The influence of antioxidants on thrombotic risk factors in healthy population

By
Indu Singh
MAppSc (Medical Laboratory Sciences)

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Publications arising from this thesis

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1. Indu Singh, Andrew Carey, Nadine Watson, Mark A. Febbraio and John A Hawley. Oxidative stress-induced insulin resistance in skeletal muscle cells is ameliorated by gamma-tocopherol treatment. Accepted for publication by European Journal of Nutrition 09 Sep 2008.


Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research programme; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

Signed:

Indu Singh

Date: ___________
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>AOX</td>
<td>Antioxidants</td>
</tr>
<tr>
<td>Ox-LDL</td>
<td>Oxidised –LDL</td>
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<tr>
<td>ONOO•</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecules</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular cell adhesion molecules</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>BH₄</td>
<td>Tetrahydrobiopterin</td>
</tr>
<tr>
<td>GP</td>
<td>Glycoproteins</td>
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>PAR</td>
<td>Protease activated receptor</td>
</tr>
<tr>
<td>Gq</td>
<td>G protein</td>
</tr>
<tr>
<td>PLCₜ</td>
<td>Phospholipase Cₜ</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol 4,5 biphosphate</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol1,4,5 triphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PtdIns 3,4,5 p₃</td>
<td>Phosphatidylinositol 3,4,5-triphosphate</td>
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<td>AA</td>
<td>Arachidonic acid</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TXA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Thromboxane A&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PGI&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostacyclin</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>TK</td>
<td>Tyrosine kinase</td>
</tr>
<tr>
<td>PUFAs</td>
<td>Polyunsaturated fatty acids</td>
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<tr>
<td>GSH-Px</td>
<td>Glutathione peroxidise</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycation end products</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
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<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidise</td>
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<td>GST</td>
<td>Glutathione S-transferase</td>
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<td>LPO</td>
<td>Lipo oxygenase</td>
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<td>PLA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Phospho lipase A&lt;sub&gt;2&lt;/sub&gt;</td>
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<tr>
<td>α-TTP</td>
<td>Alpha tocopherol transfer protein</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>VO&lt;sub&gt;2&lt;/sub&gt;max</td>
<td>Maximum oxygen uptake</td>
</tr>
<tr>
<td>FBE</td>
<td>Full blood examination</td>
</tr>
<tr>
<td>MPV</td>
<td>Mean platelet volume</td>
</tr>
<tr>
<td>RER</td>
<td>Respiratory exchange ratio</td>
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<tr>
<td>PPO</td>
<td>Peak sustained power output</td>
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<tr>
<td>EDTA</td>
<td>Ethylene-diamine-tetra acetic acid</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>SST</td>
<td>Serum separator tubes</td>
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<tr>
<td>PMT</td>
<td>Photomultiplier tube</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet-activating factor</td>
</tr>
<tr>
<td>α-T or AT</td>
<td>Alpha-tocopherol</td>
</tr>
<tr>
<td>γ-T or GT</td>
<td>Gamma-tocopherol</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>ecNOS</td>
<td>Endothelial constitutive nitric-oxide synthase</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoproteins</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
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<tr>
<td>GO</td>
<td>Glucose oxidase</td>
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Abstract

Oxidative damage has been suggested to play a key role in the pathogenesis of atherosclerosis and other cardiovascular disease (CVD). Increased free radical production induced by oxidative stress can oxidise low density lipoproteins, activates platelets, induces endothelial dysfunction and disturbs glucose transport by consuming endogenous antioxidants. Supplementation with antioxidants is known to decrease markers of oxidative stress.

Using a combination, of in vitro and in vivo experimental models, the primary aims of the studies undertaken for this thesis were to examine if different hydrophilic and lipophilic antioxidants could improve risk factors leading to thrombosis, atherosclerosis and other cardiovascular diseases through mechanism involved in platelet activity and glucose uptake by skeletal muscle myotubes in response to induced oxidative stress in normal healthy population.

The phenolic compounds of olive leaves and olive oils in the Mediterranean diet have been associated with a reduced incidence of heart disease. The active phenolic compounds in this extract (oleuropein) are part of the secoiridoid family, known for their capacity to scavenge H$_2$O$_2$. Accordingly, the aim of the first study described in Chapter Two was to determine the most effective dose of olive leaf extract that would attenuate platelet activity in healthy human subjects. Five different concentrations of extract of *Olea europaea* L. leaves ranging from 5.4 µg/mL to 54 µg/mL were investigated. Platelet function at increasing concentrations of oleuropein was determined through measures of platelet aggregation and ATP release from activated platelets. Blood analysis (*n*=11) revealed a significant dose-dependant
reduction in platelet activity with olive extract concentrations of 54 µg/mL \((p<0.001)\). ATP Release, a measure of platelet dense granule release, showed a similar pattern to the results for platelet activity \((p=0.02)\). The results from this study demonstrate that olive leaf polyphenols derived from *Olea europaea* L. leaves inhibited *in vitro* platelet activation in healthy, non-smoking male subjects.

In chapter three, the effect of exercise and antioxidant supplementation on platelet function was compared between trained and sedentary individuals. The purpose of the exercise bout was to induce oxidative stress. In this randomised, double-blind, placebo-controlled cross over study, 16 healthy, 8 trained and 8 sedentary male subjects had their diet supplemented for one week with 236 mg/day of cocoa polyphenols. All subjects underwent 1 hr of sub maximal cycling at 70% of VO\(_{2\text{max}}\) before and after 1 week of supplementation. Baseline platelet count and ATP release increased significantly \((p<0.05)\) after exercise in all subjects. Baseline platelet numbers in the trained were higher than in the sedentary \((235±37 \text{ vs. } 208±34 \times 10^9/L, p<0.05)\), whereas platelet activation in trained subjects was lower than sedentary individuals \((51±6 \text{ vs. } 59±5\%, p<0.05)\). Seven days of cocoa polyphenol supplementation did not change platelet activity compared to the placebo group. In conclusion, trained subjects had decreased activation of stimulated platelets compared to the sedentary subjects and short term cocoa polyphenol supplementation did not decrease platelet activity in response to exercise independent of prior training status.

Results of epidemiological studies show an inverse relationship between vitamin E intake and coronary disease. The activity of vitamin E is derived primarily from alpha-tocopherol (\(\alpha\)-tocopherol) and gamma-tocopherol (\(\gamma\)-tocopherol). The third study
undertaken for this thesis and described in chapter four determined the effect of 5 wk of either 100 mg/d or 200 mg/d γ-T supplementation on thrombotic risk markers including platelet function, lipid profile and the inflammatory marker C-reactive protein (CRP). In a double-blinded parallel study design 14 healthy subjects consumed 100 mg/day and 13 consumed 200 mg/d of γ-tocopherol while 12 received placebo (soybean capsules with less than 5 mg/d γ-tocopherol). Fasting pre and post supplementation blood samples were analysed for various markers of inflammation, platelet activity and lipid profile. Blood γ-tocopherol concentrations increased (p<0.05) relative to dose during the intervention period. Both groups receiving active ingredients showed lower platelet activation after supplementation (p<0.05). Subjects consuming 100 mg/d γ-tocopherol had decreased LDL cholesterol, platelet aggregation and mean platelet volume (MPV) (all p<0.05). Little effect of γ-tocopherol was observed on other parameters (i.e. CRP). These data suggest that γ-tocopherol supplementation may have a permissive role in decreasing the risk of thrombotic events by improving lipid profile and reducing platelet activity.

