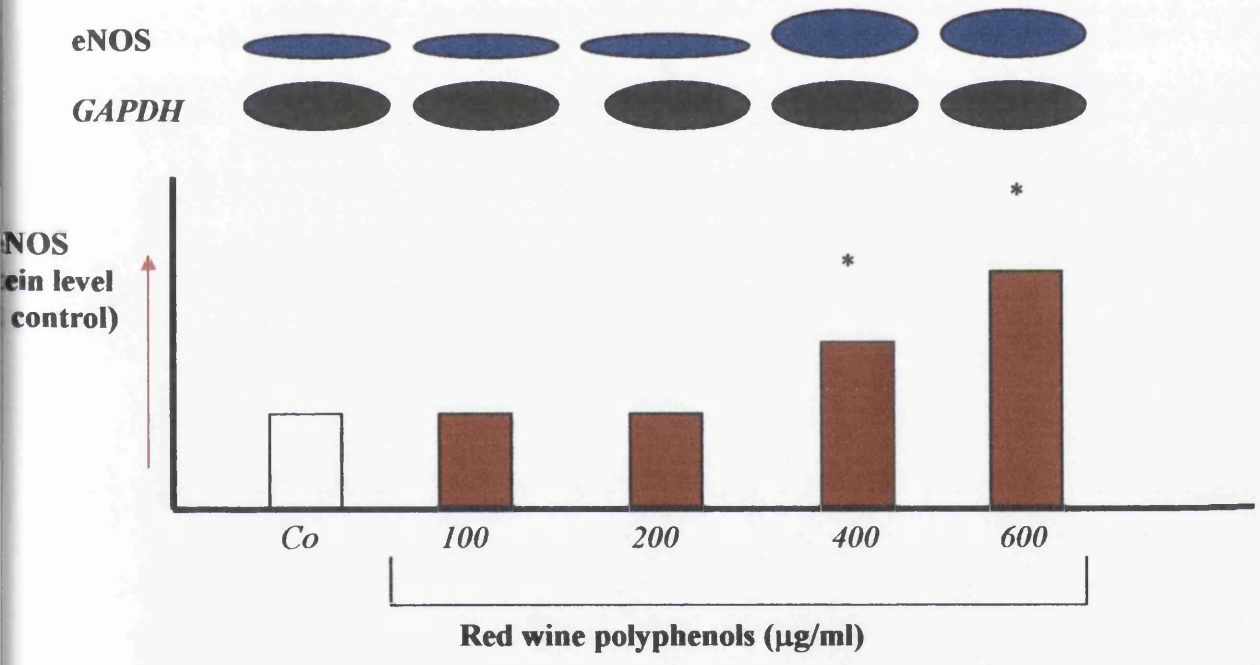
Tea polyphenols have also been shown to attenuate the development of hypertension in SHRSP (Negishi et al 2004). In one study SHRSP rats were fed either tap water (30ml/d); black tea polyphenols (3.5g/L thearubigins, 0.6 g/L flavonols and 0.4g/L catechins), or green tea polyphenols (3.5g/L catechins, 0.5g/L flavonols and 1g/L polymetric flavonoids). The intake of tea polyphenols for 3 weeks attenuated blood pressure increases measured by telemetry, in addition a marked reduction in plasma NO concentration and urinary NO excretion was observed. This data suggests that alleviation of oxidative stress by tea polyphenols diminishes ROS-mediated NO inactivation and raises the bioavailability of NO (Negishi et al 2004).

In another study (Negishi et al 2004) decreased phosphorylation of myosin light chain (MLC) in the aorta of rats fed black or green tea polyphenols occurred. This finding suggests that the inhibition of MLC phosphorylation contributes to the reduction in blood pressure. It was postulated that the phosphorylation of MLC could

be reduced by the inhibition of rho-kinase constrictor activity through an increase in NO bioavailability in the SHRSP treated with tea polyphenols (Negishi et al 2004; Chitaley et al 2002).

Leikert et al (2002) provided further data showing that an alcohol-free red wine polyphenol extract (RWPE) strongly increases NO release, eNOS activity, and eNOS expression in human umbilical vein endothelial cells, fig 13. Exposure to RWPE (100-600μg/ml) significantly increased eNOS protein levels up to 2.1 fold. Furthermore an increase in human eNOS promoter activity (up to 2-fold) in response to RWPE (18 hours, 100 to 600μg/ml) was demonstrated by a luciferase reporter gene assay.

Figure 13 schematic diagrams showing the effect of red wine polyphenols on eNOS exression. Adapted from Leikerts study (2002)



Mendes et al (2003) investigated the mechanism of vascular relaxation produced by phenolic substances from red wine. Relaxation induced by procyanin from grape seeds (GSP), anthocyanins, catechin and epicatechin were assessed in Wistar rat aortic rings either left intact or endothelium-denuded. GSP-induced relaxations were assassed in noradrenaline precontracted preparations in the presence or absence of the NO synthase inhibitor L-NAME. The preparations were incubated with reactive blue 2 (RB2, an antagonist of P2Y purinoceptors), with apyrase (an enzyme which

hydrolyses ATP and ADP) or with α-β methylene ATP (an inhibitor of ecto ATPases). The data confirmed that modest relaxations observed with cathechin and epicatechin are endothelium independent but that GSP and anthocyanins induce a relaxation which is endothelium dependent and involves the synthesis and release of NO. Inhibition of relaxation by apyrase and the increase in the presence of ecto-ATPase inhibition, suggest that GSP and anthocyanins could act via an initial release of nucleotides, which in turn could activate P2Y1 and/ or P2Y2 purinoceptors of endothelial cells, trigging the synthesis and release of NO (Mende et al 2003).

It has been known for many years that quercetin exerts systemic and coronary vasodilator effects (Perez-Vizcaino et al 2002). When given orally, it reduces blood pressure, cardiac hypertrophy, and vascular remodelling in spontaneously hypertensive rats and NO-deficient rats (Duarte et al 2001). The vasorelaxation effect of quercetin may be related to a direct action on smooth muscle cells at high concentrations, perhaps as result of cyclic nucleotide phosphodiesterase inhibition (Bertz et al 1978), although other studies suggest that it is quercetins ability to scavenge O_2^- that results in a NO sparring effect (López-López et al 2003). In an vivo study in which spontaneously hypertensive rats were fed high doses of quercetin (10mg kg^{-1}) for 5 weeks quercetin reduced the elevated blood pressure, the cardiac and renal hypertrophy and the functional vascular changes in SHR without having any effect in WKY. These effects were associated with a reduced oxidant status due to antioxidant properties of quercetin (Duarte 2001).

The anthocyanin delphinidin elicits endothelium dependent relaxant (Andriambeloson et al 1998). This action depends on the ability of delphinidin to stimulate NO production through the increase in cytosolic calcium, independently of its anti-oxidant property (Martin et al 2002). In addition, delphinidin has been recently reported to inhibit the growth of a human tumour cell line by shutting off the epidermal growth factor receptor downstream signalling cascade. Delphinidin can activate ERK-1/2 pathway resulting in an over expression of e-NOS in bovine aortic endothelial cells leading to increased NO production and protection against apoptosis (Martin et al 2002).

Several reports have suggested that flavanoid consumption may inhibit platelet aggregation (Wolfram et al 2002; Hertog et al 1996). Grape polyphenols administered to rats reduced thrombin-induced platelet aggregation (Xia et al 1998) and grape juice inhibited platelet aggregation and thrombosis in stenosed canine coronary arteries (Demrow et al 1995). Wolfram examined the effect of a 1-month consumption of 500ml black tea containing 2.0mg quercetin in healthy human volunteers. A single administration of tea did not effect plasma 8-epi-$PGF_{2\alpha}$ levels, 11-DH-TXB2, or ADP-induced platelet aggregation. However after 1-week and to a greater extent 1 month regular tea intake, there was a significant decrease in most of these parameters (Wolfram et al 2002).

Onion juice has been shown to inhibit in vitro human platelet aggregation (Goldman et al 1996). In another study dogs with mechanically damaged and stenosed coronary arteries, with periodic platelet-mediated thrombus formation followed by embolization, were administered 0.09±0.01mL/kg onion juice intravenously. This resulted in a reduction in collagen-induced whole-blood platelet aggregation (ex vivo). It was concluded that consumption of raw onion might help prevent platelet-mediated cardiovascular disorders. A greater effect was observed with dog's blood than humans in in-vitro experiments (Briggs et al 2001).

Quercetin and the flavone apigenin (2500μmol/l) significantly inhibited collagen- and ADP- induced aggregation in platelet-rich plasma (Janssen et al 1998). However lower concentrations that might occur in vivo had no effect. Moreover in a study in which 18 healthy volunteers were fed 220g onions/d providing 114mg quercetin/d, 5g dried parsley/d providing 84mg apigenin/d, or a placebo for 7 days in a randomised cross over study no significant effects of onion or parsley were found on platelet aggregation, thromboxane B_2 production, factor VII, or other hemostatic variables. It was concluded that the antiaggregatory effects of flavonoids seen in vitro are due to concentrations that cannot be attained in vivo (Janssen et al 1998)

As well as inhibiting platelet aggregation there is evidence of polyphenols reducing cell proliferation and inflammatory mediators. Phenolic acids, such as caffeic acid have been shown to protect cultured endothelial cells against the cytotoxic effects of

low-density lipoprotein (LDL) oxidation, possibly by blocking the intracellular signalling triggered by LDL oxidation (Vieria et al 1998). Caffeic acid is a specific inhibitor of nuclear transcription (NF-κB) (Natarajan et al 1996).

1.7 The super oxide scavenging ability of polyphenols

Reactive oxygen species (ROS), especially O_2^- and hydroxyl radical (OH•), are important signalling molecules in cardiovascular cells that influence both normal and abnormal cell processes, including cellular growth, hypertrophy, remodelling, lipid oxidation, modulation of vascular tone, endothelial permeability and adhesion for leukocytes (Lum et al 2001). The main flavonoids of interest are anthocyanins, flavon-3-ols, and their polymeric products flavanones, flavonols, and flavones (Haslam et al 1998). In vitro these compounds have been shown to scavenge O_2^-, OH•, and peroxyl (ROO•) (Van Acker et al 1996), inhibiting lipid peroxidation (Vinson et al 1998), and protecting LDL against oxidation (Furhman et al 1995). A 3'4'-catechol structure in the B-ring strongly enhances lipid peroxidation inhibition; this feature of flavonoids allows potent scavenging of O_2^- and $ONOO^-$ (Hu et al 2002).

Oxidative modification of LDL by free radicals particularly O_2^- is an early event in the pathogenesis of atherosclerosis. The rapid uptake of oxidatively modified LDL via scavenger receptors leads to the formation of foam cells. Oxidised LDL also has a number of other atherogenic properties. A number of mechanisms are likely to contribute to inhibition of LDL oxidation by flavonoids (Rajnarayana et al 2001). Flavonoids may directly scavenge O_2^- by acting as chain breaking antioxidants. In addition, they can recycle other chain-breaking antioxidants such as α-tocopherol by donating a hydrogen atom to the tocopheryl radical (Francel et al 1993). Transition metals such as iron and copper are important pro-oxidants, and some flavonoids can chelate divalent metal ions, hence preventing free radical formation.

The flavonol quercetin has free radical-scavenging effects (Robak et al 1988; Van Acker et al 1996), inhibits low-density lipoprotein peroxidation, and reduces the progression of atherosclerosis in vivo (Hayek et al 1997). In SHR rats, quercetin reduced the oxidative status, as indicated by lower concentrations of markers of

oxidative stress including plasma and hepatic malondialdehyde and urinary isoprostane-$F_2\alpha$ (Duarte et al 2001).

The effects of quercetin (10mg/kg) and the anti-hypertensive drug verapamil (20mg/kg/day) administered daily, on hypertension and oxidative status in different tissues in a DOCA-salt hypertension model in rats were compared (Galisteo et al 2004). Both compounds reduced systolic blood pressure and significantly reduced hepatic and renal hypertrophy. Only quercetin reduced cardiac hypertrophy and improved endothelium-dependent relaxation to acetylcholine of aortic rings from DOCA rats (Galisteo et al 2004). Increased plasma and heart thiobarbituric acid reactive substances (TBARS) and total glutathione (GSH) levels in the heart, with decreased liver glutathione peroxidase (GPX) and liver and kidney glutathione transferase (GST) activities were observed in the DOCA-salt-treated rats compared to the control animals. In the above study the antihypertensive effect of quercetin was accompanied by normalisation of plasma TBARS, improvement of the antioxidant defence systems in the heart and liver restoring total GSH levels in both organs and altered liver GST and GPX activities, and improving kidney GST activity. Verapmil treatment restored GSH levels in heart, but had no effect on the other observed alterations induced in DOCA chronic-salt administration. Thus quercetin showed both antihypertensive and antioxidant properties, while verapmil exhibited only antihypertensive effects (Galisteo et al 2004).

Cyanidin-3-O-glucoside is a scavenger of $ONOO^-$ and inhibits multiple peroxynitrite-induced processes (Serraino et al 2003). The Chilean berry Aristotelia, a rich source of polyphenols including cyanidin, had high total radical trapping potential and total antioxidant reactivity in *in* vitro antioxidant capacity tests compared with the other commercial berries lower in polyphenols (Mirand-Rottmannet al 2002). It proved effective in inhibiting copper induced LDL oxidation and showed concentration dependent protection from hydrogen peroxide-induced intracellular oxidative stress in cultured endothelial cells (Mirand-Rottmannet et al 2002).

Francel et al (1993) examined red wine inhibition of copper-catalyzed oxidation of LDL in humans. Natural flavonoids in wine were found to donate hydrogen or react

with O_2^-, hydroxyl radicals and lipid peroxyl radicals, all of which contribute to lipid preoxidation in vivo. Two possible mechanisms for this action were advanced; firstly that the phenolic compounds complexed with Cu^{2+} to reduce Cu^+, which may in turn reduce H_2O_2, and seconly that during the LDL peroxidation, phenols in wine may act as self-regenerating reducing components. The authors concluded that regular ingestion of these antioxidant phenols via red wine resulted in a collective reduction in the oxidation of lipoproteins, which may contribute to the improvement of atherosclerois and morbidity and mortality from CVD (Francel et al 1993). However the conclusions drawn from Francel's study were not supported by other data (Rajnarayana et al 2001). Miyagi et al (1997) conducted a similar study to that of Francel. Miyagi compared the effects of flavonoids from red wine and grape juice, and confirmed that both red wine and grape juice inhibited the Cu^{2+} catalysed human LDL oxidation in vitro. However, only red wine consumption resulted in LDL resistance to oxidation in vivo (Miyagi et al 1997).

This led to the question, did the polyphenols need the alcohol present in red wine to exert their therapeutic effects in vivo. Further in vivo investigations were carried out into the effect of purple grape juice on endothelial function and LDL oxidation (Stein et al 1999). Fifteen humans with an average age of 62 years, suffering from angiographically documented coronary artery disease, received a dietary supplement of 7.7ml/kg/d of purple grape juice. A significant improvement in endothelial function and reduction to the susceptibility of LDL to copper-induced oxidation was observed in these CAD patients.