The final study described in chapter five was undertaken to determine the effect of oxidative stress and gamma tocopherol treatment on glucose transport and insulin signalling in cultured rat L6 muscle cells. L6 myotubes were incubated with either 100 µM or 200 µM gamma tocopherol for 24 h and exposed to oxidative stress for 1 hr by treating with 100 mU/mL glucose oxidase (H₂O₂ generating system). 2-deoxy-D-[³H]-glucose uptake by myotubes and the phosphorylation of Akt and AS160 on amino-acid residues that are important for insulin signalling were assessed. One hour treatment with 100 mU/mL glucose oxidase significantly decreased glucose uptake both with and without 100 nM insulin stimulation (p<0.05). Pre-treatment with 100 µM and 200 µM gamma tocopherol partially protected cells from the effect of glucose
oxidase, whereas 200 µM gamma tocopherol restored both basal and insulin
stimulated glucose transport to control levels. Glucose oxidase-induced oxidative
stress did not impair basal or insulin stimulated phosphorylation of Akt or AS160, but
200 µM gamma tocopherol improved insulin-stimulated phosphorylation of these
proteins. These observations indicate that gamma tocopherol improves the free
radical defence system and partially prevents adverse effect of oxidative stress on
insulin signalling in rat L6 muscle cells.

In summary, the results from the studies undertaken for this thesis provide evidence
that antioxidant supplementation maintains normal platelet function, exerts a positive
effect on blood lipid profile and improves glucose uptake in normal healthy
asymptomatic population as well as under conditions of induced oxidative stress.
Antioxidants including foods rich in cocoa, olive and gamma tocopherol have the
potential to combat oxidative stress induced cardiovascular diseases.
Chapter One: Literature Review
Cardiovascular disease is one of the major health problems in advanced as well as developing nations (Barbato and Tzeng, 2004). According to World Health Organisation statistics, coronary heart disease currently kills more than 7 million people and costs more than $258 billion in health care services, medications, and lost productivity each year worldwide (Mackay and Mensah, 2004). Oxidative stress plays a central role in the pathogenesis of a wide range of cardiovascular related diseases, including thrombosis, Type II diabetes, hypercholesterolaemia and atherosclerosis.

Extensive research during the last 25 years has demonstrated that free radicals, particularly, reactive oxygen species and reactive nitrogen species play a cardinal role in the damage caused by oxidative stress. There is increased oxidative stress due to conditions such as diabetes (Baynes, 1991), which impairs muscle glucose uptake (Blair et al., 1999), by damaging the blood vessel walls by oxidising lipids and in turn activating platelets resulting in thrombosis and other cardiac complications (Boullier et al., 2001). The results of many epidemiological studies have demonstrated a protective role of antioxidants through a diet rich in fruits and vegetables against the development and progression of atherosclerosis and other cardiovascular disease (CVD) (Genkinger et al., 2004, Kushi et al., 1996, Law and Morris, 1998, Rimm et al., 1996). This review presents an overview of our current knowledge regarding the oxygen free radicals involved in cardiovascular complications, and the preventive endogenous as well as exogenous antioxidant defence.
In this regard, several naturally occurring products have received considerable scientific enquiry because of their potential antioxidant activity. Consuming a diet rich in natural antioxidants has been associated with prevention from and/or treatment of atherosclerosis. Bioactive components of food, which are of special interest, include the vitamin E, cocoa and olive polyphenols, all of which have antioxidant properties. Antioxidant therapy is believed to be effective in the early stages of atherosclerosis by preventing low density lipoproteins (LDL) oxidation and the oxidative lesion of endothelium. The preventive effect of dietary antioxidants can be illustrated by an improvement in impaired lipid profile and platelet dysfunction seen in response to oxidative stress. Understanding the effect of oxidative stress on cardiovascular disease, in relation to platelet activity and glucose uptake by skeletal muscle, may open new horizons for allowing effective dietary intervention strategies to recover normal homeostasis and to prevent diet-related implications.

1.1 Pathogenesis of atherosclerosis

Atherosclerosis is a disease of large and medium sized arteries that results in progressive accumulation of lipids within intima of smooth muscle cells. Growth of atherosclerotic lesions causes narrowing of blood vessel lumen and affects the arterial wall (Ross, 1993, Vardaxis, 1994, Chan, 1998). Atherosclerosis is a multifactorial disease and accounts for the majority of premature deaths in Western, developed countries (Poulter, 1999).

Early asymptomatic atherosclerosis lasting for years is characterised by the deposition of intracellular and extra cellular lipids and by the appearance of macrophages and T-lymphocytes in the vessel intima. As macrophages and smooth-muscle cells below the endothelial cells accumulate lipids, they acquire a foamy
appearance visible as fatty streaks (Figure 1.1) (Davies and Woolf, 1993). These flat, fatty lesions may be transformed into raised fibro lipid plaques and ultimately into a fibroatheroma, which has a characteristic microanatomy of a core of extra cellular lipid covered on the luminal side by a thick fibrous cap. Surrounding the core are lipid-laden foam cells, while ischaemia in the necrotic core initiates angiogenesis (Paul et al., 2004).

![Figure 1.1 oxidative modifications of low density lipoproteins (LDL) and its implication in atherosclerotic development. Native LDL is oxidatively modified by reactive oxygen species (ROS) generated by resident vascular cells (e.g. smooth muscle cells, macrophages, endothelial cells etc) in the subendothelial space. Oxidised LDL promotes foam cell formation, endothelial dysfunction and injury, eventually leading to the development and progression of atherosclerotic plaques.](image)

Native LDL is oxidatively modified by reactive oxygen species (ROS) generated by resident vascular cells (e.g. smooth muscle cells, macrophages, endothelial cells etc) in the subendothelial space (Figure 1.1). Oxidised LDL promotes foam cell formation, endothelial dysfunction eventually leading to the development and progression of...
atherosclerotic plaques. Although oxygen is a crucial molecule to all living organisms, it can be toxic for endothelial cells. Perturbations to oxidative balance can lead to induction of oxidative stress by ROS. Collectively, cells residing in atherosclerotic lesions may produce ROS that contribute to oxidative modification of LDL, which in turn is believed to promote foam cell formation (Figure 1.1), accelerating the progression and growth of atherosclerotic plaques (Rein et al., 2000b, Kuhn and Chan, 1997, Juliano et al., 1997, Hertog and Hollman, 1996, Chan, 1998).