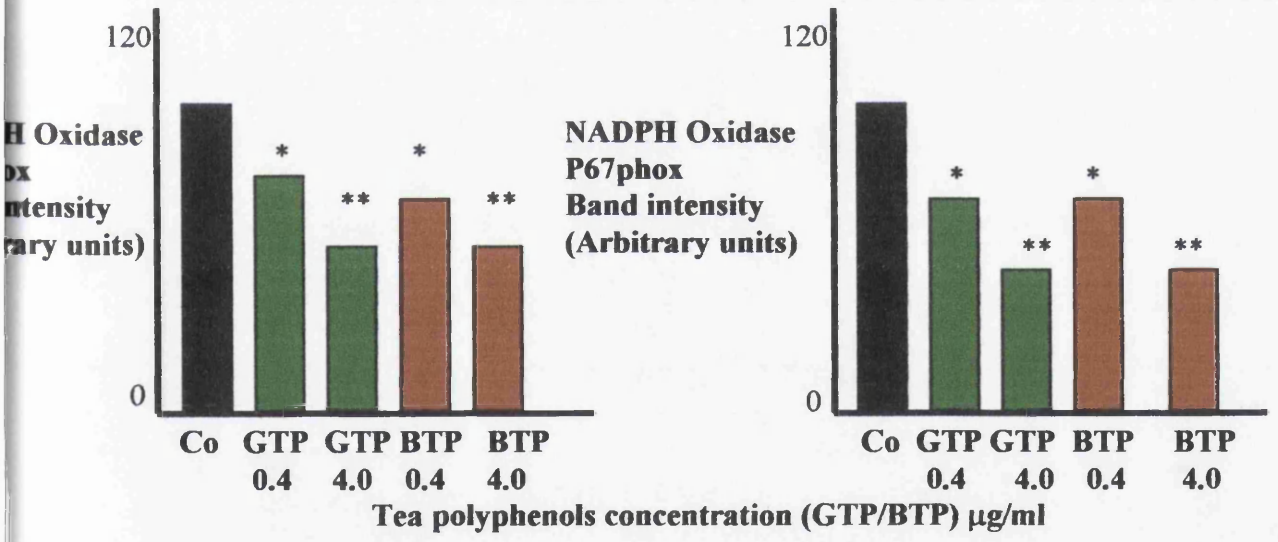
Duffy et al showed that short- and long-term consumption of black tea reversed endothelial vasomotor dysfunction in patients with coronary artery disease (Duffy et al 2001). The daily tea polyphenol intake (2.6mg/d) was quite low (Mukamal et al 2002), thus the free radical scavenging ability of tea polyphenols should be limited. This lead Ying et al (2004) to hypothesize that the effect of tea polyphenols on ROS is mainly due to the regulation of enzymes related to ROS formation and degradation in vascular endothelial cells. To test this hypothesis, protein expression of NADPH oxidase subunits p22phox and p67phox, cytosolic Cu/Zn SOD (SOD-1), and catalase in human endothelial cells were evaluated. The results showed that tea polyphenols at 0.4μg/ml and 4.0μg/ml (from either green tea or black tea, table 1.2) down- regulated

NADPH oxidase p22phox and p67phox expressions in a concentration dependent manner at the concentrations examined, and up-regulated the expression of catalase, fig 14 (Ying et al 2004).

Table 1.2 Components of Tea Polyphenols. Adapted from Ying et al (2004)

	Green tea Polyphenols (GTP) %	Black Tea Polyphenols (BTP) %
Catechin	70	8
Flavonols	10	10
Thearubigins	-	70
Theaflavins	-	12
Polymeric Flavonoids	20	-

Figure 14 schematic diagram showing regulation of NAD(P)H oxidase subunits by tea polypheols. Adapted from Ying et al (2004)



** P<0.01 versus control (Co)
* P<0.05 versus control

Quercetin has also been shown to inhibit several O_2^- generating enzymes such as xanthine oxidase (Chang et al 1993), and the neutrophil membrane NADPH oxidase complex (Tauber et al 1984).

1.8 Potential of quercetin and raspberry polyphenols in cardiovascular protection

Raspberries have been reported to have a number of heath benefiting properties, recently receiving great media attention (Telegraph, Times, Daily Mail, Daily Express June 2004). They contain quercetin and are also an important source of anthocyanidins particularly cyanindin (Rice-Evans et al 1996). In addition raspberries contain high concentration of ellagic acids, which have been reported to have anticarcinogenic and antioxidant effects (Macheix et al 1996).

Raspberry extracts have shown high antioxidant potential in several studies (Mullen et al 2002, Käkhönen et al 2001). Raspberry extracts were effective in inhibiting the formation of H_2O_2 in bulk methyl linoleate. In an earlier study a phenolic extract of red raspberries inhibited human LDL and liposome oxidation (Heinonen et al 1998). Raspberries have shown a remarkably high scavenging activity toward chemically generated active oxygen species (Constantino et al 1992; Wang et al 2000). However scant information is available on the contribution of different phenolic subgroups or single compounds in this observed activity (Käkhönen et al 2001).

Mullen et al (2002) examined the polyphenol compounds in Glen Ample raspberries (Rubus idaeus L.), by gradient reverse phase HPLC and identified eleven anthocyanins. Significant quantities of ellagitannin, sanguiin H-6 and another ellagitannin were present together with lambertianin C at lower concentrations. The flavaonols quercetin-3-rutinoside, quercetin-3-glucoside and querctin-3-glucoronide were present along with kaempferol glucoronide conjugate and a putative xyloside conjugate of methylquercetin, traces of ellagic acid and its sugar conjugates were also detected, table 1.3. In addition, raspberry juice is reported to contain catechins (Arts et al 2000). Raw raspberry extracts also contain non-phenolic substances such as sugars, organic acids, proteins and pigments (Macheix et al 1990).

Table 1.3 Summary of polyphenols detected in extracts of Glen Ample Raspberries following HPLC analysis (Adapted from Mullen et al 2002)

Polyphenol Compound	Wave length λmax(nm)
cyanidin-3,5-diglucoside	519
cyanidin-3-sophoroside	519
cyanidin-3-(2g-glucosylrutinoside)	519
cyanidin-3-glucoside	519
cyanidin-3-sambubloside	519
pelagonidin-3-sophoroside	503
cyanidin-3-xylosylrutinoside	519
cyanidin-3-rutinoside	519
pelagonidin-3-(2G-glucosytrutinoside)	503
pelagonidin-3-glucoside	503
pelagonidin-3-rutinoside	503
lambertianin C	250
sanguiin H-6	250
ellagic acid-pentose conjugate	361
ellagic acid-pentose conjugate	365
ellagic acid	365
quercetin-3-rutinoside (rutin)	365
quercetin-3-glucoside	365
quercetin-3-glucuronide	365
methylquercetin-pentose conjugate	365
ellagic acid acetylxyloside	360
kaempferol glucuronide	365
ellagic acid acetylarabinoside	360

Anthocyanins like other flavonoid members such as quercetin are effective antioxidants (Stinzing et al 2004). The anthocyanin cyanidin present in high concentrations in raspberries like quercetin has a high TEAC value indicating high antioxidant status, table 1.4 (Rice-Evans et al 1996). Anthocyanins have the ability to scavenge reactive oxygen species (Kong et al 2003); and protect low-density lipoproteins from oxidation thus improving cardiovascular performance (Ghiselli et al 1998). Some anthocyanins such as delphidin but not malvin or cyanidin have been reported to show endothelium-dependent vasorelaxation (Andriambelson et al 1998), which can improve endothelium function. However low bioavailability of anthocyanidins appears to cast doubt on their ability to exert proposed beneficial effects. This prompted McDougall and collegues from the Scottish Crop Research Institute (SCRI) to investigate the gut metabolism of Glen Ample raspberry anthocyanidins, using an in vitro digestive procedure adapted from the method outlined by Gil-Izquierdo et al (2002), which was adapted from the work of Miller et al (1981). McDougall provided evidence that the active components in at least eight of the anthocyanins, plus other polyphenols reported being present in Rubus idaeus L, were not broken down by digestion

Table 1.4. Hierarchy of Trolox Antioxidant Activities of Polyphenols (Adapted from Rice-Evans et al 1996)

Compound	Family	TEAC (mM)
Epicatechin gallate	Flavonol	4.9 ± 0.02
Epigallocatechin gallate	Flavanol	4.8 ± 0.06
Quercetin	Anthocyanidin	4.7 ± 0.1
Delphinidin	Anthocyanidin	4.44± 0.11
Cyanidin	Flavonol	4.4 ± 0.12
Epigallocatechin	Anthocyanidin	3.8 ± 0.06
Keracyanin	Flavanol	3.25± 0.1
Myricetin	Hydroxybenzoate	3.1 ±0.30
Galleic acid	Anthocyanidin	3.01±0.05
Ideain	Flavonol	2.9 ±0.03
Morin	Flavanol	2.55±0.02

In further studies Mullen and collegues examined the antioxidant and vasodilatory activities of raspberry polyphenol fractions. The major peak of antioxidant activity was found in the sanguiin H-6 fraction. Calculations for the overall antioxidant activity showed, the vitamin C fraction reduced $1.6x10^{17}$ radicals/g fresh weight (fw), the ellagitannin fractions reduced $3.2x10^{17}$ radicals/gfw and the anthocyanin fractions reduced $3.8x10^{17}$ radicals/gfw (Mullen et al 2002).

Vasodilatory assays were also under taken. The anthocyanins exhibited little activity, while the fractions that contained the lambertiannin C and sanguiin H-6 showed major activity. They were shown to be vasodilators in rabbit aorta (Mullen et al 2002).

Quercetin is the predominant polyphenol in the diet (DeVries et al 1998) and is found in fruits, vegetables, nuts seeds, flowers and bark. There is epidemiological evidence for a protective effect against CVD from foods providing 16 to 24mg/day of quercetin (Knekt et al 1996). In addition to raspberries the main dietary sources of quercetin are onion, lettuce, broccoli, cranberry, apple skins, other berries, olives, tea and red wine (Rice-Evans et al 1996). Quercetin and its metabolites have been reported in plasma and urine, and quercetin has been reported to have a long half-life of 20-72hours in man (Warden et al 2001). Quercetin has been reported to exert a number of properties protective against cardiovascular disease, this fact combined with evidence that it is bioavailable in humans makes it an exciting polyphenol to investigate in relation to CVD. Although prospective randomised clinical trials are lacking several studies using animal models support these potential protective effects of quercetin in cardiovascular disease (Middleton et al 2000). Quercetin exerts systemic and coronary vasodilator effects (Duarte et al 2001; Perez-Vizcaino et al 2002). When given orally it reduced blood pressure, cardiac hypertrophy, and vascular remodelling in spontaneously hypertensive rats and NO-deficient rats (Duarte et al 2001). It also exerts free-radical-scavenging effects (Robak et al 1988; Van Acker et al 1996). It inhibits low-density lipoprotein per oxidation, and reduces the progression of atherosclerosis in vivo (Hayek et al 1997). Quercetin has a high Trolox Antioxidant Activity value (TEAC), which represents high antioxidant potential compared to the other polyphenols, Table 1.4 (Rice Evans et al 1996).

When Itoigawa (1999) tested different flavonoids for positive inotropic effect on guinea pig papillary muscle. Quercetin showed the most potent intrinsic activity, and produced the strongest inotropic responses among the different flavonoids. The relative order for potency of the tested flavonoids was quercetin> morin = kaempferol>cateolin = apigenin > fisectin = galangin (Itoigawa et al 1999).

The interactions between quercetin and NO yield a confusing picture. Concentrations of quercetin present in the average diet are unlikely to have any effect in reducing NO availability. However quercetin has been reported to scavenge NO at very high non-physiological concentrations. At concentrations >100μM quercetin inhibits the activity of the endothelial nitric oxide synthase (eNOS) and the neuronal and inducible isoforms of NOS (Chiesi et al Schwaller 1995). In vivo, high doses of quercetin (above 200mg/kg/d for 10 days) increased the activity, but not the expression, of vascular eNOS, whereas at low doses (5 and 10mg/kg/d for 5 weeks) no changes were observed in vascular eNOS, inducible NOS expression or total NOS activity (Duarte et al 2001). Quercetin directly scavenges O_2^- (Robak et al 1988). In SHR rats quercetin reduced the oxidative status, as indicated by lower concentrations of markers of such oxidative stress such as plasma and hepatic malondialyde and urinary isoprostane-$F_2\alpha$ (Duarte et al 2001). By reducing O_2^- concentrations, quercetin might be expected to protect NO from O_2^- driven inactivation. Quercetin improved the endothelial function in SHR rats (Duarte et al 2001), possibly by the enhancement of NO bioavailability. At lower concentrations that would occur in vivo, quercetin is believed to increase NO bioavailability because of its ability to scavenge O_2^-.

2. Aims

2.0 AIMS

As discussed raspberries contain a range of polyphenols that have antioxidant properties. A number of these polyphenols have been identified in plasma and could contribute to the cardiovascular protective effects of fruit and vegetables, although most studies utilised high concentrations of polyphenols which would be difficult to achieve in the normal diet. The aim of this study is to investigate whether the raspberry polyphenols and two purified quercetins improve endothelium function by decreasing superoxide levels, increasing nitric oxide bioavailability and decreasing platelet aggregation, at biological relevant concentrations.

The following plant polyphenols extracts were studied;

- Crude Glen Ample raspberry extract (Rubus idaeus L.)
- Rubus idaeus L. processed through an in vitro digestion procedure
- Quercetin-3-glucoronide
- Quercetin-3'-sulphate

And

- Ascorbic acid as a positive control.

3. Materials and Methods

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Test compounds- Plant extract polyphenols (PEP)

The four PEP investigated in this study were a crude raspberry extract (RE), the same raspberry extract after processing in an artificial gut system (GR) both supplied by D. Stewart and G. McDougall from the Scottish Crop Research Institute (SCRI-Invergowrie, Dundee), plus two purified polyphenols quercetin-3-glucuronide (QG) and quercetin-3'-sulphate (QS) supplied by A. Crozier at the division of Biochemistry and Molecular Biology University of Glasgow. The PEP were examined together with ascorbic acid (AA) (supplied by Sigma) as a positive control.

3.1.2 Preparation of crude Raspberry Extract- carried out by SCRI

The raspberries were homogenized in an equal volume of acidified acetonitrite- this gives good extraction of soluble phenolics without solubilising too much protein and cell wall polymers. The extract is precipitated with 50% then 75% ethanol to remove pectins, which would cause viscosity problems. This has the advantage that effectively all of the vitamin C in the extracts is oxidised-therefore any effects seen is the result of the polyphenols.

3.1.3 Preparation of Gut raspberry carried out by SCRI

Crude RE could be metabolised during digestion, therefore some potential active polyphenols could be destroyed while others could be formed during the digestive process. Therefore studies were undertaken using a RE, which had undergone in vitro digestion to determine whether digestion resulted in loss of activity.

Diallysis tubing, containing the sample of interest is placed in chemical solutions that mimic the chemical reactions of the gastric and pancreatin digestion system, and maintained at physiological pH and temperatures. The dialysis bag restricts movement of the particles to intra-pore diffusion only. In vitro gastric digestion is stimulated by

pepsin. After gastric digestion is complete, pancreatin and bile salts plus sodium bicarbonate solutions are added to represent pancreatin digestion (as described in Gil-Izquierdo et al, 2002). Once the dialysis phase is complete, samples are ready for HPLC/MS (High Performance Liquid Chromatography/ Mass Spectrometry) analysis, which allows identification of the polyphenols present in the extract.

Phenolic compounds were detected with a PDA (photo diode array) detector, which records wavelengths between 230 nm and 600 nm. In order to identify the unique compound(s) present in a particular sample MS/MS (or MS^2) feature of the HPLC/MS apparatus is used. The MS/MS breakdown pattern appears as a series of peaks on the mass spectrum with decreasing mass/charge ratios. Analysis of data was performed using Qual Browser software.

3.1.4 Preperation of the quercetin compounds carried out by the Division of Biochemistry and Molecular Biology University of Glasgow

QG was purified from green beans (Phaseolus vulgaris). Raw beans were freeze dried and powdered. Extracts were then homogenized with 70% methanol and cooled on ice, and then filtered through filter paper. The fractions were then partially evaporated to remove methanol. The resultant aqueous phase was defatted by serial extraction with hexane. The aqueous layer was fractionated in two portions on a polyamide column; neutral flavonols were eluted with methanol/ ammonia. The purity of the quercetin fraction was checked by HPLC/MS and was found to be greater than 97%.

QS was chemically synthesized. Quercetin (1g; 3mmol) and sulphamic acid (0.58g; 6mmol) were heated in pyridine (5ml; 80°C; 1h) with continuous stirring. After cooling the reaction medium was diluted with 5% potassium acetate (100ml; pH 7.2)

and extracted with ethyl acetate. The precipitate from the ethyl acetate extract was isolated by filtration and purified on Sephadex G-10 using a gradient on methanol in water as solvents. The purity of the synthesis was checked by LCMS and was found to contain >97% quercetin-3'-sulphate.

3.1.5 Concentrations of test compounds

The stock solution of the RE supplied by SCRI contained 1809µg/ml polyphenols. RE contained a mixture of polyphenols therefore a molar concentration could not be calculated. For this reason the concentrations of all the PEP have been expressed as µg/ml to allow for comparisons between PEP. The stock solution of GR was 1785 µg/ml dissolved in water.

The stock solution of QG equalled 4808 µg/ml (10mmol/l) and was dissolved in DMSO, and QS 30482µg/ml (80mmol/l) was dissolved in DMSO.

AA was made up in Krebs to 1761µg/ml (10mmol/l).

3.1.6 Tissue preparation

In vitro studies were carried out to measure O_2^- levels and nitric oxide (NO) bioavailability in vascular tissue. Female SHRSP were used for the majority of the studies. Some experiments were undertaken in normotensive Wister Kyoto Rats (WKY) to allow for comparison between effects of PEP in 'normal tissue' and tissue from animals exposed to high levels of oxidative stress. The animals were obtained from colonies established in Glasgow by brother-sister mating.

The animals were sacrificed by an overdose of halothane. The abdominal and thoracic arteries were carefully removed for O_2^- measurement. The carotid arteries were

removed for NO bioavailability organ bath studies. The arteries were transported to the laboratory in Krebs solution.

3.1.7 Buffers

Krebs Solution

The Krebs buffer used through out this study had the following composition mmol/litre: Sodium Chloride (NaCl) 130, Potassium Chloride (KCl) 4.69, Sodium Hydrogen Carbonate ($NaHCO_3$) 14.9, Potassium Dihydrogen Orthophosphate (KH_2PO_4) 1.18, Glucose 5.5, Magnesium Sulphate-7-hydrate ($MgSO_4$) 1.17, Calcium Chloride ($CaCl_2$) 1.6, Ethylenediamine Tetraacetic Acid (EDTA) 0.03 and indomethacin 0.02. Indomethacin inhibits any prostanoid-mediated effects.

3.1.8 Solutions for superoxide assays

Xanthine oxidase (XO)-0.09g/10ml in distilled water (1U/10ml), Bis-N-Methylacridinium Nitrate (Lucigenin) ($5x10^{-3}M$) 0.0030g/100ml in distilled water and xanthine ($2x10^{-8}M$) 0.0030g/100ml ethanol.

3.1.9 Chemicals

All chemicals were supplied by Fisher Chemicals, BDH AnalaR® or Sigma (Dorset, United Kingdom), unless otherwise stated in the text.

3.1.10 Plastic ware

All plastic ware (pipettes, eppendorf tubes, centrifuge tubes, etc.) were purchased from Gibco. Packard Bioscience Company supplied the polythene (PE) vials used for O_2^- measurement.

3.2 Methods

3.2 Measurment of Superoxide

3.2.1 Xanthine/ Xanthine Oxidase Lucigenin chemiluminescences

Xanthine oxidase (XO) is capable of generating O_2^- and H_2O_2 by reducing O_2, when supplied with its substrates xanthine or hypoxanthine (White et al 1996).

The effect of PEP on O_2^- levels generated by xanthine/ XO was investigated by lucigenin chemiluminescence. Serial dilutions of the stock PEP were prepared in Krebs. AA was examined at the following final concentrations- 9 to 0.0009 μg/ml. RE at 9 to 0.0009 μg/ml, GR at 9 to 0.0009 μg/ml, QG 2 to 0.0002 μg/ml and QS 3 to 0.0003 μg/ml. The PEP were added to miniature 6ml polyethylene (PE) vials containing 2ml Krebs. XO 0.002U, lucigenin15μmol/l and xanthine 800nM were added to the PE vial and chemiluminescence read in a TRI-CARB 2100 TR liquid scintillation analyser (Packard Bio Sciences), set to count at 10-second intervals for three minutes. Counting was initiated immediately after introduction of XO. The results obtained in the presence of PEP were compared to standards in which no PEP was present to determine if PEP had reduced O_2^- levels. A blank containing only 15μmol/l lucigenin was also included.

The blank was subtracted from all readings. The data obtained was expressed as a percentage of O_2^- inhibited in the presence of PEP compared to the control, the calculation is shown below;

$$A = \frac{\text{PEP} - \text{blank}}{\text{Control-blank}} \times 100$$

A = % O_2^- detected in presence of PEP
100 – A = % inhibition of O_2^- by PEP

3.2.2 Assessment of superoxide levels in the aorta of SHRSP

Lucigenin chemiluminescence was also used to investigate O_2^- levels in the abdominal and thoracic arteries of SHRSP, in the presence and absence of PEP. The arteries from the SHRSP were cleaned and cut in to 4mm rings weighing approximately 0.01g and placed into a PE vial containing 2ml Krebs. The weight of the tissue was recorded and the artery ring was incubated for one hour with PEP.

AA was made up to a final concentration of 130 μg/ml (738μmol/l), RE 90 μg/ml, GR 90μg/ml, QG 240 μg/ml (500μmol/l) and QS 310 μg/ml (809μmol/l). The control consisted of 100 μl Krebs.

After the hour's incubation, O_2^- was quantified against a standard curve generated from xanthine and XO and 15μmol/l lucigenin. Combinations of xanthine 100-1000nM and XO 0.002U generate O_2^- and produce transient chemiluminescence signals dependent on xanthine concentration. The reduction of oxygen by XO can occur by both univalent and divalent pathways. The percentage reduced to superoxide depends on pH and other experimental conditions. The yield of O_2^- under our experimental conditions has been determined previously and is shown below in Table 3.1.

Table 3.1 Yield of Super oxide for given quantities of xanthine and xanthine oxidase

O_2^- Concentration	Krebs	Xanthine	Xanthine Oxidase	Lucigenin
0nM	2ml	0μl-0nM	20μl/0.002U	60μl
28nM	2ml	10μl-100nM	20μl/0.002U	60μl
56nM	2ml	20μl-200nM	20μl/0.002U	60μl
84nM	2ml	30μl-300nM	20μl/0.002U	60μl
112nM	2ml	40μl-400nM	20μl/0.002U	60μl

These values were used to calibrate the chemiluminescent signal. Total luminescence was quantified by integration of the areas under the curve generated. The calibration procedure was carried out before each experiment.

In our studies lucigenin (15μmol/l) was incubated with the tissue for 6 mins prior to counting. Results were expressed as nmol/mg wet weight/min.

Lucigenin itself has been reported to generate O_2^- at higher concentrations (Skatchov et al 1999). This is more of a problem in the presence of high concentrations of NAD(P)H and does not effect basal measurements O_2^- in tissues to any significant effect (Berry et al 2000).

3.2.3 Potential quenching of chemiluminescence

It was necessary to insure that the plant compounds were having a genuine effect on O_2^- levels and not simply quenching the light because of their deep colour and viscous make up. This was a particular concern for RE, which had very strong colour in its concentrated form.

Tritiated noradrenaline ([^{3}H] NA) was added to 2mls of sintillation fluid in a PE vial. The radioactive emission from [^{3}H] NA is captured by the sintillation fluid (sintillent), which converts it into light that the liquid scintillation analyser records.

RE because of its pigment could be absorbing light, which would result in lower counts. Alternatively the RE pigment if it reacted with the lucigenin could produce pseudo counts; the result would be more counts. No change in counts per minute (CPM) would indicate that the RE was having no effect.

3.2 Measurement of Nitric Oxide

3.2.4 Organ bath studies

The carotid arteries were cleaned of connective tissue and cut into 2mm rings. Then the rings were mounted in an individual organ baths containing 10mls of Krebs solution for the measurement of isometric tension (the isometric tension studies were performed using a force transducer and recorded using a Mac Lab dedicated computer). The rings were suspended between two wire hooks attached to an isometric force transducer and were stretched to achieve approximately 1g of resting tension (determined from previous work to produce optimal length-tension of this tissue). The baths were maintained at 37°C and bubbled with 95% Oxygen and 5% Carbon dioxide during the experiment.

Equilibration of 30 minutes was allowed before exposing the tissues to a test dose of 3μmol/l phenylephrine (PHE) (an α1 adrenergic receptor agonist) and to 3μmol/l carbachol (CARB) (a muscarinic agonist). After washing out thoroughly the tissues were contracted with 10mmol/l KCL twice with a 20 minute wash out in between. After the second constriction to KCl, another wash out was carried out and approximately 30 minutes was allowed for the tissues to return to resting tone before further studies were undertaken.

A cumulative does-response curve to PHE (0.01μmol/l to 10μmol/l) was then constructed which allowed the vessels to reach a stable plateau, from this point relaxation to CARB (0.01μmol/l to 10μmol/l) was recorded. The tissues were then washed out thoroughly before the test compounds were added.

The PEP were added to give the following final concentrations; RE 90, 18, 1.8, 0.36 and 0.036μg/ml, GR 1.8μg/ml, QG 50μg/ml (100μmol/l) and 10 μg/ml (21μmol/l) and QS 60 μg/ml (157 μmol/l), 12 μg/ml (31μmol/l) and 3μg/ml (8 μmol/l). AA was studied at 18μg/ml (100μmol/l). In the WKY studies only RE was tested at the following concentrations; 18 and 1.8μg/ml.

The combined effect of QG and QS was also investigated. The two compounds were added at concentrations, which had no effect on nitric oxide bioavailability when

added alone. Two concentrations were studied; QG 10μg/ml with QS 3μg/ml and QG 3μg/ml (6.3μmol/l) with QS 0.5μg/ml (1μmol/l).

Additional rings were incubated with vehicle, krebs buffer for RE, GR and AA. DMSO for QG and QS.

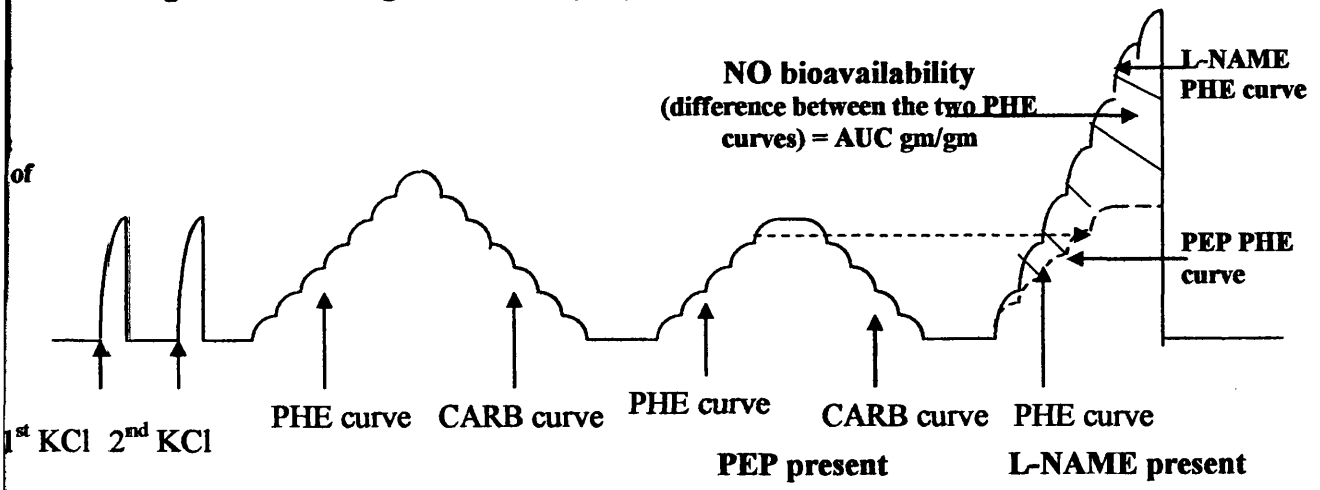
The artery rings were incubated with one of the PEP for one hour. After the incubation period the concentration response curves to PHE (0.01μmol/l to 10μmol/l) and CARB (0.01μmol/l to 10μmol/l) were repeated. After wash out the vessels were incubated with 100μmol/l N^G-Nitro-L-Arginine methyl ester (L-NAME) a nitric oxide synthtase inhibitor. After 20 min incubation a concentration response curve to PHE (0.01μmol/l to 10μmol/l) was again constructed.

The contractile responses to KCl at the start of the experiment allowed standardisation of the results obtained from the different rings in the different organ baths. The increase in tension caused by PHE in the presence of L-NAME compared to in the presence of the PEP provides a measure of basal NO bioavailability. NO bioavailability was calculated for each ring over the full PHE concentration-response curve and for each sample and was expressed as the area between the two curves (AUCgm/gm), shown in fig 3.1 page 61.Calculation shown below;

$$\left[\frac{\text{PHE + L-NAME}}{\text{KL}}\right] - \left[\frac{\text{PHE+PEP}}{\text{KL}}\right] = \frac{X}{2} = \textbf{AUC gm/gm}$$

(Measurement of NO bioavailability)

3.1- Diagram illustrating nitric oxide (NO) bioavailability measurement



3.2 Measurment of Platelet Aggregation

3.2.5 Platelet aggregation

25mls of blood from healthy human volunteers was collected to investigate the effect of PEP on platelet aggregation. The antecubital blood was mixed with 3.15% of sodium citrate to obtain a ratio of 9:1 (blood: anti-coagulant).

The mixture was then centrifuged at 1000 rev/min (centrifuge-MSE MISTRAL 2L) for 18 minutes to obtain platelet rich plasma (PRP). The PRP was pipetted off and collected in an empty centrifuge tube. The reminder of the sediment was spun again at 2000 rev/min for 10 minutes to obtain platelet poor plasma (PPP). The PPP was collected and the sediment from the final spin containing the red blood cells was discarded.

A 450µl aliquot of PPP in a glass vial was placed in the aggregometer. The aggregometer (Chrono-Log corporation Aggregometer) was adjusted so that the PRP gave no light transmittance and PPP gave 100% light transmittance.

The PEP or vehicle (DMSO or Krebs) were added to 450µl aliquots of PRP in glass vials. RE was tested at a final concentration of 20 and 100µg/ml, GR 20 and 100µg/ml, QG 260µg/ml (540µmol/L), QS 340µg/ml (890µmol/L) and AA 235µg/ml (1334µmol/L) and 100µg/ml (556µmol/L).

Adenosine Diphosphate (ADP) was the aggregating agent added to the samples at concentrations of 0.3, 0.6, 1.2, 2.5, 5.0, 10, 20 mmol/l to promote aggregation. The rate and degree to which dispersed platelets in a sample of plasma form clumps (aggregate) after the addition of ADP was examined. The aggregating of platelets causes the sample to become colourless (less turbid). The aggregometer measures the change in turbidity and prints a graphic recording of the results (paper tracer supplied by Servogor Belmont Instruments) as a percentage of platelet aggregation.

3.3 Statistical Analysis

3.3.1 In vitro scavenging of O_2^- (generated by xanthine/ xanthine oxidase)

EC_{50} values of PEP for scavenging O_2^- were obtained using the Prism programme. ANOVA was used to test for over all differences between EC_{50} values for the different PEPs followed by paired t-tests with Bonferroni correction. P values < 0.05 were regarded as significant. n=3-6 per group. Results are expressed mean ± S.E.M. (standard error of the mean).

3.3.2 Scavenging of O_2^- generated in SHRSP aortic tissue

The O_2^- levels in arteries form SHRSP were analysed using paired t-tests comparing levels in the absence and presence of PEP in vessels from each animal using Minitab. n=6-10 per group. Results are expressed mean ± S.E.M.

3.3.3 Nitric Oxide bioavailability

Unpaired t-tests using Minitab were carried out comparing NO bioavailability in the presence of PEP and the appropriate vehicle with Bonferroni correction tests for multiple comparisons. A value of $P < 0.05$ was regarded as significant. n= 6-10 per group results expressed mean ± S.E.M.

3.3.4 Platelet Aggregation

EC_{50} values for aggregation in the presence and absence of PEP were calculated using the statistics package Prism. Paired t-tests were carried out to compare values in the absence + presence of PEP in blood from the same individual. n= 7 per group all results expressed mean ± S.E.M.

4.Results

4. RESULTS

4.1 Scavenging capacity of plant extract polyphenols (PEP) for super oxide

Polyphenols have been reported to exert free radical scavenging properties; they scavenge free radicals, including O_2^- and hydrogen peroxide (Robak et al 1998; Van Acker et al 1996). Oxidative stress occurs when the production of damaging ROS overwhelms the antioxidant defences (Halliwell et al 1989). Endothelial dysfunction tends, not to be due to disruptions in the production of NO, but to increased inactivation of NO by excessive production of O_2^- (Hamilton et al 1997; Boulomié et al 1997)

The aim of these studies is to investigate the ability of selected PEP to scavenge O_2^-.

The O_2^- scavenging ability of a crude raspberry extract (RE) and a raspberry extract that had been processed through an artificial gut system (GR) were examined. Two purified polyphenols, quercitin-3'-sulphate (QS) and quercetin-3-glucuronide (QG) were also examined. Ascorbic acid (AA) acted as the positive control.

The scavenging capacity of the PEP for O_2^- was measured in two ways; firstly the ability of the PEP to reduce O_2^- levels when O_2^- was generated in vitro by xanthine and xanthine oxidase (XO) was examined; secondly the ability of the PEP to reduce the elevated levels of O_2^- in the aorta of a hypertensive rat model was examined. O_2^- levels were determined for both methods using lucigenin chemiluminescence.

The results were expressed in μg/ml. This was to allow for comparison between the PEP. For the raspberry extracts a μmol/l concentration could not be calculated because they contained a mixture of polyphenols.

4.1.2 Potential Quenching by plant extract polyphenols (PEP)

Before measurement of O_2^- by lucigenin chemiluminescence could be carried out it had to be determined if the PEP, because of their strong pigmentation, could interfere

with the lucigenin chemiluminescence. The pigment could quench the light, which would result in lower counts, or the PEP pigment could produce chemiluminesence giving artificially high results. To investigate these possibilities, counts per minute (CPM) from a standard amount of tritiated noradrenaline ([^{3}H] NA) were recorded in the presence and absence of the raspberry extract (RE).