The mural thrombosis is a critical part of the later progression of the atherosclerotic lesion and is probably the major event leading to vascular occlusion. If the thrombus is not occlusive, it becomes incorporated into the plaque and is organized by invading macrophages, endothelial cells, and smooth muscle cells, thereby further compromising the lumen of the vessel. The sequence of fissure, thrombus formation, organization, and incorporation into the plaque may occur repeatedly (Bult et al., 1999). Once compensatory vascular remodelling becomes inefficient, this type of plaque may cause narrowing of the lumen. The ultimate stage, the complicated plaque, may arise either from a fissure in the fibrous cap, or from intraplaque haemorrhage. Antioxidant agents have been proposed to arrest these effects (Chan, 1998).

Thromboembolic events following plaque fissure are a major cause of clinically manifest acute ischaemic syndromes. Major mechanisms leading to coronary thrombosis include frank rupture of a plaque's fibrous cap, intraplaque haemorrhage, and superficial erosion of the endothelium. Plaque rupture occurs when the mechanical stresses in the fibrous cap exceed a critical level that the tissue cannot withstand (Lee and Libby, 1997). Furthermore, activated macrophages in
atherosclerotic lesions kill smooth muscle cells in their vicinity either by lytic damage leading to necrosis or by inducing apoptosis (Kockx, 1998, Kockx et al., 1996a, Kockx et al., 1996b). As smooth muscle cells are central to the biosynthesis and maintenance of the fibrous cap, their numbers may become insufficient to repair the degradation. Well known accelerating risk factors for atherosclerosis include hypercholesterolaemia, hypertension, diabetes, and smoking.

In hypercholesterolaemia induced atherosclerosis, a causal role has been attributed to oxidized-LDL (ox-LDL) (Witztum, 1993, Hamilton, 1997). Oxidation of lipoproteins flooding the intima may result from the production of reactive oxygen intermediates, particularly peroxynitrite (ONOO−) or from 15-lipoxygenase activities in the endothelial cells. Ox-LDL is, in turn, cytotoxic to endothelial cells due to the metal-catalysed production of free radicals from lipid hydroperoxides contained in the modified lipoprotein particle (Thomas et al., 1993). Ox-LDL accumulates in the endothelial cells, intima, and smooth muscle cells and is chemotactic for monocytes and T-lymphocytes. Newly formed antigenic determinants (epitopes) in ox-LDL elicit cell-mediated and humoral immune responses (Libby and Hansson, 1991). Minimally modified LDL stimulates the endothelial and smooth muscle cells to secrete monocyte chemotactic protein-1 and growth factors involved in the differentiation and proliferation of monocytes.

In addition, ox-LDL may, synergistically with cytokines, promote mononuclear leukocyte adhesion to the endothelium through the induction of vascular cell adhesion molecules and intercellular cell adhesion molecules (Khan et al., 1995, Kume et al., 1992). Monocyte-derived macrophages internalize ox-LDL through scavenger receptors. As these receptors are not down regulated by the intracellular
cholesterol level, massive cholesterol accumulation occurs and the macrophages transform to foam cells (Jialal et al., 2004). Ox-LDL cholesterol increases the synthesis of caveolin-1 (Cav-1; the principal structural protein in caveolae that binds cholesterol), which inhibits production of nitric oxide (NO) by inactivating endothelial nitric oxide synthase (eNOS) (Kinlay et al., 2001). Conversely, normal release of NO prevents oxidative modification of LDL cholesterol (Rubbo et al., 2002). Under healthy conditions, low concentrations of NO are continuously involved in a variety of physiological functions, which include the regulation of blood pressure, blood flow and platelet aggregability (Moncada et al., 1991). However, in atherosclerosis there is impaired generation of NO in the vascular endothelium, leading to vascular dysfunction (Harrison, 1997). This probably co-exists with the expression of inducible nitric oxide synthase (iNOS) in the plaque, generating excessive amounts of NO and leading to an interaction with oxygen-derived radicals, generation of ONOO⁻, and further impaired vascular function.

### 1.1.1 Endothelial Dysfunction

The major risk factors for atherosclerosis include hypercholesterolaemia, hypertension, diabetes, and smoking (Cai and Harrison, 2000). These conditions are associated with endothelial dysfunction, which itself is associated with reduced endothelial generation of NO. Vascular injury begins with endothelial dysfunction and activation. Endothelium dysfunction is considered as an early indicator of atherosclerosis, and precedes atherosclerotic plaque. Endothelium dysfunction can be detected at both the conduit and microvascular levels in patients with coronary risk factors, even though there may be no evidence of structural coronary artery disease (McFadden et al., 1991, Vrints et al., 1992a, Vrints et al., 1992b).
Endothelial dysfunction is at the core of microvascular complications, and correlates closely with insulin resistance (Cleland et al., 2000). Endothelial dysfunction might itself contribute to platelet hyperactivity and to insulin resistance (Scherrer and Sartori, 2000); indeed, it could precede the onset of insulin resistance in populations at risk for diabetes (Caballero et al., 1999). A direct role of insulin in microvascular physiology is also recognized. For example, insulin stimulates the expression and activation of endothelial NO synthase (Lazar et al., 1995). In addition to being a potent vasodilator, NO is a key vasoprotective agent influencing pathogenic pathways including oxidative stress, smooth-muscle proliferation, lipid per oxidation, and chemo attraction and monocyte adhesion. Even though insulin is a potent vasoactive hormone, under basal circumstances it has limited net hemodynamic effects (Vicent et al., 2003).

Endothelial dysfunction of the microvasculature has also been associated with exercise-induced myocardial ischaemia in patients without haemodynamically significant CAD of the epicardial arteries, suggesting that endothelial dysfunction of the microcirculation may contribute to ischaemia when myocardial oxygen demand is increased (Zeiher et al., 1995). The enhanced ROS production at sites of injury has multiple consequences for endothelial function. Increased adhesion molecule expression attracts monocytes, which transmigrate and become macrophages and produce much higher amounts of ROS. Reactive oxygen species can also promote vascular leakage, which is important in ischemia–reperfusion-induced vascular injury and the induction of inflammatory responses.
1.1.1.1 Role of Inflammation in CVD