These studies were carried out in the presence of 18μg/ml and 180μg/ml of RE. Tritiated noradrenaline ([^{3}H] NA) +/- PEP was added to 2 mls of scintillation fluid and CPM recorded, as described in methods 3.2.3.

Table 4.1A Effect of raspberry extract (RE) on counts per minute (CPM) produced by [^{3}H] NA

n=3 per group. Results expressed as mean counts per minute (CPM) ± standard error of the mean (S.E.M).

SAMPLES	CPM ± S.E.M
[^{3}H] NA	276 ± 29
[^{3}H] NA + 18μl/ml RE	213 ± 21
[^{3}H] NA + 180μl/ml RE	239 ± 7

There was no significant difference in CPM between RE and control samples, Table 4.1A.

It was concluded that RE pigment was not having a significant quenching effect on [^{3}H] NA and was not having a generalised artifactual effect when using scintillation counting.

The potential quenching effect of the PEP was analysed further comparing baseline counts per minute (CPM) in the presence of PEP and 15μmol/l lucigenin with controls containing only 15μmol/l lucigenin. RE and GR were tested at the following concentrations 9, 0.9, 0.09μg/ml.

Table 4.2B Effect of RE and GR on basal lucigenin counts

The effect of RE and GR on basal lucigenin counts are expressed as mean counts per minute (CPM) ± S.E.M. n= 4-7 per group. Paired t-tests were then carried out, comparing PEP with the control, $P<0.05$ was regarded as significant.

PEP	PEP plus 15 µmol/ml lucigenin (CPM)	Control 15 µmol/ml lucigenin (CPM) n=4-7	P value
RE 9 µg/ml	16217 ± 718	17368 ± 666	0.255
RE 0.9 µg/ml	16227 ± 913	17368 ± 666	0.329
RE 0.09 µg/ml	17865 ± 222	18628 ± 309	0.101
GR 9 µg/ml	18229 ± 579	17368 ± 666	0.342
GR 0.9 µg/ml	17297 ± 816	17368 ± 666	0.947
GR 0.09 µg/ml	19896 ± 298	19571 ± 135	0.376

Paired t-tests showed no significant effect of PEP on base line counts. Therefore the use of lucigenin to measure the O_2^- scavenging ability of the PEP was continued.

4.1.3 Scavenging capacity of PEP on O_2^- generated by xanthine/ xanthine oxidase

O_2^- was generated by 800nM xanthine and 0.002U XO in the presence and absence of PEP and measured by chemiluminescence using 15µmol/l lucigenin. The PEP were made up in a 1:10 serial dilution and tested at five different concentrations as described in the methods section 3.2.1.

All the PEP showed the ability to scavenge O_2^- in a concentration dependent manner. At the highest concentrations tested all PEP showed near maximum inhibition of O_2^- (100% inhibition).

From the concentration response curves, RE, GR and QG appeared to be the most potent in inhibiting O_2^- levels and AA showed the least potency, figure 4.1C page 68.

EC_{50} values for scavenging O_2^- were calculated using the package prism and are shown in table 4.3D. QG and GR had the lowest EC_{50} values, indicating greater potency for scavenging O_2^- (GR EC_{50} 0.012μg/ml and QG EC_{50} 0.012μg/ml). AA had the highest EC_{50} 0.178μg/ml indicating least potency for scavenging O_2^-. It must be noted that the slope of GR was shallower than QG, this could reflect the different compounds present in the GR compared to a pure compound such as QG. No significant differences were found between EC_{50} values for the different PEP and AA using ANOVA. However when unpaired t-tests were carried out with bonferoni correction three of the PEP (QG, GR and RE) differed significantly from AA, $P<0.005$.

Table 4.3D; PEP EC_{50} values for scavenging O_2^- generated by xanthine/ xanthine oxidase

Plant Extract	**EC_{50} μg/ml**	**Number per group (n)**	**95% Confidence Interval**
Quercetin Glucuronide	0.012	4	0.006-0.022
Gut Raspberry	0.012	5	0.0055-0.024
Raspberry extract	0.026	6	0.012-0.059
Quercetin Sulphate	0.047	5	0.020-0.103
Ascorbic Acid	0.178	6	0.089-0.415

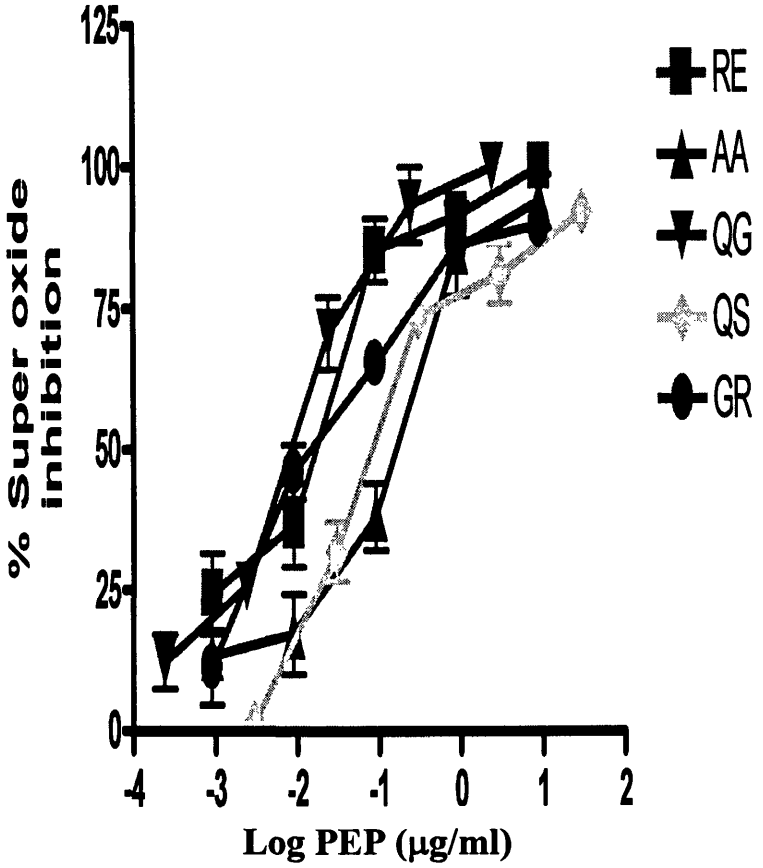


Figure 4.1C Scavenging capacity of PEP of O_2^- generated by xanthine/ xanthine oxidase

Concentration response curves showing the ability of the PEP to scavenge O_2^-, generated by 800nM xanthine and 0.002U xanthine oxidase.

4.1.4 Effect of PEP on the elevated O_2^- levels in SHRSP aortic tissue

The SHRSP, is a well-characterised model of hypertension and has a reduced NO bioavailability which is the result of the scavenging of NO by the elevated concentration of vascular O_2^- (Kerr et al 1999).

Thoracic and abdominal 4mm artery rings of SHRSP were used to assess the effect of PEP on O_2^- levels in vitro, using 15μmol/l lucigenin, as described in 3.2.2 of the methods section. The tissues were incubated with the PEP or vehicle for one hour prior to measuring O_2^- levels.

Vehicle and PEP treated rings were studied in parallel to allow for differences in O_2^- levels in aortas from different animals. There was a slight tendency for all the PEP, with the exception of GR, to reduce the elevated O_2^- levels compared to their controls after an hour's incubation, fig 4.2E. However when paired t-tests were carried out no significant differences were observed $p>0.05$.

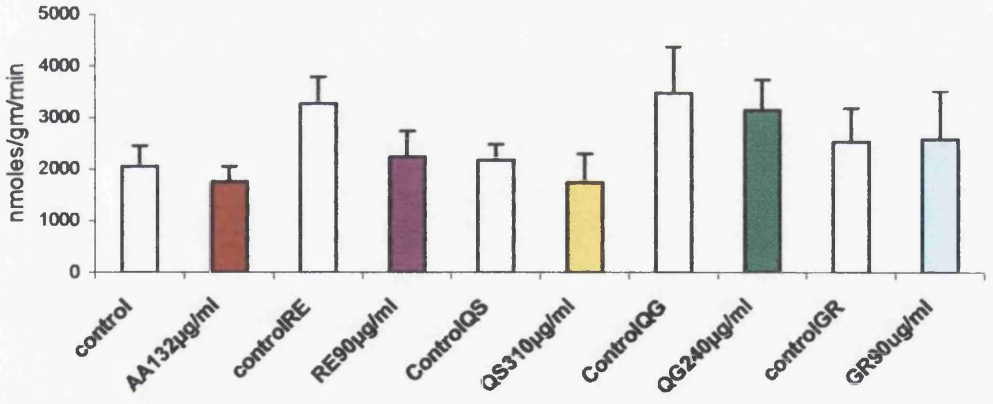


Figure 4.2E Effect of the PEP on the elevated O_2^- levels in SHRSP arteries.
Counts per minute (CPM) are measured and read against a standard curve to give O_2^- levels in nmoles. Results are expressed as nmol/gram wet weight tissue/min ± S.E.M.

4.1.5 Summary of the scavenging capacity of the PEP for O_2^-

The results showed that all the PEP in this study incuding AA could scavenge O_2^- generated from xanthine and XO in vitro.

However none of the PEP were able to reduce the elevated levels of O_2^- in the arteries of SHRSP. This may suggest that PEP have the ability to scavenge extracellular O_2^- but not intracellular O_2^-, this could be due to poor lipid solubility of the PEP.

4.2 Plant extract polyphenols (PEP) effect on Nitric Oxide (NO) bioavailability

Previous studies have reported that polyphenols can increase nitric oxide bioavailability in vivo (Andriambelson et al 1998, Fitzpatrick et al 1993) but often only at very high concentrations. Endothelial NO is essential for healthy endothelium function; it is responsible for vasorelaxation, inhibition of platelet aggregation, leukocyte adhesion and smooth muscle cell proliferation and migration. A reduction in NO can result in endothelium dysfunction; therefore the ability to increase NO bioavailability might be anticipated to have cardiovascular protective effects.

Most studies have examined the ability of PEP to enhance NO release in response to agonists such as acetylcholine and bradykinin. However the effect of PEP on basal levels of NO may have more physiological relevance. Measuring the effect of PEP on changes in isometric tension in vitro may give a closer approximation to this than studying relaxation to acetylcholine.

In this study the effects the PEP on basal NO bioavailability were assessed in organ bath experiments using 2mm carotid artery rings from SHRSP and Wistar-Kyoto rats (WKY). Responses to phenylephrine were assessed in the presence and absence of the NOS inhibitor L-NAME as detailed in 3.2.4 methods. The carotid arteries were incubated with the PEP for an hour before the phenylephrine curve was constructed.

The PEP investigated were two raspberry extracts, a crude extract (RE) and an extract that had been processed through an artificial gut system (GR), the two purified polyphenols quercitin-3'-sulphate (QS) and quercetin-3-glucuronide (QG) were studied and ascorbic acid acted as a positive control. In addition the combinational effects of the two purified polyphenols were investigated.

4.2.1 Effect of raspberry extract on NO bioavailability in SHRSP arteries in vitro

A series of concentrations were examined for RE and a concentration response curve was obtained. Significant increases in NO bioavailability were seen from 90-0.36μg/ml RE compared with the control krebs buffer, fig 4.3F page 73. The active ingredients of RE are still not known, nor has the effect of digestion on the extract been determined. Therefore experiments were repeated on a raspberry extract that had been processed through an artificial gut system (GR). Due to time constraints only one concentration of GR was examined. The GR was examined at 1.8μg/ml, one of the lower concentrations of the RE to show a significant effect on NO bioavailability. GR was also found to significantly increase nitric oxide bioavailability, and appeared to be at least as potent as the original extract at the same concentration. This may indicate that the active ingredients of RE are not lost during digestion. It is also possible that additional active compounds are formed during the digestive process.

AA at 18μg/ml (100μmol/l) also increased NO bioavailability significantly compared to the control. The effect was similar to that observed with RE at 18μg/ml but the study was not designed to compare AA and RE in detail.

4.2.2 Effect of QS and QG on NO bioavailability in SHRSP arteries in vitro

Both the two purified polyphenols QS and QG significantly increased NO bioavailability compared to their vehicle DMSO, unpaired t-tests $p<0.05$, fig 4.4G p74.

The effects were concentration dependent except in the case of QS. At 60μg/ml QS showed no significant effect on increasing NO bioavailability, yet at 12μg/ml a significant increase in NO availability was observed.

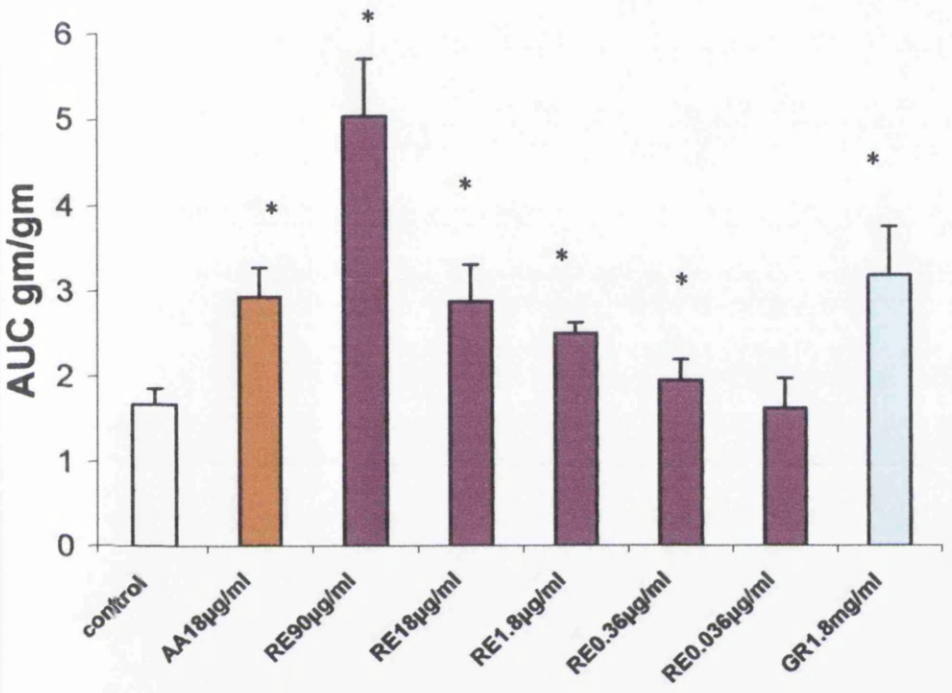


Figure 4.3F Effect of raspberry extract (RE) and a gut raspberry extract (GR) on Nitric Oxide bioavailability in the arteries from SHRSP

Basal NO availability was measured as the difference in contractile response to phenylephrine in the presence and absence of the NOS inhibitor L-NAME. NO bioavailability was expressed as the difference between the two curves AUC gm/gm.

The results are expressed as mean ± S.E.M.

* Indicates a significant difference between the raspberry extracts and the control, using unpaired t-tests with boneferroni correction tests for multiple comparisons using Minitab. $p < 0.05$ was regarded as significant.

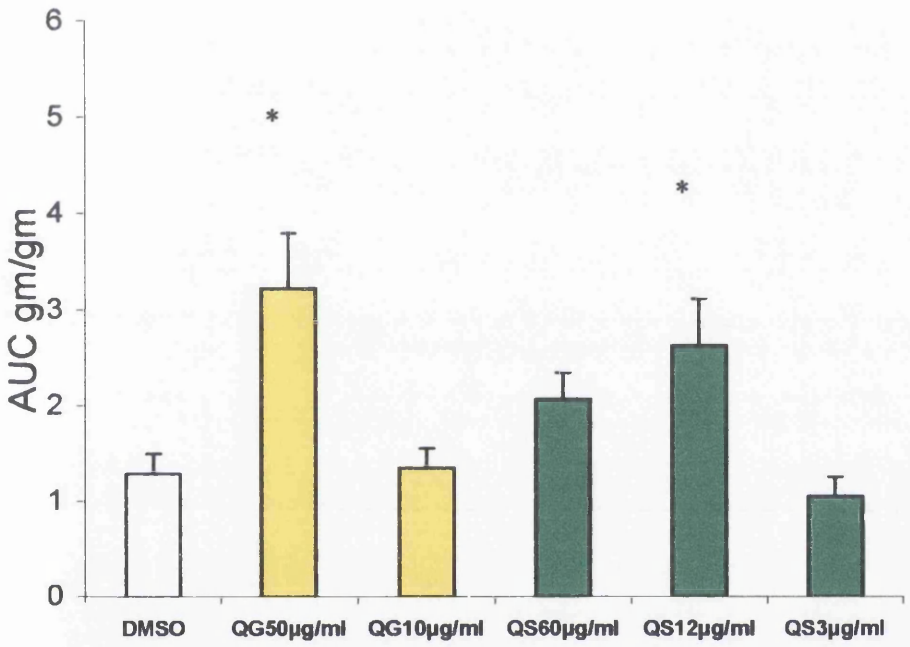


Figure 4.4G Quercetin-3-glucoronide (QG) and quercetin-3'-sulphate (QS) effect on Nitric Oxide bioavailability in the arteries from SHRSP

Basal NO availability was measured as the difference in contractile response to phenylephrine in the presence and absence of the NOS inhibitor L-NAME. NO bioavailability was expressed as the difference between the two curves AUC gm/gm.