Epidemiological and clinical studies have linked inflammatory markers to the risk of future cardiovascular events (Ridker, 2004). White blood cells are part of the body's immune defense system, and their levels are an indirect measure of inflammation in the body. Studies examining links between CVD and inflammation have focused mostly on a molecule called C-reactive protein (CRP) as a marker of inflammation. CVD is associated with elevated markers of systemic inflammation, including CRP and members of the coagulation cascades. Systemic inflammation has been reported to be present before any evidence of myocardial infarction (Lowe, 2001). This observation was first interpreted as an inflammatory response to the developing atheromatous vascular damage. However, evidence to suggest that systemic inflammation causes atherosclerosis is supported by the observation that patients with pre-existing inflammatory diseases have a dramatically increased risk of CVD at younger ages. Patients with autoimmune diseases such as rheumatoid arthritis and lupus have accelerated rates of atherosclerosis (Kao et al., 2003). Elevated CRP levels have also been associated with endothelial dysfunction in the form of inappropriate vascular constriction/relaxation. CRP has been shown to cause induction of endothelial adhesion proteins ICAM-1, VCAM-1, E-selectin, P-selectin, and angiotensin type 1 receptor (Pasceri et al., 2000, Verma et al., 2003). Additionally, CRP has been implicated in the activation of endothelial NF-κB, induction of endothelial IL-1β, PAI-1, IL-6, TNF-α, monocyte chemoattractant protein-1 (MCP-1), endothelin-1, and tissue factor, and inhibition of endothelial NO synthase and NO signalling (Labarrere and Zaloga, 2004).

Polyphenols express anti-inflammatory activity by modulation of pro-inflammatory gene expression such as cyclooxygenase, lipoxygenase, nitric oxide synthases and
several pivotal cytokines, mainly by acting through nuclear factor-kappa B and mitogen-activated protein kinase signaling (Yoon and Baek, 2005, Kim et al., 2004 and Stangl et al., 2007).

1.1.2 Endothelial dysfunction and nitric oxide

Nitric Oxide released by the vascular endothelium acts as endothelium-derived relaxing factor, and the elucidation of its biosynthetic pathway has opened a whole new area in the quest for understanding cardiovascular disease and the ways in which it may be prevented (Palmer et al., 1987, Moncada et al., 1991, Feelisch et al., 1994, de Meyer and Herman, 1997). A reduction in the generation and bioavailability of NO has been shown to occur in several disorders, including atherosclerosis.

Endothelium is not merely an inert, single-cell lining covering the internal surface of blood vessels, but plays a crucial role in regulating vascular function (Landmesser et al., 2004). As the major regulator of vascular homeostasis, the endothelium maintains the balance between vasodilation and vasoconstriction, inhibition and stimulation of the proliferation and migration of smooth muscle cells, and thrombogenesis and fibrinolysis. Endothelial dysfunction leads to the disruption of this balance and causes damage to the arterial wall (Landmesser et al., 2004, Luscher and Barton, 1997, Kinlay et al., 2001, Davignon and Ganz, 2004). Enhanced superoxide ion (O$_2^-$) levels play an important role in the pathophysiology of atherosclerosis (Figure 1.2). For instance, O$_2^-$ may inactivate NO and diminish its bioavailability, thus inducing endothelial dysfunction (Kojda and Harrison, 1999). OONO$^-$ induces lipid peroxidation and protein nitration and in the process damages vascular cells and wall (Figure 1.2).
On the other hand $O_2^-$ is instrumental in uncoupling of eNOS which results in decreased NO production and increased $O_2^-$ concentration (Figure 1.2). Increased vascular production of ROS promotes the oxidative degradation of the critical eNOS cofactor tetrahydrobiopterin (BH$_4$), leading to eNOS uncoupling and the consequent reduced production of NO and the increased production of superoxide anion from the enzyme (Vasquez-Vivar et al., 2002, d'Uscio et al., 2003, Landmesser et al., 2003).

There is indirect evidence to suggest that eNOS uncoupling contributes to endothelial dysfunction and increased production of superoxide anion in the oxidative stress of ischaemia/reperfusion injury (Huk et al., 1997), hypercholesterolaemia (Pritchard et al., 1995), hypertension (Mollnau et al., 2002), diabetes (Guzik et al., 2002), and heart failure (Dixon et al., 2003).
Figure 1.2 Flow diagram illustrating the multifactorial origin, the intermediate pathways and final detrimental consequences of an excess of superoxide anion ($O_2^-$) in the vascular system. Adapted from (Zalba et al., 2006).

A defect in NO production or activity has been proposed to be a major mechanism of endothelial dysfunction and a contributor to atherosclerosis (Verbeuren et al., 1986, Davignon and Ganz, 2004). Impaired production or activity of NO leads to events or to actions such as vasoconstriction, platelet aggregation, smooth muscle cells proliferation and migration, leukocyte adhesion, and oxidative stress (Endres et al., 1998). However, endothelial dysfunction is likely to be a multifactorial process. Increased vascular production of ROS or superoxide anion has been demonstrated in all major conditions predisposing to atherosclerosis (Cai and Harrison, 2000, Landmesser and Harrison, 2001, Krotz et al., 2004, Schulz et al., 2004).
1.1.3 Role of Platelets in Primary Haemostasis

Blood platelets play a pivotal role in the development of atherosclerosis and are often found at sites of atherosclerotic lesions, closely associated with neutrophils, monocytes, macrophages and endothelial cells that produce ROS capable of modulating platelet function, via normal cellular metabolism.

Haemostasis is the physiological defence against blood loss and involves tight control of clot formation (Figure 1.3) (Schneider et al., 1998). Platelets and coagulation factors under normal circumstances do not directly interact with endothelial cells lining the vessel walls and only do so when the endothelial lining is breached as a consequence of trauma or atherosclerotic plaque disruption (Hoffbrand and Pettit, 1993, Veijio et al., 1999).

![Figure 1.3](image_url)

**Figure 1.3** Integrated concept of cell-based haemostasis. Adapted from (Coller, 1992, Hirsh and Weitz, 1999).
A delicate balance exists between the interaction of platelets, coagulation factors and the fibrinolytic system to maintain haemostatic integrity. The net result of these interactions is stabilisation of the platelet plug by fibrin, followed by cross-linking of fibrin by factor XIIIa to form an insoluble fibrin clot.

Vascular breach releases tissue factor (TF) and factor VIIa from endothelial cells (initiating release of clotting factors and activation cascade leading to blood clot formation). These factors activate factor X and V, which in turn lead to thrombin formation necessary for platelet activation and to convert fibrinogen to fibrin (Figure 1.3). Tissue factor released from damaged endothelial cells activates the extrinsic clotting system, generating thrombin, which serves two main functions. The first is the primary role of thrombin in conversion of fibrinogen to fibrin and resultant clot formation. Secondly, there is self-amplification of thrombin via feedback activation of Factors V and VIII. An increase in negatively charged phospholipids (e.g. phosphatidylserine) causes membrane modification, permitting assembly of a ‘tenase’ (Factor X activating complex) and a prothrombinase (prothrombin activating complex) (Bennett, 2001, Dracup and Cannon, 1999, Veijio et al., 1999). Amplification of thrombin generation enhances platelet activation, aggregation, fusion and coagulation (Figure 1.3).