The results are expressed as mean ± S.E.M.

* Indicates a significant difference between the quercetin extracts and the control (DMSO), using unpaired t-tests with boneferroni correction tests for multiple comparisons using Minitab. $p < 0.05$ was regarded as significant.

4.2.3 QG and QS combined effects on NO bioavailability in SHRSP in vitro studies

A greater effect on NO bioavailability was observed when the two quercetins were added to the organ baths together. The two quercetins were initially added in combination at concentrations which had previously been shown to have no effect on their own, QG 10μg/ml (21μmol/L) and QS 3 μg/ml (8μmol/L). A significant increase in NO bioavailability was observed. The two quercetins were then added at a lower concentrations QG 0.3μg/ml (6 μmol/l) and QS 0.5μg/ml (1μmol/l) and a significant increase in NO bioavailability was again observed. If time had permitted still lower concentrations would have been tested to determine the lowest effective concentrations, fig 4.5H page76.

4.2.4 Effect of RE on NO bioavailability in WKY arteries in vitro

The effect of the PEP on NO bioavailability in control Wistar-Kyoto rat arteries (WKY) was also investigated to determine if PEP increased or improved NO bioavailability out-with the hypertensive animal model.

Only 18μg/ml and 1.8μg/ml RE were examined. No significant difference in NO bioavailability was found compared to the control Krebs buffer, at either concentration, fig 4.6I page 77.

It should be noted however under control conditions basal NO bioavilability was higher in arteries from WKY than SHRSP (2 vs. 1.6 respectively). This is consistent with previous work and is believed to be related to lower O_2^- levels in WKY arteries (McIntyre et al 1997).

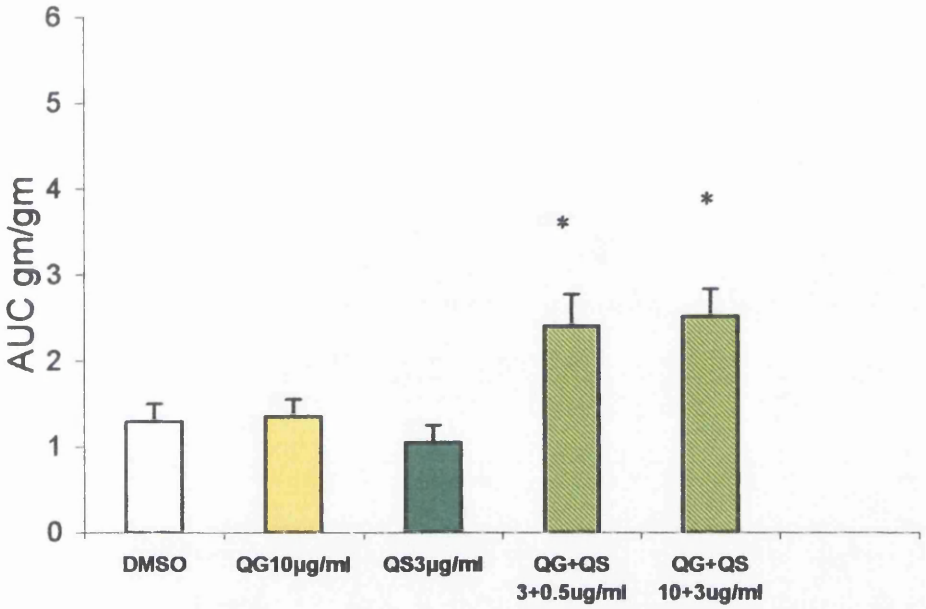


Fig 4.5H Quercetin-3-glucoronide (QG) and quercetin-3'-sulphate (QS) combinational effect on Nitric Oxide bioavailability in the arteries from SHRSP

Basal NO availability was measured as the difference in contractile response to phenylephrine in the presence and absence of the NOS inhibitor L-NAME. NO bioavailability was expressed as the difference between the two curves AUC gm/gm.

The results are expressed as mean ± S.E.M.

* Indicates a significant difference between the quercetin extracts and the control (DMSO) using unpaired t-tests with boneferroni correction tests for multiple comparisons using Minitab. $p < 0.05$ was regarded as significant.

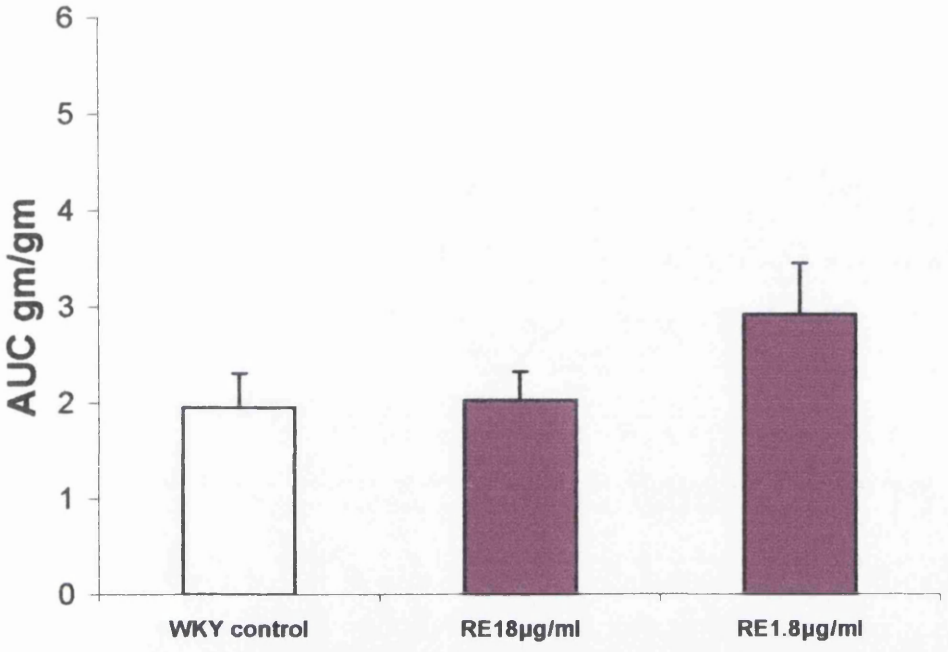


Fig 4.6I Crude Raspberry extracts (RE) effect on nitric oxide bioavailability in the carotid arteries from WKY

Basal NO availability was measured as the difference in contractile response to phenylephrine in the presence and absence of NOS inhibitor L-NAME. NO bioavailability was expressed as the difference between the two curves AUC gm/gm.

The results are expressed as mean ± S.E.M.

There was no significant difference between the RE and the control treated WKY arteries.

4.2.5 Summary of PEP effect on NO bioavailability in vitro studies

All PEP treated vessels showed a significant increase in NO bioavailability compared to their vehicle treated in the carotid arteries of SHRSP.

4.3 Plant extract polyphenols (PEP) effect on Platelet Aggregation (PA)

Inhibition of platelet aggregation by polyphenolic compounds has been proposed to contribute to a reduction in heart disease and stroke in people consuming high concentrations of fruit and vegetables (Hertog et al 1996). However many studies suggest that the antiaggregatory effects of flavonoids seen in vitro are due to concentrations that cannot be attained in vivo (Janssen et al 1998).

The aim of this study was to determine whether any of the PEP, which had been shown to increase NO bioavailbility in SHRSP carotid arteries, were able to attenuate platelet aggregation.

25mls of blood was collected from healthy volunteers. PA was stimulated by 0.3-20mmol/l Adenosine Diphosphate (ADP) and recorded by an aggregometer, in the presence of PEP or its vehicle (DMSO or Krebs) as explained in 3.2.5 of methods section.

4.3.1 Raspberry extracts effect PA

RE and GR were investigated at 20μg/ml and 100μg/ml. Only the RE at 100μg/ml showed a significant reduction in PA compared to the appropriate controls, fig 4.7J.

From the ADP dose response curves EC_{50} values were calculated for the raspberry extracts. RE at 100μg/ml was the only PEP to show a significantly lower EC_{50} indicating greater potency in inhibiting PA compared to its vehicle, table 4.4M page 84.

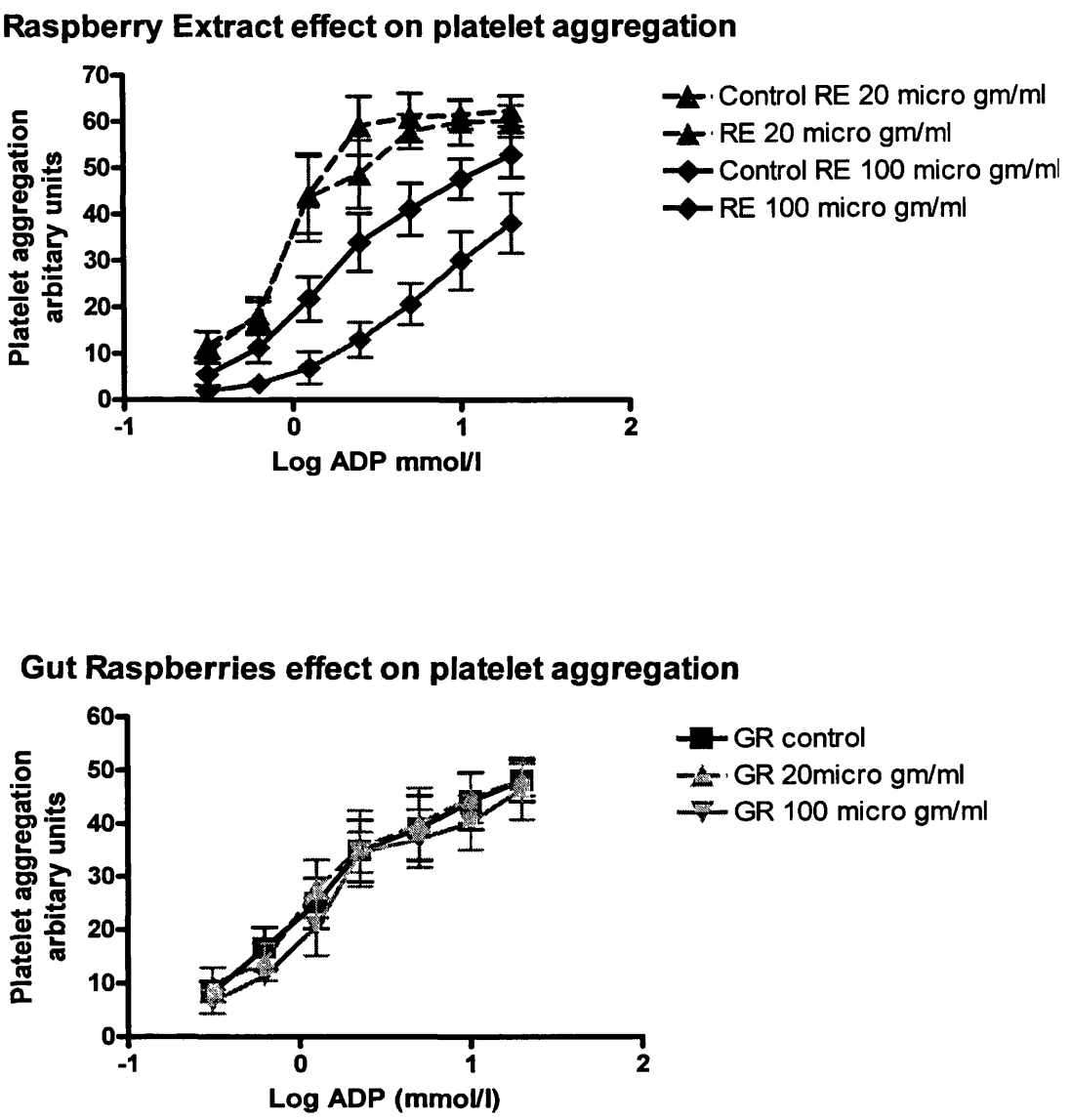


Figure 4.7J Inhibition of platelet aggregation by crude raspberry extract (RE) and an extract after processing by an artificial gut system (GR).

Platelet Aggregation was stimulated by 0.3-20mmol/l ADP and recorded by an aggregometer, using platelet rich plasma from healthy human volunteers, in the presence of raspberry extract or it's vehicle (Krebs buffer).

Results were calculated using the prism package and are expressed as a mean ± S.E.M. n= 7 per group.

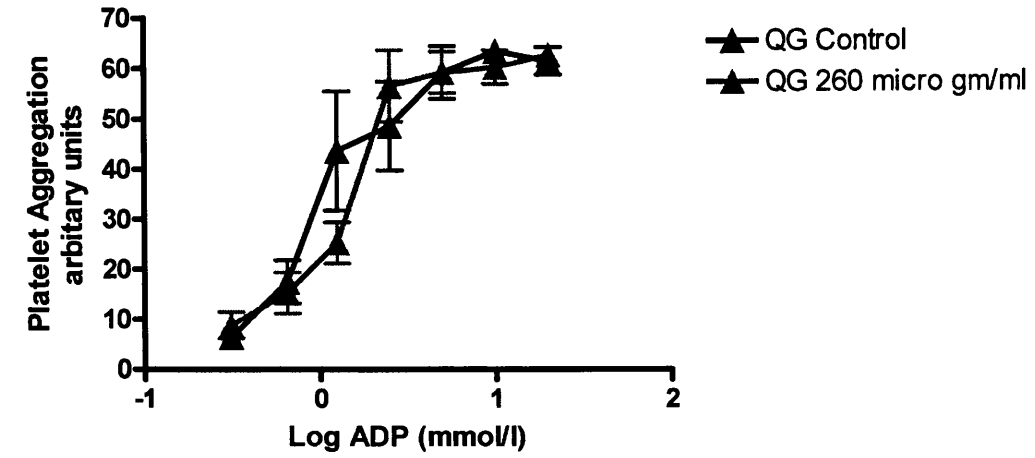
4.3.2 Quercetins effect on PA

Initial studies showed that DMSO, the vehicle for QG and QS had a strong inhibitory effect on PA. The effects of QG and QS on PA were therefore compared to those of DMSO alone in each subject.

QG was examined at a concentration of 260μg/ml (540μmol/l) and QS 340μg/ml (890μmol/l), the concentrations were chosen, as they were similar to the concentrations used by others (Wolfram et al 2002). From the ADP concentration response curves (fig 4.8K) the EC_{50} values for QS and QG and their control DMSO were calculated, Table 4.4M page 84. Paired t-tests showed the difference between the quercetins EC_{50} value and their control DMSO was not significant $P>0.05$. As no effect of either QG or QS was observed and the concentrations were already much higher than could be achieved in vivo, no further experiments were carried out, fig 4.8K page 82.

4.3.3 Ascorbic Acids effect on PA

The effect AA on PA was examined at the following concentrations; 235μg/ml (1334μmol/l) and 98μg/ml (556μmol/l). At neither concentration was an inhibitory PA effect observed, fig 4.9L page 83. From the ADP concentration response curves the EC_{50} values were calculated and AA compared to the control. No significant difference was observed, paired t-test $P>0.05$, table 4.4.M. Both concentrations of AA used were too high to have any relevance for in vivo studies; therefore further experiments at higher concentrations of AA were not carried out.