1.1.4 Platelet Physiology: Mechanisms of Platelet Adhesion, Activation and Aggregation

Platelet adhesion, activation and aggregation, constitute the pre-developmental stages of an arterial thrombus. At the site of arterial injury, the endothelial barrier is broken and platelets adhere to exposed collagen, von Willebrand factor (vWF is a large plasma protein and is also present on the surface of platelets serving as a hook.
Injury

Shear Forces

Adhesion
- vWF
- Thrombin
- Collagen
- Fibronectin

Activation
- Membrane changes
- Granule secretion
- GPIIb/IIIa expression
- Multiple agonists
- Feedback loops

Aggregation
- GPIIb/IIIa mediated
- Fibrinogen
- vWF

Thrombus

Figure 1.4 Key mediators in platelet adhesion, activation & aggregation. See text for further details.

Activated platelets degranulate and secrete chemotaxins, clotting factors and vasoconstrictors, thereby promoting thrombin generation, vasospasm, and additional platelet accumulation. The release of internally stored ADP and thromboxane amplifies the process of platelet activation by secondary feedback loops. Activated platelets also change shape resulting in cell membrane changes, which are important in further aggregation and coagulation (Figure 1.4). Previously inactive GPIIb/IIIa receptors on the platelet membrane undergo structural modification and become available for fibrinogen and vWF binding. In patients with atherosclerotic stenosis, shear stress can be abnormally elevated and can directly activate platelet
aggregation and/or amplify all of the three-platelet steps, mediated through vWF and ADP-receptors (Figure 1.4).

1.1.4.1 Platelet Membrane Glycoproteins: Adhesion Molecules
Platelet membrane glycoproteins (GP) are now known to be adhesion molecules identified in other cell types in the late 80’s (Pischel et al., 1988, Roth, 1992, Phillips et al., 1988, Hsu-Lin et al., 1984). They are present on the platelet surface or inserted into the alpha-granule membrane (Figure 1.4), and play a key role in normal platelet adhesion or aggregation.

1.1.4.2 Membrane Glycoproteins in Platelet Physiology
Physiological agonists that may be present at the vascular lesion site include thrombin, collagen and ADP (Ruggeri, 1994). In response to these agonists, a spectrum of specific activation dependent modifications of platelet antigens can be detected. This process leads to an intracellular signal transduction cascade involving ion fluxes and activation of the cytoskeleton. The end result is secretion of endosomes, which causes reorganisation and conformational changes of surface receptor expression through inside-out signalling. Platelet activation leads to an altered expression of already constitutively expressed surface glycoproteins (figure 1.5). Increased numbers of GPIIb-IIIa complexes and reduced numbers of GPIb-IX complexes result from bi-directional trafficking of these glycoproteins between the cell surface, the surface-connected canalicular system and intracellular storage (Michelson and Barnard, 1987).

Inside-out signalling leads to conformational changes of GPIIb-IIIa complexes, exposing conformation-dependent activation epitopes with high affinity for their ligands (Sims et al., 1991).
The release reaction of platelets is associated with the neo-expression of α-granule glycoproteins such as CD62P or CD63. Measuring the expression of these antigens on circulating platelets reflects not only the activation state of the platelets, but also to what extent secretion has occurred.

Platelets adhere to other platelets through fibrinogen attachment at GPIIb-IIIa receptors on platelet surface and form a plug at site of injury by their GPIb receptors interacting with von Willebrand factor (vWF), while GPVI, GPIa/IIa and GPIV interact with collagen released from the damaged endothelial wall (Figure 1.5).

1.1.4.3 Adhesion

Trauma or plaque disruption causes platelet adherence to exposed subendothelial structures (e.g. collagen), via interactions with glycoprotein GP Ib-IX-V complex on the platelet membrane and von Willebrand factor (vWF) under conditions of high
shear stress (Fredrickson et al., 1998). Interactions mediated by GP Ia/IIb occur under conditions of low shear stress. Occupancy of this receptor by collagen, activates platelets (Figure 1.5), thereby causing fusion and exocytosis of alpha and dense granule contents (e.g. ADP, ATP, serotonin, calcium (Ca$^{2+}$), fibronectin, β-thromboglobulin, thrombospondin, P-selectin etc.) (Lefkovits et al., 1995, Puri and Colman, 1997, Schneider et al., 1998, Hirsh and Weitz, 1999). Adhesion causes microtubule depolymerisation, pseudopodia extension and loss of the discoid appearance of platelets (Reed et al., 2000).

1.1.4.4 Activation

During agonist-induced platelet activation, energy-dependent fusion of both alpha and dense granules with the plasma membrane and expression of granule proteins (e.g. P-selectin), promotes and propagates platelet adhesion to endothelial and white cells (Schneider et al., 1998). Binding of thrombin to its receptor, protease activated receptor (PAR) activates the alpha unit of Gq (G protein) via tyrosine phosphorylation, thereby producing inside-out signalling by activating phospholipase C$\beta$ (PLC$\beta$) (Shattil et al., 1998). Activated PLC$\beta$ hydrolyses phosphatidylinositol 4,5 bisphosphate (PIP$_2$), producing the second messengers inositol 1,4,5 triphosphate (1,4,5-IP$_3$) and sn-1, 2 diacylglycerol (sn-1, 2 DAG) (Figure 1.6).
Figure 1.6 Cascade of events leading to platelet activation, aggregation and its inhibition upon agonist binding to its specific cellular receptor, tyrosine kinase (TK) activation subsequently phosphorylates and activates phospholipase C (PLC). This cleaves phosphatidylinositol 4,5 bisphosphate (PIP2), resulting in the generation of inositol 1,4,5 triphosphate (1,4,5-IP3) and sn-1, 2 diacylglycerol (Sn-1, 2 DAG). Coupled with increased Ca2+, the latter activates protein kinase C (PKC). As a result, PLA2 is activated and arachidonic acid (AA) is produced. Activation of cyclooxygenase (COX) produces the proaggregatory agent thromboxane (TXA2), which functions to stimulate tyrosine kinase (TK) activity via autocrine signalling, dense granule release, further platelet activation and aggregation. Prostaglandin (PGI2) released from endothelial COX inhibits adenylate cyclase, thereby increasing intracellular cAMP levels, decreasing Ca2+ levels, thus inhibiting platelet aggregation. Prostaglandin produced by platelets can inhibit aggregation via activation of platelet adenylate cyclase. Human platelet adenylate cyclase is stimulated by prostaglandin E1 (PGE1). Adapted from (Shattil et al., 1998)
IP₃ mobilises Ca²⁺ from internal stores. Sn-1, 2 DAG and the released Ca²⁺ activate protein kinase C (PKC) and subsequently promote PLA₂ activation (Shattil et al., 1998, Schneider et al., 1998, Savage et al., 2001). Hydrolysis of membrane phospholipids via PLA₂ activation yields arachidonic acid (AA) and thromboxane A₂ (TXA₂) (Figure 1.6) (Savage et al., 2001). PKC has been implicated in assisting the exocytosis of substances from platelet granules (Selheim et al., 2000).

Synergism between PKC and Ca²⁺ levels help regulate platelet exocytosis of microparticles (e.g. AA), which are metabolised via the cyclooxygenase (COX) pathway (Reed et al., 2000). Arachidonate is oxygenated via platelet COX to produce prostaglandins D₂ (PGD₂), G₂/H₂ (PGG₂/H₂) and thromboxane (TXA₂). Despite PGD₂ inhibition of platelet activation, the net effect of platelet activation by PGG₂/H₂ and TXA₂ is greater (Holmsen, 1994). In contrast, endothelial COX produces PGI₂. Platelet lipid peroxidation products (12-hydroxyeicosatetraenoate or 12 HETE) undergo further processing by neutrophils and red cells to yield certain hydroeicosatetraenoic acids of altered biological function. It has been hypothesized that transcellular transfer of AA may amplify or modulate the consequences of platelet activation, possibly extending to other cells in the growing thrombus (Barry et al., 1997).

As thrombus grows there is further release of ADP, serotonin and TXA₂ stimulating coagulation. ADP released from platelet granules recruits additional platelets, causing platelet cohesion and thrombus growth promotion (Veijio et al., 1999, Hoffbrand and Pettit, 1993, Hirsh and Weitz, 1999). Platelet activation and secretion of growth factors stimulate smooth muscle cell proliferation, thereby accelerating atherosclerosis (Schneider et al., 1998). Collagen-induced platelet activation
releases $\text{H}_2\text{O}_2$, which activates tyrosine kinase (TK), consequently resulting in phosphorylation and activation of phospholipase $\text{C}_{\gamma}2$ (PLC$_{\gamma}2$) (Pignatelli et al., 1998). This results in an increase in intracellular $\text{Ca}^{2+}$ levels, accumulation of PtdIns 3,4P2 and irreversible platelet aggregation (Savage et al., 2001, Selheim et al., 2000).

### 1.1.4.5 Aggregation

Collagen and thrombin stimulation of platelet prostaglandin (PG) production results in the production of TXA$_2$. TXA$_2$ triggers the release reaction by lowering cyclic adenosine monophosphate levels (cAMP). Additional ADP and TXA$_2$ released stimulate secondary aggregation. Endothelial PGI$_2$ inhibits the release reaction by increasing cAMP concentrations, thereby regulating platelet aggregation (Figure 1.5) (Hoffbrand and Pettit, 1993, Bennett, 2001). ADP, TXA$_2$, thrombin and collagen bind to specific receptors, trigger signalling pathways and act synergistically in activating and modulating GP IIb/IIIa receptors for fibrinogen and vWF (Hirsh and Weitz, 1999, Phillips et al., 2001). Each fibrinogen molecule binds two platelets, creating a bridging network of platelet aggregates. Fibrin incorporation produces a fibrin clot (Hirsh and Weitz, 1999, Veijio et al., 1999, Bennett, 2001).

### 1.1.5 Atherosclerotic Involvement in Arterial Thrombosis

Atherosclerosis causes coronary insufficiency, manifesting clinically as coronary artery disease (CAD). Dietary factors have been implicated as potential risks for atherosclerotic development. As a result, dietary modification has been one of the strategies adopted in secondary prevention and management of this disease (Hoffbrand et al., 1999, Vardaxis, 1994). Oxidative stress induced by free radicals has been associated with atherosclerotic development (Berliner et al., 1995). Atherosclerosis increases with age. However, it frequently escapes detection because of its insidious and asymptomatic behaviour. Of particular concern is the
socioeconomic impact inflicted by the high incidence of mortality and morbidity associated with atherosclerosis and its complications (Ross, 1993).

Atheroma-narrowed arteries are characterised by high levels of shear stress capable of inducing platelet activation and aggregation (Schneider et al., 1998). Plaque content, integrity of the fibrous cap, and haemodynamic stress determine the occurrence of plaque fissure (Vardaxis, 1994, Veijio et al., 1999). Although endothelial cells synthesize anti-thrombotic and anti-haemostatic substances, thrombosis ensues when prothrombotic stimuli (e.g. vWF, collagen and TF) and mitogens are released during plaque ulceration. Interaction of thrombogenic substances with blood elements results almost immediately in thrombin-mediated platelet dependent thrombosis (Gallus, 1986, Hoffbrand and Pettit, 1993, Hoffbrand et al., 1999, Veijio et al., 1999). After reaching a considerable mass and under high shear conditions induced by stenotic lesions, thromboembolism occurs (Hoffbrand et al., 1999).

1.1.5.1 The relationship of CVD to reactive oxygen species

Reactive oxygen species or free radical formation is associated with the normal natural metabolism of aerobic cells. The oxygen consumption inherent in cell growth leads to the generation of a series of oxygen free radicals. The interaction of these species with molecules of a lipid nature produces new radicals, hydroperoxides and different peroxides (Aust and Sringen, 1982). Lipid peroxidation in biological membranes is a highly destructive phenomena produced when reactive oxygen species and free radicals attack the double bonds of polyunsaturated fatty acids (PUFAs) to yield lipid peroxides (Halliwell et al., 1995).
This group of radicals, either superoxide, hydroxyl or lipid peroxides, may interact with biological systems in a prominent cytotoxic manner (Bors et al., 1990b, Bors et al., 1990a). These free radicals have been suggested to lead to oxidation of low density lipoprotein (LDL), which in turn promotes foam cell formation and accelerates the progression of atherosclerosis (Kuhn and Chan, 1997) (Figure 1.7).

![Figure 1.7](image)

**Figure 1.7** Foam cell formation in early atherosclerosis. Reactive oxygen species (ROS) produced by other cells during normal cell metabolism modifies native low density lipoprotein (LDL) into oxidised LDL which then causes monocyte chemotaxis, foam cell formation, endothelial cell damage and dysfunction. The deposition of foam cells brings about the fatty streaks which forms the initial atherosclerotic lesion. ROS, reactive oxygen species; LDL, low-density lipoprotein. Adapted from (Kuhn and Chan, 1997)

The sensitivity of LDL to oxidative stress depends on an appropriate balance between the amount of PUFAs and antioxidant concentrations. The LDL particle itself contains various antioxidants (e.g. tocopherols, β-carotene, ubiquinol 10, criptoxantine) which protect it from non-enzymatic oxidation (Esterbauer et al., 1992). Data from Visioli et al. suggests that massive lipid peroxidation only occurs when the antioxidant pool is depleted from the LDL particle (Visioli and Galli, 1994).