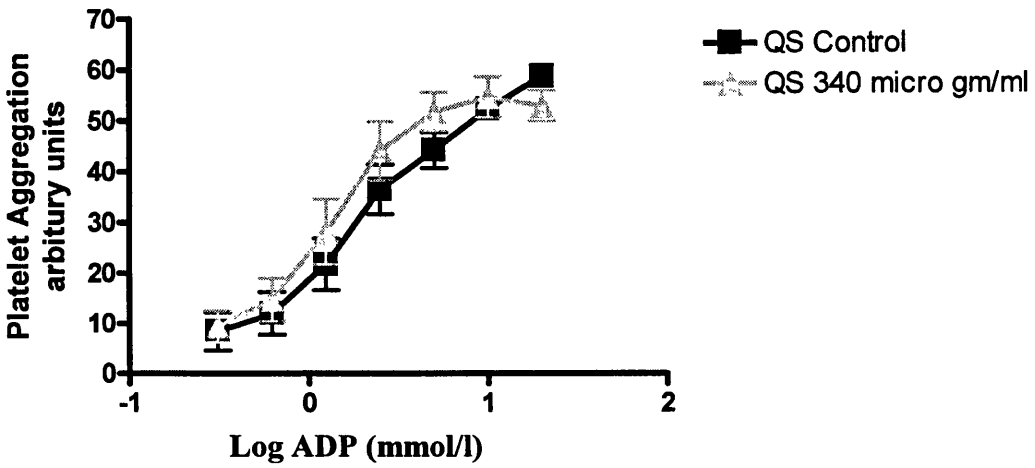


Fig 4.8K Inhibition of Platelet Aggregation by quercetin-3-glucoronide (QG) and quercetin-3'-sulphate (QS)

Platelet Aggregation was stimulated by 0.3-20mmol/l ADP and recorded by an aggregometer, using platelet rich plasma from healthy human volunteers, in the presence of quercetin or it's vehicle (DMSO).

Results were calculated using the prism package and are expressed as a mean ± S.E.M. n= 7 per group. No significant difference was found between the EC_{50} values of the two quercetins and their vehicle when paired t-tests were carried out. $P>0.05$.

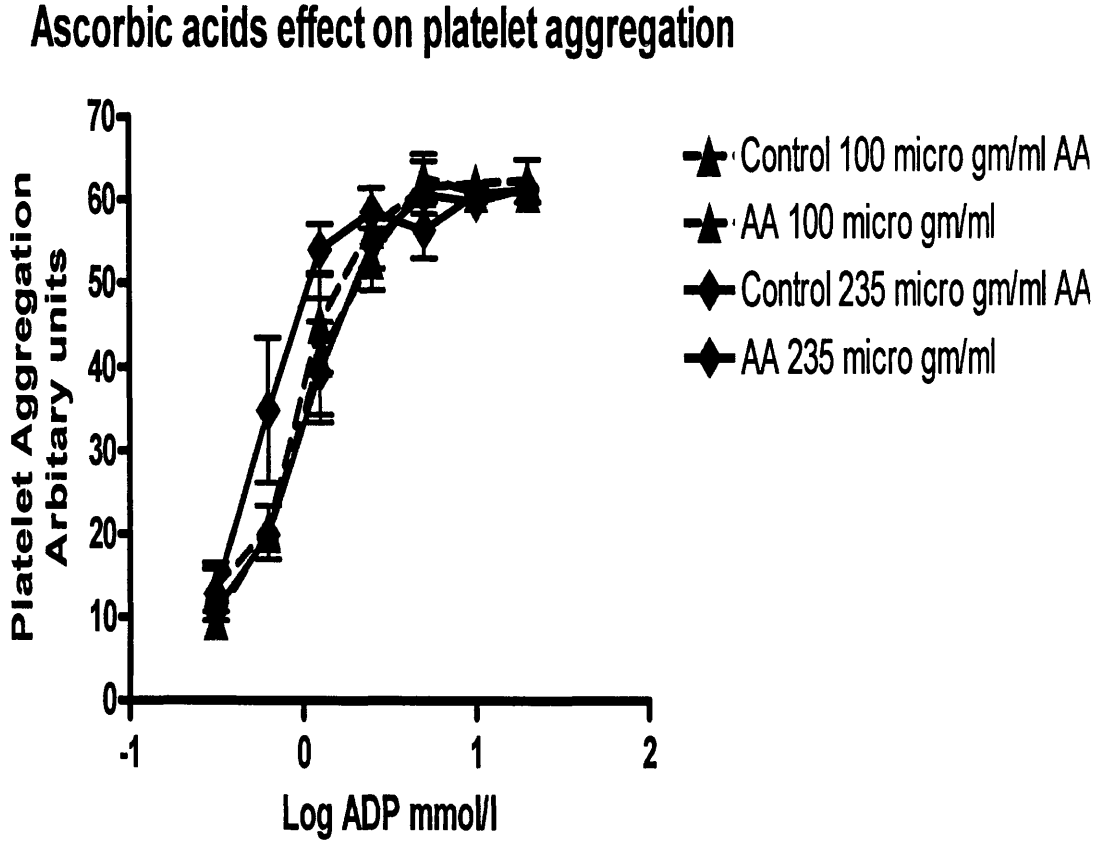


Fig 4.9L Inhibition of Platelet Aggregation by Ascorbic Acid (AA)

Platelet Aggregation was stimulated by 0.3-20mmol/l ADP and recorded by an aggregometer, using platelet rich plasma from healthy human volunteers, in the presence of ascorbic acid or it's vehicle (krebs buffer).

Results were calculated using the prism package and are expressed as a mean ± S.E.M. n= 7 per group.

No significant difference was found between EC_{50} of AA and it's vehicle when paired t-tests were carried out, $P>0.05$.

Table 4.4M Inhibition of Platelet Aggregation (PA) by plant extract polyphenols (PEP)

PA was stimulated by 0.3-20mmol/l Adenosine Diphosphate (ADP) and recorded by an aggregometer, using 25mls of blood collected from healthy human volunteers, in the presence of PEP or its vehicle (DMSO or Krebs). Results are shown for the highest concentration of PEP examined.

The ADP concentration response curves were constructed using the prism package, and EC_{50} values of the PEP and their respective vehicle calculated. Paired t-tests were carried out to test for significance, $P<0.05$ was regarded as significant.

PEP	Number per group (n)	EC_{50} PEP µg/ml	EC_{50} Vehicle µg/ml	P value
Ascorbic Acid (AA)	7	0.023	0.498	0.155
Raspberry Extract (RE)	7	1.617	7.512	0.001
Quercetin Sulphate (QS)	7	0.898	2.19	0.137
Gut Rasberry (GR)	7	1.18	0.98	0.59
Quercetin Glucuronide (QG)	7	1.047	0.657	0.701

RE (at100µg/ml) was the only PEP at to show a significant reduction than its vehicle on platelet aggregation.

4.3.4 Summary of the effect of the plant extract polyphenols on platelet aggregation

Blood was taken from healthy volunteers. As a result of time constraints, it was not possible to obtain ethical permission for taking blood from hypertensive patients, and not enough blood could be obtained from SHRSP for the PA experiment to work.

RE at a concentration of 100μg/ml was the only PEP tested to show a significant reduction in platelet aggregation compared to it's control krebs buffer, in platelet rich plasma from healthy human volunteers. However for RE the concentration required to inhibit PA was very high and unlikely to be achieved in vivo.

5. Discussion

5. Discussion

All the PEP tested in this study scavenged O_2^- when it was generated in vitro by xanthine/xanthine oxidase (XO). The order of potency for the PEP determined by the EC_{50} values is as follows; (the lowest EC_{50} indicating greatest potency) quecertin-3-glucoronide EC_{50} = 0.012μg/ml, gut raspberry 0.12μg/ml, crude raspberry extract 0.026μg/ml, quercetin-3'-sulphate 0.047μg/ml and ascorbic acid 0.178μg/ml. When ANOVA was carried out comparing all groups no significant differences were found. However when PEP were compared to AA using unpaired t-tests with bornferoni correction, QG, RE and GR had significantly lower EC_{50} values ($P<0.05$) but the EC_{50} QS did not differ to that of AA.

In contrast to the studies with xanthine/ XO no effect was observed with any of the PEP on scavenging elevated O_2^- levels ex vivo from SHRSP arteries. As most of the O_2^- in SHRSP is generated intra-cellularly, these results may suggest that these PEP only scavenge extra-cellular O_2^-, this could be due to poor lipid solubility of the PEP.

On a weight basis the PEP in this study appeared to be more potent at scavenging extra-cellular O_2^- than AA. This is consistent with previous studies, where the antioxidant capacities, measured as oxygen radical absorbance, of some flavonoids including quercetin have been reported to be several times stronger on a molar basis than AA (Cao et al 1997, Wang et al 1997). Quercetin has a very high trolox equivalent antioxidant activity (TEAX) value (4.7mM) compared to other polyphenols (Rice-Evans et al 1996), which would be consistent with the high EC_{50} value of purified QG. Alternatively quercetin has been reported to be a strong inhibitor of xanthine oxidase (IC_{50}=7.23μmol) (Chang et al 1993); this could explain why it is a potent inhibitor of O_2^- when it is generated by xanthine oxidase but not in vascular tissue from SHRSP. In SHRSP O_2^- is generated predominantly from NAD(P)H oxidase and eNOS thus inhibition of XO would have very little effect on O_2^- production in the arteries from SHRSP. Quercetin binds to the reactive site of xanthine oxidase, which prevents the production of O_2^-, the 3-hydroxyl group on benzopyranane from quercetin results in reduction of the binding affinity (Lin et al 2002).

All PEP where able to increase NO bioavailability ex vivo in SHRSP. On a weight basis RE appeared the most potent. QG appeared to be the least potent; however the studies were not designed to make direct comparisons between the groups. The observation that QG appeared the least potent compared to the other PEP in the NO bioavailability studies is the opposite finding to the O_2^- studies, in which QG was found to be one of the more potent scavengers of O_2^- generated by xanthine oxidase. The different ranking of potency (of the PEP) between the two studies would be consistent with other mechanisms in addition to O_2^- scavenging contributing to NO production.

QS unlike the other PEP did not show a concentration dependent response in increasing NO bioavailability. This could occur due to type two errors due to small sample size. However this finding is consistent with previous studies, where at concentrations >100μM quercetin has been reported to scavenge NO production, through the ability to inhibit the endothelial nitric oxide synthase and the neuronal and inducible forms of NOS (Chiesi et al 1995). However at lower concentrations of quercetin (<100μM) no effects were observed on either vascular eNOS or inducible NOS expression or total NOS activity (Duarte et al 2001), and quercetins ability to scavenge O_2^- was believed to have an NO sparring effect.

QG and QS have been reported in plasma and urine in man at concentrations of around 7-10μM (Grafe et al 2001), and have reported to have a long half-life-20-72 hours (Walle et al 2001). When both QG and QS were combined, increases in NO bioavailability were observed which were not seen when QG and QS were added alone. The concentration of the two quercetins when combined which showed the ability to increase NO bioavailability was getting close to concentrations that had been reported to be found in plasma. If time had permitted further experiments would have been carried out using even lower concentrations. In previous studies reporting vasodilator effects of polyphenols the concentrations used were frequently so high that they could never be achieved in vivo. In one study reporting vasodilatory effects 3.5g/l thearubigins, 0.6 g/l flavanols and 0.4g/l catechins were used (Negishi et al 2004), while in the study of Leikets et al (2002), 100-600μg/ml red wine polyphenol were used. In our study QG 3μg/ml (6 μM) and QS 0.5μg/ml (1 μM) caused a

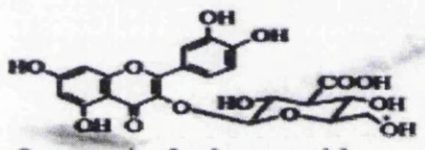
significant increase in NO bioavilabilty which would suggest that vasodilatory effects may be achieved at concentrations that could be attained in vivo.

GR increased NO bioavailability to a similar extent, as RE, at 1.8μg/ml suggesting that the digestive process does not destroy the active vasodilatory raspberry components. However it is still not known if the gut would absorb the active components. Mullen et al (2002) reported lambertianin C and sanguiin H-6 were the two main raspberry polyphenols to show the major vasodilatory activity in rabbit aortas. It is interesting to compare these two structures with the two quercetins, which are present in raspberries but were found to have minimal vasodilatory effect in comparison with lambertianin C and sanguiin H-6 fig 5.0 page 90. The major peak of antioxidant activity was found in the sanguiin H-6 fraction (Mullen et al 2002), making this polyphenol an interesting compound for further cardiovascular studies.

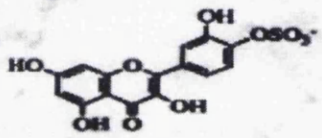
Assuming that biologically significant concentration of polyphenols can be achieved in the diet an additional consideration is their stability. Freezing + thawing have been shown to have no effect on the antioxidant capacity of polyphenols whereas levels of AA antioxidant activity decline under the same conditions (Mullen et al 2002).

Figure 5.0; Structures of Quercetin-3-glucoronide, Quercetin-3'-sulphate, Sanguiin H-6 and lambertianin C

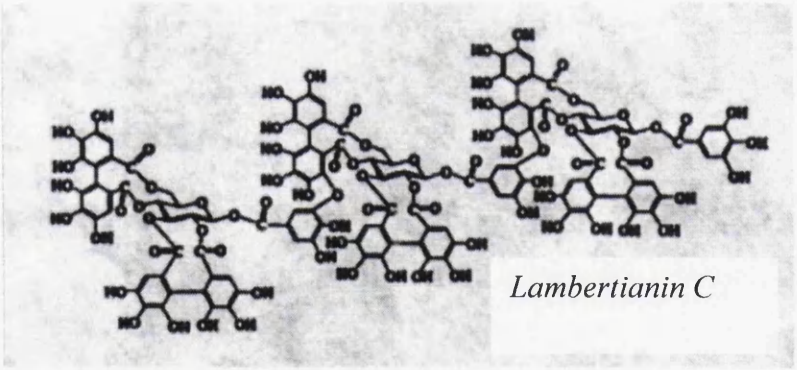
From the structure, Sanguiin H-6 and Lamertianin C are a lot larger compared with the quercetins. They both contain many hydroxyl groups, which previous research has reported to have important implications in the therapeutic properties that polyphenols possess, particularly in scavenging free radicals. This may explain why Lambertianin C and sanguiin H-6 in RE have been found to be more potent vasodilators than quercetin. The fact that quercetin-3-glucoronide showed more effect in scavenging O_2^- when it was generated by xanthine oxidase than the RE, could be due to the fact that it is a smaller molecule and has greater affinity, therefore able to block the reactive binding site of the xanthine oxidase enzyme.



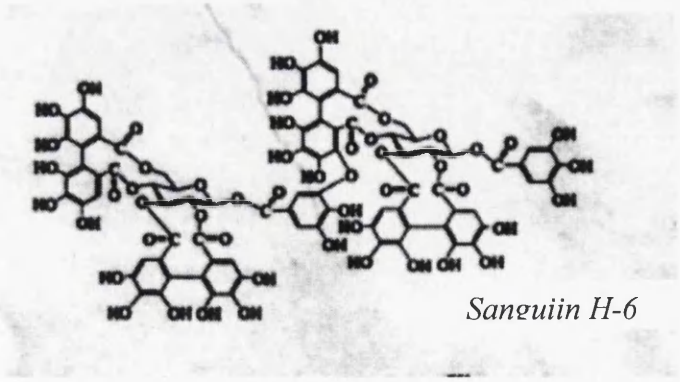
Quercetin-3-glucoronide



Quercetin-3'-sulphate



Lambertianin C



Sanguiin H-6

In the platelet aggregation (PA) experiments only RE had any effect. This appears to be in contrast to the studies on NO bioavailability. Only healthy volunteers could be investigated in this study. Time constraints did not permit ethical approval being obtained for studies in patients, therefore PA effects were not examined in patients with high oxidative status in whom the effects of PEP on PA might have been greater. In the NO bioavailability studies increases NO bioavailability in the presence of RE was only observed in the SHRSP, not the WKY controls. The same could hold true for the PA experiments it is possible that PEP only show thereupeutic effects when the oxidative status is raised.

Different polyphenol components in the RE could be responsible for the inhibitory PA effects than the ones that increase NO bioavailability. GR did not show any effect in inhibiting platelet aggregation; this suggests that the compounds in RE that inhibit PA could be broken down by digestion. It has been reported that polyphenols compounds ability to inhibit PA is not exclusively related to their ability to increase NO bioavailability. Results obtained by incubation of human platelets or animal cells with isolated flavonoids suggest that flavonoids inhibit PA by inhibition of cycooxygenase activity (Laughton et al 1991). Other studies have suggested that flavonoids may have an effect on the concentrations of plasma coagulation or fibrinolysis factors such as fibrinogen, factor VII, and plasminogen (Beretz et al 1978). It would be interesting to investigate purified sanguiin H-6 on PA, as this compound may only be responsible for the vasodilatory effects seen with RE and have no effect on reducing PA.

In the quercetin PA studies the results were consistent with other findings that at lower concentrations (<2500μmol/l), quercetin showed no anti-aggregatory effects (Janssen et al 1998).

To conclude these studies confirm that the PEP examined can scavenge O_2^- and increase nitric oxide bioavailability. In addition RE showed a reduction in PA. The benificaial effects of PEP on NO bioavailability and PA could not be related directly to their efficacy as O_2^- scavengers.