Antioxidants have been proposed to chelate the hydrogen peroxide ($\text{H}_2\text{O}_2$) produced, bringing about a decrease in platelet aggregation (Figure 1.8) (Pignatelli et al., 1998).
Figure 1.8 Cascade of arachidonic acid metabolism. Following stimulation by agonist like collagen, there is burst of the hydrogen peroxide ($H_2O_2$) (Pignatelli et al., 1998). $H_2O_2$ activates the enzyme phospholipase C that brings about arachidonic acid metabolism and platelet aggregation. Antioxidants target the $H_2O_2$ produced, reducing it, thus preventing phospholipase C activation and a decrease in platelet aggregation. Adapted from (Scalbert and Williamson, 2000).

1.2 Oxidative stress

Oxidative stress is defined in general as excess formation and/or insufficient removal of highly reactive molecules such as ROS and reactive nitrogen species (RNS) (Turko et al., 2001, Maritim et al., 2003). Oxidation reactions are an essential part of normal metabolism as oxygen is the ultimate electron acceptor in the electron flow system that produces ATP (Davies, 1993). Problems may arise when electron flow and energy production become uncoupled so that oxygen free radicals, that is, ROS, are produced (Nohl et al., 2005).

ROS are continuously produced within the cell as a result of mitochondrial electron transfer processes or as bioproducts of the enzymes xantine oxidase, lipoxygenases...
and cyclooxygenases (Szocs, 2004). They can also be generated as a consequence of the intracellular metabolism of foreign compounds, toxins or drugs by cytochrome P450, monoxygenases, or because of exposure to environmental factors such as excessive iron salts or UV irradiation (Ichihashi et al., 2003). External sources of oxidative stress include ageing, smoking, alcohol, environmental pollution, all of which increase production of free radicals more than required for normal body metabolism and endogenous antioxidants are not enough to neutralise them.

Other sources of ROS are macrophages and neutrophils that contain enzymes, such as NADPH oxidase complex, which are able to generate superoxide radicals and hydrogen peroxide (Rosen et al., 1995). ROS, thus play different positive roles \textit{in vivo}, being involved in energy production, phagocytosis, cell growth and intercellular signalling regulation. However, they may also be highly damaging, as they can attack biological macromolecules, namely, lipids, proteins and DNA, induce oxidation and cause membrane damage, enzyme inactivation and DNA damage (Halliwell and Gutteridge, 1999, Valko et al., 2004). When the level of ROS exceeds the antioxidant capacity of the cell, the intracellular redox homeostasis is altered and oxidative stress ensues (Halliwell, 1999). Under these conditions external source of antioxidants may be required to combat oxidative stress related damages.

ROS include free radicals such as superoxide (‘\(O_2^−\)’), hydroxyl (‘\(\text{OH}\)’), peroxyl (‘\(\text{RO}_2^−\)’), hydroperoxyl (‘\(\text{HRO}_2^−\)’), as well as nonradical species such as hydrogen peroxide (\(\text{H}_2\text{O}_2\)) and hydrochlorous acid (HOCI) (Turko et al., 2001, Evans et al., 2002a). RNS include free radicals like nitric oxide (‘\(\text{NO}\)’), nitrous oxide (‘\(\text{NO}_2^−\)’), as well as nonradicals such as peroxynitrite (ONOO\(^−\)), nitrous oxide (\(\text{HNO}_2\)) and alkyl peroxynitrates (RONOO) (Evans et al., 2002a, Turko et al., 2001). Of these reactive
molecules, $'O_2^-$, $'NO$ and ONOO$^-$ are the most widely studied species and play important roles in the diabetic cardiovascular complications.

1.2.1 What causes oxidative stress?
Oxidative stress is the damage to cells caused by oxidation. All forms of life maintain a reducing environment within their cells. The cellular redox environment is preserved by enzymes that maintain the reduced state through a constant input of metabolic energy. Disturbances in this normal redox state can cause toxic effects through the production of peroxides and free radicals that damage components of the cell such as lipids and DNA. Other conditions, such as toxic substance exposure, smoking and asphyxia, obesity, diabetes and excessive sudden exercise, likewise induce oxidative stress. The oxidized lipid products generated as a consequence of these conditions are highly reactive and cause damage to cells and cell membranes. Thus, increased oxidative stress accompanied by reduced endogenous defences may play a role in the pathogenesis of a number of diseases.

Production of one ROS or RNS may lead to the production of others through radical chain reactions. As summarised in Figure 1.9, $'O_2^-$ is produced by one electron reduction of oxygen by several different oxidases including NAD(P)H oxidase, xanthine oxidase, cyclooxygenase and even eNOS under certain conditions as well as by the mitochondrial electron transport chain during the course of normal oxidative phosphorylation, which is essential for generating ATP (Evans et al., 2003, Griendling and FitzGerald, 2003a, Griendling and FitzGerald, 2003b, Taniyama and Griendling, 2003). Under normal conditions, $'O_2^-$ is quickly eliminated by antioxidant defence mechanisms. $'O_2^-$ is dismutated to H$_2$O$_2$ by manganese superoxide dismutase (Mn-SOD) in the mitochondria and by copper (Cu)-SOD in the cytosol (Evans et al., 2003).
Figure 1.9 Generation of reactive species in diabetes. Highlighted are some of the most important ROS and RNS in vascular cells. Oxygen is converted to $\cdot$O$_2$ via the activation of enzymatic and nonenzymatic pathways, which is then dismutated to H$_2$O$_2$ by SOD. H$_2$O$_2$ can be converted to H$_2$O by catalase or glutathione peroxidase (GSH-Px) or to $\cdot$OH after reaction with metals like iron (Fe). Glutathione reductase regenerates glutathione (GSH). In addition, $\cdot$O$_2$ reacts rapidly with $\cdot$NO to form ONOO$^-$. Adapted from (Johansen et al., 2005).

H$_2$O$_2$ is converted to H$_2$O and O$_2$ by glutathione peroxidase (GSH-Px) or catalase in the mitochondria and lysosomes, respectively. H$_2$O$_2$ can also be converted to the highly reactive $\cdot$OH radical in the presence of transition elements such as iron (Figure 1.9).

In vasculature, NO is normally produced from L-arginine by eNOS (Turko et al., 2001, Evans et al., 2002). NO mediates endothelium-dependent vasorelaxation through its action on guanylate cyclase in vascular smooth muscle cells. $\cdot$NO also displays antiproliferative properties and inhibits platelet and leukocyte adhesion to vascular endothelium (Turko et al., 2001, Evans et al., 2002). $\cdot$NO is therefore considered a
vasculoprotective molecule. However, \(^\cdot\)NO easily reacts with superoxide, generating the highly reactive molecule ONOO\(^-\), and triggering a cascade of harmful events (Turko et al., 2001, Evans et al., 2002, Vega-Lopez et al., 2004). Its chemical environment, i.e. presence of \(^\cdot\)O\(_2\)^-, determines whether \(^\cdot\)NO exerts protective or harmful effects.