QS and QG have the potential to offer cardioprotecitive effects. Both showed the ability to scavenge O_2^- and increase NO bioavailability at low concentrations, especially when combined. The RE also showed CVD therapeutic potential, but whether the active ingredients in the RE and GR are absorbed still needs to be determined.

Future work is planned in SHRSP, to determine if dietary PEP are able to scavenge O_2^- and increase NO bioavailability in vivo. Studies feeding onion extracts and raspberry extracts are currently underway. Further work both in vitro and in vivo using purified polyphenols such as lambertiannin C and sanguiin H-6, plus other purified polyphenols with reported cardio-protective effects, would aid a greater understanding into which chemical structures of polyphenols have clinically sought after cardiovascular protective properties.

6. References

6. References

American Heart Association; 1999 Heart and Stroke Statistical Update. Dallas, Texas.

Andriambelson E, Magnier C, Haan-Achipoff G, Lobstein A. Natural dietary polyphenolic compounds cause endothelium-dependent vasorelaxation in rat thoracic aorta. **J Nutr** 1998; 128: 2324-2333.

Appel LJ, Moore TJ, Obarzaniek E. A clinical trial of the effect of dietary patterns on blood pressure. **N Engl J Med** 1997; 336: 1117-1124.

Arts ICW, Vande-Putte B, Hollman PLH. Catechin contents of foods commonly consumed in the Netherlnads 1. Fruits, vegetables, staple foods and processed foods. **J Agric Food Chem** 2000; 48:1746-1751.

Baursachs J, Bouloumié A, Fraccarollo D, Hu K, Basse K, Ertl G. Endothelial dysfunction and chronic myocardial infarction despite increased vascular endothelial nitric oxide synthase and soluble guanylate cyclase expression/ role of enhanced vascular superoxide production. **Circulation** 1999; 100: 292-298.

Bazzano L, He J, Ogden L, Loria C, Vupputuri S, Myers L, Whelton P. Fruit and vegetable intake and risk of cardiovascular disease in US adults: the first National Health and Nutrition Examnation Survey Epidemiologic Follow- up Study[1-3]. **Am J Cin Nutr** 2002; 76: 93-99.

Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. **Proceedings of the National Academy of Sciences of the United States of America** 1990; 87: 1620-1624

Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. **Am J Physiol** 1996;271:C1424-C1437.

Berry C, Hamilton CA, Brosnan MJ. Investigations into the sources of superoxide in human blood vessels: angiotensin II increases superoxide production in human internal mammary arteries. **Circulation** 2000; 101: 8-12.

Bertz A, Anton R & Stoclet JC. Flavonoid compounds are potent inhibitors of cyclic AMP phosphodiesterase. **Experientia** 1978; 34:1054-1055.

Bobik A. Intracellular signalling pathway regulating vascular NAD(P)H oxidase and hypertension: an opputrtunity for development of novel antihypertensive agents. **Hypertension** 2003; 21:859-861.

Bouloumié A, Bauersachs J, Linz W. Endothelial dysfunction coincides with an enhanced nitric oxide synthase expression and superoxide anion production. **Hypertension** 1997; 30: 1517-1524.

British heart foundation (BHF-Statistics Database)
Website;www.heartstats.org

Broukouych V, Storizgk E, Oman J, Tomboulin P, Malinski T. Direct electrochemical measurement of nitric oxide in vascular endothelium. **J Pharm & Biochem anal** 1999; 19:135-143.

Burt UL, Whelton P, Roccelila EJ. Prevalence of hypertension in US adult population: results from the Third National health and nutrition examination survey. **JAMA** 2002; 287:356-359.

Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. **Circ Res** 2000; 87: 840-844.

Calver A, Collier J, Vallance P. Inhibition and stimulation of nitric oxide synthesis in the human forarm arterial bed of patients with insulin dependent diabtetes. **J Clin Invest** 1992; 90: 2548-2554.

Cao G, Sofic F, Prior RC. Antioxidant and pro-oxidant behaviour of flavonoids structur activity relationship. **Free Radic Biol Med** 1997; 22: 749-760.

Cathcart MK. Regulation of super oxide anion production by NADPH oxidase in monocyte/ macrophages contributes to atherosclerosis. **Arterioscler Thromb Vasc Biol** 2004; 24:23-28.

Celermajer DS, Sorensen KE, Gooch VM, Spiegeehalter DJ, Miller OI. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. **Lancet** 1992; 340:1111-1115.

Chang WS, Lee YJ, Lu FL & Ching HC. Inhibitory effects of flavonoids on xanthine oxidase. **Anticancer Res** 1993; 13:2165-2170.

Chiesi M & Schwaller R. Inhibition of constitutive endothelial NO-synthase activity by tannin and quercetin. **Biochem Pharmacol** 1995;14:495-501.

Chislom GM, Hazen SC, Fox PL, Cathcart MK. The oxidation of lipoproteins by monocyte-macrophage biochemical and biological mechanisms. **J Biol Chem**. 1999; 274: 25959-25962.

Chitaley K & Webb RC. Nitric Oxide induces dilation of rat aorta via inhibition of Rho-kinase signaling. **Hypertension** 2002; 39: 438-442.

Cifuentes ME, Rey FE, Carretero OA, Pagano PJ. Upregulation of p67(phox) and gp91(phox) in aortas from angiotensin II-infused mice. **Am J Physiol Heart Circ Physiol** 2000; 279: H2234-H2240

Constantino L, Albasino A, Rastelli G, Benvenuti S. Activity of polyphenolic crude extracts as scavengers of super oxide radicals and inhibitors of xanthine oxidase in plants. **Planta Med** 1992; 58: 342-344.

Creager MA, Cooke JP, Mendelsohn ME. Impaired vasodilation of forarm resistance vessels in hypercholesterolemic humans. **J Clin Invest** 1990; 86: 228-234.

De Logeril M, Salen P, Martin J, Monjaud I, Delaye J, Mamelle N. Mediterranean diet, traditional risk factors, and the rate of cardiovascular complications after myocardial infarction: final report of the Lyons Diet health study. **Circulation** 1999; 99: 779-785.

Demrow HS, Slane PR, Folts. Administration of wine and grape juice inhibits in vivo platelet activity and thrombosis instenosed canine coronary arteries. **Circulation** 1995; 91:1182-8.

De Vries J.H.M, Hollman P.C.H, Meyboom S, Baysman M.N.C.P, Zock P.C, Van Staveren W.A., Katan M.B. Plasma concentrations and urinary excretion of the antioxidant flavonols quercetin and kaemperol as biomarkers for dietary intake. **Am J Clin Nutr** 1998; 68: 60-65.

Diaz MN, Frei B, Vita JA, Keaney JF. Antioxidants and atherosclerotic heart diseases. **N Engl J Med** 1997; 337:408-416.

Diaz-Velez GR, Garcia-Ineivis S, Mendza-Ramis E, Hernandez-Lopez E. Increased malondialdehyde in peripheral blood of patients with congestive heart failure. **Am Heart J** 1996; 131: 146-152.

Dimmeler S, Zeiher AM. Exercise and cardiovascular Health. Get active to "AKTivate" Your endothelial Nitric Oxide Synthase. **Circulation** 2003; 107: 3118-3120.

Dixon LJ, Morgan DR, Hughes SM, McGrath LT, Naylace ES, Plumb RD, Devine A. Functional consequences of Endothelial Nitic Oxide synthase uncoupling in congestive heart failure. **Circulation** 2003; 107: 1725-1728.

Duarte J, Perez-Palencia R, Vargas F, Ocete MA, Perez-vizcanio F, Zarzuelo A & Tamargo J. Antihypertensive effects of the flavonoid quercetin in spontaneously hypertensive rats. **Br J Pharmachol** 2001; 133: 177-124.

Duffy SL, Keaney JF, Holbrook M, Goke N, Swerdloff PL, Frei B, & Vita JA. Short and long-term black tea consumption reverses endothelial dysfunction in patients with coronary artery disease. **Circulation** 2001;104:151-156.

Fennell J. Adenoviral-Mediated Vascular Gene Tansfer: a strategy to manipulate the nitric oxide/superoxide pathway. **University of Glasgow PhD thesis** 2002.

Flesch M, Schwarz A, Böhm. Effects of red and white wine on endothelium-dependent vasorelaxation of rat aorta and human coronary arteries. **Am J physiol** 1998; 275: H1183-H1190.

Fitzpatrick DF, Hirschfield SL & Coffey RG. Endothelium dependent vasorelaxing activity of wine and other grape products. **Am J Physiol** 1993;265:H774-H778.

Francel EN, Kanner J, German JB, Packs E, Kinsella JE. Inhibition of oxidation of human low-denstiy lipoprotein by phenolic substances in red wine. **Lancet** 1993;341:454-7.

Frei B, England L, Ames B. Ascorbate is an outstanding antioxidant in human blood plasma. **Proc Natl Acad Sci USA** 1989; 86: 6377-6381.

Fuhrman B, Lavy A, Aviram M. Consumption of red wine with meals reduces the susceptibility of human plasma and low-density lipoproteins to lipid peroxidation. **Am Soc Clin Nutr** 1995;61:545-554.

Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. **Nature** 1980; 288:373-376.

Galisteo M, García-Saura MF, Jiménez. Effects of chronic quercetin treatment on antioxidant defence systems and oxidative status of deoxycorticosterone acetate-salt-hypertensive rats. **Molec Cell Biochem** 2004;259: 91-99.

Gao Z, Haung K, Xu H. Protective effects of flavonoids in the roots of scutellaria baicalensis Georgi against hydrogen peroxide-induced oxidative stress in HS-SYSY cells. **Pharmacol Res** 2001; 43(2): 173-178.

Ghiselli A, Narindi M, Baldi A, Scaecini C. Antioxidant activity of different phenolic fractions sepertaed from an Italian red wine. **J Agric Food Chem** 1998; 46: 361-367.

Gil-Izquierdo A, Zatrilla P & Thomas-Barberan FA. An in vitro method to stimulate phenolic compound release from food matrix in the gastrointestinal tract. **European Food Research Technology** 2002; 214: 155-159.

Goldman IL, Kopelberg M, Debaene JEP & Schwartz BS. Antiplatlet activity of onion (Allium cepa) is sulphar dependent. **Thromb. Haemostasis** 1996; 76: 450-452.

Gover R, Rabelink TJ. Cellular regulation of endothelial nitric oxide synthase. **Am J Physiol Renal Physiol** 2001; 280: F193-F206.

Grafe R, Wittig J, Mueller S, Reithling A-K, Vehleke B, Drewélow B, Pforte H, Jacobash G, Derendorf H & Veitm. Pharmacokinetics and bioavailability of quercetin glycosides in humans. **J Clin Pharmacol** 2001; 41: 492-499.

Greindling KK, Minieri CA, Ollerenshaw JD, Alexander RW. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. **Circ Res** 1994; 74: 1-8.

Grunfield S, Hamilton CA, Mesaros S. Role of superoxide in the depressed nitric oxide production by the endothelium of genetically hypertensive rats. **Hypertension** 1995; 26: 854-857

Gryglewski RJ, Palmer RM, Moncada S. Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. **Nature** 1986; 320: 454-456.

Halliwell BH & Gutteridge JMC. Elevated mitochondrial cytochrome p450 2E1 and glutathione s-transferase A4-4 in stretozotocin induced diabetic rats: tissue specific variations and roles in oxidative stress-complications. **Free radicals in biology and Medicine Oxford University Press** 1989; 281: 1309-1312.

Hamilton CA, Berg G, McIntyre M, McPhaden AR, Reid JL, Dominicak AF. Effects of nitric oxide and superoxide on relaxation in human artery and vein. **Atherosclerosis** 1997; 133: 77-86.

Harbor ME, Balentine DA, Bouwens LC. The chemistry of tea flavonoids. **Crit Rev Food Sci Nutr**. 1997;37:693-704.

Harris K, Matthews. Intractions between Autonomic Nervous System Activity and Endothelial Function: a model for the Development of Cardiovascular Disease: **Psychosomatic Medicine** 2004; 66:153-164.

Haslam E. Practical polyphenols. From Structural to Molecular Recognition and Physiological Action. Cambridge University Press: Cambridge, UK 1998: p412

Hayek T, Fuhrmann B, Vaya J, Rosenblat M, Belinky P, Coloeman R, Elis A, Avirman M. Reduced progression of the atherosclerosis in apolipoprotein E-deficient mice after consumption of red wine, or its polyphenols quercetin or catechin, is associated with reduced susceptibility of LDL to oxidation and aggregation. **Arterioscler Thromb Vasc Biol** 1997; 17:2744-2752.

Heart Protection Study Group. MRC/BHF heart protection study of antioxidant vitamin supplementation in 20 536 high-risk individuals: a randomised placebo-controlled trial. **Lancet** 2002; 360: 23-33.

Heim K, Tagliaferro A, Bobilya D. Flavonoid antioxidants; chemistry, metabolism and structural-activity relationships. **J Nutr Biochem** 2002;13:572-584.

Heinonen IM, Meyer AS, Frankel EN. Antioxidant activity of berry phenolics on human low-density lipoprotein and liposome oxidation. **J Agric Food Chem** 1998; 46: 4107-4112.

Heitzer T, Krohn K, Albers S, Meinertz T. Tetrahydrobiopterin improves endothelium-dependent vasodilation by increasing nitric oxide activity in patients with Type II diabetus mellitus. **Diabetologia** 2000; 43: 1435-1438.

Hertog MGL, Hollman PCH. Potential health benefits of the dietary flavonal quercetin. **Eur J Clin Nutr** 1996; 50: 63-71.

Hu F. Dietary pattern analysis: a new direction in nutritional epidemiology. **Curr Opin Lipidol.** 2002; 13: 3-9.

Huang PL, Huang Z, Mashimo H. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. **Nature** 1995; 377: 239-242

Ignarro LJ. Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium derived relaxing factor produced from arteries and veins is nitric oxide. Proceedings of the **National Academy of Sciences of the United States of America** 1987; 84:9265-9269.

Itoigawa M, Takeya K, Ito C, Furu Kawa H. Stucture activity relationship of cardiotonic flavonids in guinea pig papillary muscle. **J Ethnopharmacol** 1999; 65: 267-72.

James WPT, Ferro-Lozz A, Isakson B, Szotak LB. Healthy Nutrition: Preventing nutrient related diseases in Europe Copenhagen: **WHO regional publication.** European Series. NO24 1988.

Janssen K, Mensink RP, Cox FJJ, Harryvan JL, Hovenier R, Hollman P & Katan MB. Effects of the flavonoids quercetin and apigenin on hemostasis in healthy volunteers: result from an in vitro and a dietary supplement study. **Am J Clin Nutr** 1998; 67: 255-62.

John JH, S Ziebland, Yudkin P, Roe LS. Effects of fruit and vegetable consumption on plasma antioxidant concentrations in a randomised controlled trial. **Lancet** 2002; 359: 1969-74.

Joshipura K, Hu F, Manson J. The effect of fruit and vegetable intake on risk for coronary heart disease. **Ann Intern Med** 2001; 134: 1106-1114.

Kähkönen MP, Hopia AI, & Heinonen M. Berry phenolics and their Antioxidant activity. **J Agric Food Chem** 2001; 49; 4076-4082.

Kerr S, Brosnan MJ, Mc Intyre, Reid JL, Dominiczak AF, Hamilton CA. Superoxide anion production is increased in a model of genetic hypertension: role of the endothelium. **Hypertension** 1999; 33: 1353-1358.

Khaw K, Barrett-Connor E. Dietary potassium and stroke-associated mortality: a 12 year prospective population study. **N Engl J Med** 1987; 316: 235-40.

Kitts D. Bioactive substances in food: identification and potential uses. **Can J Physiol Pharmacol**. 1994; 72: 423-424.

Knekt P, Jarvinen R, Reunanen A, Maatela J. Flavonoid intake and coronary mortality in Findland: cohort study. **BMJ** 1996; 342:1007-1111.

Kong J-M, Chia C-S, Goh N-K, Chia T-F & Brouillard R. Analysis and biological activities of anthocyanins. **Phytochemistry** 2003; 64:923-933.

Koshland DE. The molecule of the Year. **Science** 1992;258:1861-1861.

Kris-Etherton, Hecker KD, Borianome A. Bioactive compounds in foods. Their role in the prevention of cardiovascular disease and cancer. **Am J Med** 2002; 113 (9B): 715-885.

Kuo R, Baxter GT, Thompson SH. NO is necessary and sufficient for egg activation and fertilization. **Nature** 2000;406:633-636.

Landmesser ULF, Harrison DG. Oxidant stress as a marker for cardiovascular events. Ox marks the spot. **Circulation** 2001; 104:2638-2640.

Laughton MJ, Evans PJ, Morney MA, Hoult JR, Halliwell B. Inhibition of mammalian S-1 poxy genose and cyclo-oxygenase by flavonids and phenolic dietary additives relationship to antioxidant activity and to iron-reducing ability. **Biochem Pharmacol** 1991; 42:1673-81.

Leikert J, Räthel, Wohlfart P. Redwine polyphenols enhance nitric oxide synthase expression and subsequent nitric oxide release from endothelial cells. **Circulation** 2002; 106:1614-1617.

Lin C-M, Chen C-S, Chen C-T, Liang Y-C & Lin J-K. Molecular modeling of flavonoids that inhibit xanthine oxidase. **Biochem Biophys Res Commun** 2002; vol 294: 167-172.

Lincoln J, Hoyle CHV, Burnstock G. Nitric Oxide in health and Disease. **Cambridge: Cambridge University Press,** 1997;33-41.

Liu S, Manson J, Lee I. Fruit and vegetable intake and risk of cardiovascular disease: the Womens Health study. **Am J Clin Nutr** 2000; 72: 922-28.

López-López G, Morerro L, Coyollando A, Galisteo M, Ibarra M, Duarte J. Nitric oxide (NO) scavenging and NO protecting effects of quercetin and their biological significance in vascular smooth muscle. **Mol Pharmacol** 2004; 65:851-859.

Loscalzo J. Nitric oxide Insufficiency, Platelet Activation and Arterial Thrombosis. **Circ Res** 2001; 88: 756-762.

Lum H, Roebuk KA. Oxidant stress and endothelial cell dysfunction. **Am J Physiol** 2001; 280: C719-41.

Luscher TF, Barton M. Biology of the endothelium. **Clin Cardiol** 1997;20:II-3-10.

Macheix J-J, Fleuriet A, Billot J. Fruit Phenolics, CRC Press: Boca Raton, FL, 1990.

Marletta MA, Yoon PS, Iyengar R, Leaf CD, Wishok JS. Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. **Biochem** 1988; 27:8706-8711.

Martin S, Andriambeloson E, Takeda K, Andriantsitohaina R. Red wine polyphenols increase calcium in bovine aortic endothelial cells: a basis to elucidate signalling pathways leading to nitric oxide production. **Br J Pharmacol** 2002; 135:1579-87.

McIntyre M, Hamilton CA, Rees MD, Reid JL, Dominicak AF. Sex differences in the abundance of endothelium nitric oxide in a model of genetic hypertension. **Hypertension** 1997; 30: 1517-1524.

Mendes A, Desgranges C, Chéze C, Vercauteren J, Freslon J-L. Vasorelaxant effects of grape polyphenols in rat isolated aorta. Possible involvement of a purinergic pathway. **Fund & Clinical Pharmacol** 2003;17:673-681.

Meydani M. Dietary antioxidants modulation of aging and immune-endothelial cell interaction. **Mechanisms of Ageing and Development** 1999; 111:123-132.

Miller DD, Schricher BR, Rasmassen RR & Van Campen D. An in vitro method for estimation of iron availability from meals. **Am J Clin Nutr** 1981; 34:2248-2256.

Milstein S, Kausic Z. Oxidation of tetrahydropbiopterin by peroxynitrite implications for vascular endothelial function. **Biochem Biophys Res Commun** 1999; 263: 681-684.

Mirand-Rottermann S, Aspillaga AA, Pérez D, Vasques L, Martinez ACF & Leighton F. Juice and phenolic fractions of the Berry Aristotelia chilensis inhibit LDL oxidation in vitro and protect human endothelial cells against oxidative stress. **J Agric Food Chem** 2002;50:7542-7547.

Miyagi Y, Miwa K & Inoue H. Inhibition of low density lipoprotein oxidation by flavonoids in red wine and grape juice. **Am J Cardiol** 1997; 80: 1627-1631.

Mukamal KJ, Maclure M, Muller JE, Sherwood JB & Mittleman MA. Tea consumption and mortality after acute myocardial infarction. **Circulation** 2002; 105:2476-2481.

Mullen W, Stewart A, Lean M, Gardner P, Duthie G & Crozier A. Effect of freezing and storage on the phenolics, ellagitannins, flavonoids, and antioxidant capacity of red raspberries. **J Agric Food Chem** 2002; 50:5197-5201.

Mullen W, Lean M, Crozier A. Rapid charecterization of anthocyanins in red raspberry fruit by high-perfromance liquid chromatography coupled to single quadrupole mass spectrometry. **Journal of Chromatography A** 2002; 966:63-70.

Mullen W, McGinn J, Lean M, MacLean M, Gardner P. Ellagitannins, Flavonoids, and other Phenolics in Red Raspberries and their Contribution to Antioxidant Capacity and Vasorelaxation Properties. **J Agric Food Chem** 2002; 50:5191-5196.

Münzel T, Harrison DG. Increased superoxide in heart failure: biochemical baroflex gone awry. **Circulation** 1999; 100:216-218.

Murad F. Discovery of Some of the Biological Effects of Nitric Oxide and its role in cell signaling (Nobel lecture). **Angewate Chemie International Edition** 1999;38:1856-1868.

Murphy MP, Packer MA, Scarlett JL, Martin SW. Peroxynitrite: a biologically significant oxidant. **Gen Pharmac** 1998; 31: 179-186.

Nakazono K, Watanabe N, Matsuno K, Sasaki J, Sato T, Inoue M. Does superoxide underlie the pathogenesis of hypertension? **Pro Natl Acad Sci U S A** 1991; 88: 10045-10048

Natarajan K, Singh S, Burke TR, Grunberger D, Aggaval B. Caffeic acid phenelthyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF-kB. **Pro Natl Sci.** USA 1996; 93:9090-9095.

Negishi H, Xu J-W, Ikeda K, Njelekela M, Nara Y & Yamori Y. Black and Green Tea Polyphenols Attenuate Blood Pressure Increases in Stroke-Prone Spontaneously Hypertensive Rats. **J Nutr** 2002; 134:38-42.

Ness AR, Powles JW. Fruit and vegetables, and cardiovascular disease: a review. **Int J Epidemiol** 1997; 26:1-3.

Ohara Y, Peterson TE, Harrison DG. Hypercholesterolemia increases endothelial superoxide anion production. **J Clin Invest** 1993; 91:2546-2551.

Orallo F, Alwares E, Camina M. The possible implication of trans resveratol in the cardio-protective effects of long-term moderate wine consumption. **Mol Pharmacol** 2002; 294-302.

Packer JE, Slater TF, Wislson RL. Direct observation of a free radical interaction between vitamin E and vitamin C. **Nature** 1979; 278:737-738.

Palmer RM, Ferrigie AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. **Nature** 1987; 327:524-526.

Pannala AS, Chan TS. O'Brien, Rice-Evans CA. Flavonoid B-ring chemistry and antioxidant activity: fast reaction kinetics. **Biochem Biophys Res Commun** 2001;282:1161-1168.

Panza JA, Casino PR, Kilcoyne CM, Quyyumi AA. Role of endothelium-derived nitric oxide in the abnormal endothelium-dependent vascular relaxation of patients with essential hypertension. **Circulation** 1993; 87: 1468-1474.

Perez-Vizcaino F, Ibarra M, Cogolludo AL, Duarte J, Zaragoza-Arnaez F. Endothelium-independent vasodilator effects of the flavanoid quercetin and its

methylated metabolites in rat conductance and resistance arteries. **J Pharmacol Exp Ther** 2002; 302:66-72.

Prascal K, Guputu JB, Kulara J, Bharadwy B. Oxygen free radicals in volume overload heart failure. **Mol Cell Biochem** 1992; 111: 55-59.

Raha S, McEachern GE, Myint AT, Robinson BH. Superoxides from mitochondrial complex III: the role of manganese superoxide dismutase. **Free Radical Biology & Medicine** 2000; 29 –80, 2000

Rajagopalan S, Kurz S, Munzel T. Angiotension II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/DADPH oxidase activation. Contribution to alterations of vasomotor tone. **J Clin Invest** 1996; 97: 1916-1923

Rajnarayana K, Sirpal Reddy M, Chaluvadi, Krishna DR. Bioflavonoids classification, pharmacological, biochemical effects and therapeutic potential. **Indian J Pharmacol** 2001;33:2-6.

Ramsay LE, Williams B, Johnston GD. British Hypertension Society guidelines for hypertension management 1999: summary. **BMJ** 1999; 319: 630-635.

Rang HP, MM Dale, Ritter JM. Parmacology 4th Edition. Church Livingston 1999, Chapter 11: p188.

Rechner AR, Kuhnler G, Bremmer P, Hubard GP, Moore KP, & Rice-Evans CA. The metabolic fate of dieatary polyphenols in humans. **Free Radical Biology & Medicine** 2002; Vol 33: 220-235.

Rein D, Lotito S, Holt R, Keen C, Schmitz H, Fragu C. Epicatechin in human plasma: in vitro determination and effect of chocolate consumption on plasma oxidation status. **J Nutr** 2000: 130:21095-21145.

Renaud S & de Lorgeril M. Wine alcohol. Platelets, and French paradox for cornory heart disease. **Lancet** 1992; 339: 1523-1526.

Rice-Evans C, Miller NJ & Paganga G. Structure-Antioxidant activity relationships of flavonoids and phenolic acids. **Free Radical Biology & Medicine** 1996;7;933-956.

Robak J and Grylewski RJ. Flavonids are scavengers of superoxide anions. **Biochem Pharmacol** 1988;37:837-841.

Rotondo S, de Gaetano G. Protection from cardiovascular disease by wine and its derived products: epidemiological evidence and biological mechaninsms. **World Rev Nutr Diet** 2000; 87: 90-113.

Salonen RM, Nygssonen K, Kaikkonen J, Porkala-Sorataho E. Six year effect of combined vitamin C and E. Supplementation on Atherosclerotic progression. The antioxidant supplementation in atherosclerosis prevention (ASAP) study. **Circulation** 2003; 107: 947-953.

Schachinger V, Britten MB, Zeiher AM. Prognastic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. **Circulation** 2002; 101: 1899-906.

Serraino I, Dugo L, Dugo P, Mondello L, Mazzon E, Dugo G. Protective effects of cyaniding-3-*O*-glucoside from blackberry extract against peroxynitrite-induced endothelial dysfunction and vascular failure. **Life Sciences** 2003; 73: 1097-1114.

Skatchkov MP, Sperling D, Hink U. Validation of lucigenin as a chemiluminescent probe to monitor vascular superoxide as well as basal vascular nitric oxide production. **Biochem & Biophys Res Commun** 1999; 254;14-24, 1999.

Singleton VL. Wine phenols. In: Linskens HF, Jackson JF. **Modern Methods of Plant Analysis; vol 6. Berlin: Springer-Verlag,** 1988: 173-218.

Spiekermann S, Landmesser Ulf, Dikalov S, Bredt Martin Gamez Graciela, Tatge Helma. Electron Spin Resonance Characterization of Vascular Xanthine and NAD(P)H Oxidase Activity in patients With Coronory Artery Disease. **Circulation** 2003; 107: 1383-1389.

Stein JH, Keevil JG, Wiebe DA, Aeschliman S & Folts JD. Purple grape juice improves endothelial function and reduces the susceptibility of LDL cholesterol to oxidation in patients with coronary heart disease. **Circulation** 1999;100:1050-1055.

Stinzing FC & Carle R. Functional properties of anthocyanins and betalins in plant foods and in human nutrition. **Trends in Food Science and Technology** 2004; 15: 19-38.

Stroes E, Kastelein J, Cosentino F. Tetrahydrobioptrin restores endothelial function in hypercholesterolemia. **J Clin Invest** 1997; 99: 41-46

Stryer. Biochemistry 4th Edition. New York.WH Freeman and company 1995:p732

Synder H, Jaffery SR & Zakhary R. Nitric Oxide and carbon monoxide: parallel role as neural messengers. **Brain Res Rev** 1998; 26:167-175.

Taddei S, Agostino V, Mattei, Ghiadoni L. Aging and endothelial function in normotensive subjects and patients with essential hypertension. **Circulation** 1995;91:1981-1987.

Tauber AI, Fay JR & Marletta MA. Flavonoid inhibition of the human neutrophil NADPH-oxidase. **Biochem Pharmacol** 1984; 33:1367-1369.

Tiefenbacher CP, Bleeke T, Vahl C, Amann K, Vogt A, Kubler W. Endothelial dysfuction of coronary resistance arteries is improved by tetrahydrobiopterin in atherosclerosis. **Circulation** 2000; 102: 2172-2179.

Tribble D. Antioxidant consumption and risk of coronary heart disease: emphasis on vitamin C, vitamin E, and beta-carotene: a statement for healthcare professionals from the American Heart Association. **Circulation** 1999; 99: 591-5.

Truelssen T, Grøback H, Schnohr P & Boysen G. Intake of beer, wine and spirits and risk of stroke. The Copenhagen City Heart Study, Stroke 1998; 29: 2467-2472.

Ulbricht TT & Southgate DA. Coronary heart disease. Seven dietary factors. **Lancet** 1991; 338: 985-992.

Ülker S, Pascal P. Vitamins reverse endothelial dysfunction through regulation of eNOS and NAD(P)H oxidase activities. **Hypertension** 2003; 41: 534-539.

Van Acker S, De Groof M, Van den Berg D, Tromp M. Quercetin chemical explanation of the antioxidant activity of flavonoids. **Chem Res Toxicol** 1996;9:1305-1312.

Verma S, Buchanana M, Anderson T. Endothelial function testing as a biomarker of vascular disease. **Circulation** 2003; 108: 2054-2059.

Viera O, Escorgneil-Blanc I, Meilhac O. Effect of dietary phenolic compounds on apoptosis of human cultured endothelial cells induced by oxidized LDL. **Br J Pharmacol** 1998; 123: 565-73.

Vinson JA, Dabbagh Y. Effect of green and black tea supplementation lipids, lipid oxidation and fibrinogen in the hamster: mechaninsms for epidemiological benefits of tea drinking. **FEBS lett** 1998;433:44-46.

Walle T, Walle UK, Halash KA. Carbon dioxide is the major metabolite of quercetin in humans. **J Nutr** 2001; 131: 2648-2652.

Wang H, Cao G, Prior RL. Total antioxidant capacity of fruits. **J Agric Food Chem** 1997; 44: 701-705.

Wang SY, Jiao H. Scavenging capacity of berry crops on superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. **J Agric Food Chem** 2000; 48: 5672-5676.

Wever R, Boer P, Hijmering M. Nitric Oxide production is reduced in patients with chronic renal failure. **Atheriosc Thromb Vasc Biol** 1999; 19: 1168-1172.

White CR, Darly-Osmar U, Berrington WR, Adams MC, Jeri Z, Cor I, Thompson AJ, Parks DA, Tarpey M & Freeman BA. Circulting plasma xanthine oxidase contributes to vascular dysfunction in hypercholesterlolemic rabbits. **Pharmacology** 1996; Vol 93: 8745-8749.

World Health Organisation (WHO). Report of WHO study group. Diet Nutrition and the prevention of chronic diseases. **World Health Organ Tech Rep Ser** 1990; 191.

WHO. Cardiovascular disease: prevention and control, 2004 Website;www.who.int/dietphysical activity/facs/cvd/en/

Wolfram RM, Oguogho A, Efthimiou Y, Budinsky AC, Sinzinger. Effect of black tea on (iso-)prostaglandins and platelet aggregation in healthy volunteers. **Prostglandins, Leukotrienes and Essential Fatty Acids** 2002; 66(5-6): 529-533.

Wu KK, Thigarajan P. Role of endothelium in thrombosis and hemostasis. **Annu Rev Med** 1996; 47:315-31.

Xia Y, Tsui Al, Berka V, Zweier JC. Superoxide generation from endothelial nitric oxide synthase. A Ca2+/ Calmodulin-dependent and tetrahydrobiopterin regulatory process. **J Biol Chem** 1998; 273:25804-25808.

Xu A, Vita JA, Keaney JF. Ascorbic acid and gluthione modulate the biological activity of S-nitrosoglutathione. **Hypertension** 2000;36:291-295.

Ying C-J, Sun X-F, Zhang S-L, Zhang X-P, Mao L-M, Zuo X-Z and Yao P. ROS-related enzyme expressions in endothelial cells regulated by tea polyphenols. **Biomed Enviro Sci** 2004; 17:33-39.

Zanetti M, Zwacka R, Engelhart J, Katusic Z, O'Brein T. Superoxide anions and endothelial cell proliferation in nomoglycemia and hyperglycemia. **Artioscler Thromb Vasc Biol** 2001; 21:195-200.

Zino S, Skeaff M, Williams S, Mann J. Randomised controlled trial of effects of fruit and vegetable consumption on plasma concentrations of lipids and antioxidants. **BMJ** 1997; 314:1787-1791.