### 1.2.2 Why are reactive species bad?

While ROS are generated under physiological conditions and are involved to some extent as signalling molecules and defence mechanisms, in phagocytosis, neutrophil function, and shear-stress induced vasorelaxation, excess generation in oxidative stress has pathological consequences including damage to proteins, lipids and DNA. ROS can stimulate oxidation of LDL, and ox-LDL, which is not recognised by the LDL receptor, can be taken up by scavenger receptors in macrophages leading to foam cell formation and atherosclerotic plaques (Boullier et al., 2001). Several damaging pathways in diabetes including accelerated formation of advanced glycation end products (AGE), polyol pathway, hexosamine pathway and PKC, all of which have been proven to be involved in micro- and macrovascular complications, can be activated by O\(_2\)^-. In endothelial cells, H\(_2\)O\(_2\) mediates apoptosis and pathological angiogenesis (Taniyama and Griendling, 2003). Furthermore, \(^\cdot\)O\(_2\)^- immediately reacts with \(^\cdot\)NO generating cytotoxic ONOO\(^-\) and this reaction itself has several consequences. First, ONOO\(^-\) alters function of biomolecules by protein nitration as well as causing lipid peroxidation (Turko et al., 2001). For example, potassium channels, which regulate the vasorelaxation response, are inhibited by nitration (Liu and Gutterman, 2002, Liu et al., 2002b). Increased levels of nitrotyrosine are associated with apoptosis of myocytes, endothelial cells and fibroblasts in diabetes (Turko et al., 2001). Second, ONOO\(^-\) causes single-strand DNA breakage, which in turn activates nuclear enzyme poly (ADP-ribose) polymerase (PARP) (Soriano et al.,
Third, it decreases \(^{\cdot}\)NO bioavailability causing impaired relaxation and inhibition of the antiproliferative effects of \(^{\cdot}\)NO (Maritim et al., 2003). Furthermore, ONOO\(^{-}\) oxidizes tetrahydrobiopterin (BH\(_4\)), an important cofactor for NOS, and causes uncoupling of NOS, which produces \(^{\cdot}\)O\(_2\) instead of \(^{\cdot}\)NO (Maritim et al., 2003). ROS-induced peroxidation of membrane lipids alters the structure and the fluidity of biological membranes, which ultimately affects function (Maritim et al., 2003, Griendling and FitzGerald, 2003a, Griendling and FitzGerald, 2003b, Taniyama and Griendling, 2003). All these pathological modifications contribute to the pathogenesis of vascular dysfunction.

1.2.3 Oxidative stress and Coronary Heart Disease

Considerable research interest has developed in determining the role of free radical–mediated damage seen in many major medical disorders, in particular CHD and diabetes (Baynes, 1991). Oxidative stress results from an imbalance between oxidant production (or the formation of reactive oxygen species) and antioxidant defences (Maritim et al., 2003). Plasma markers of oxidative stress are increased in CHD, diabetes or in the presence of its classic risk factors (Cai and Harrison, 2000, Chisolm and Steinberg, 2000, Harrison et al., 2003, Maritim et al., 2003) and as a result of sudden exercise by sedentary population (Belviranli and Gokbel, 2006).

1.2.3.1 Oxidative stress due to Diabetes lead to Coronary Heart Disease

Type 2 diabetes mellitus (T2DM) induces a number of cardiovascular disorders, including platelet hyperactivity and hyperaggregability (figure 1.10), that are associated to an increased oxidant production (Sobol and Watala, 2000). Oxidative stress in T2DM was thought to be a result of free radicals generated during autoxidation of glucose (Miyata et al., 1999). Increased levels of ROS in T2DM was implicated to contribute to a hypercoagulable state (Collier et al., 1992), and there is
evidence for the accumulation of oxidation products prior to the development of diabetes (Matteucci and Giampietro, 2000).

Metabolic dysfunction occurring in diabetes mellitus is capable, through increased oxidative stress, protein kinase C (PKC) and receptor for advanced glycation end products (RAGE) activation, of inducing activation of endothelial cells (EC) and platelets, which causes a switch toward a pro-thrombotic, pro-inflammatory condition and contributes to the pathogenesis of atherosclerosis (Figure 1.10).

Numerous adverse effects on the vascular system are associated with increased oxidative stress. The oxidation of vulnerable cell membrane unsaturated lipids (Evans et al., 2002) may modulate diverse signal transduction pathways (Harrison et al., 2003, Suzuki et al., 1997), leading to numerous adverse effects implicated in the pathogenesis of atherosclerosis. These include increased expression of cell adhesion molecules, induction of proinflammatory pathways, activation of matrix metalloproteinase, vascular smooth muscle cell proliferation and death, endothelial dysfunction, and lipid peroxidation (LDL oxidation). Apart from the global effects associated with increased oxidative stress described above, more specific effects also occur. LDL is an important target of oxidation, and oxidative modification of LDL is a key step in the pathogenesis of atherosclerosis (Witztum and Steinberg, 2001).
Therefore, as well as the general harmful effects on the vasculature, more specific effects may be seen at the lipoprotein level, which are dependent on the properties of the lipoprotein molecules. Previous observations suggest that HDL exhibits antioxidant properties (Durrington et al., 2001), and increasing plasma glucose is associated with increased oxidative stress (Davi et al., 1999). Interestingly, increased triglyceride concentrations are also associated with the increased preponderance of small, dense LDL, which is more labile to oxidation (Weinbrenner et al., 2003).

1.2.3.2 Oxidative stress due to sudden exercise lead to Coronary Heart Disease

Oxidative stress induced by acute strenuous exertion by normally sedentary population may interfere with platelet responsiveness by promoting ox-LDL-mediated platelet activation and by decreasing platelet-derived nitric oxide bioactivity (Di
Massimo et al., 2004). The exact mechanisms and the regulatory pathways involved in the effects of acute exercise on platelet function are not completely understood. Increases in plasma levels of catecholamines (Ohri et al., 1983, Ikarugi et al., 1997), changes in the performance of α-adrenergic receptors, increases in platelet count (Ohri et al., 1983, Ricci et al., 1991), prostacyclin/thromboxane A₂ (PGI₂/TxA₂) imbalance (Drygas, 1988), impaired sensitivity of platelets to PGI₂ and nitric oxide (Sakita et al., 1997, Drygas, 1988, Kishi et al., 1992, Sinzinger and Fitscha, 1986), changes in plasma lipoprotein profile (Agren et al., 1991, Baumstark et al., 1993), oxidative stress and changes in antioxidant status are mechanisms potentially involved in exercise-induced platelet responses (Salvemini and Botting, 1993, Wang et al., 2000a). Hemodynamic disturbances and increase in shear stress, as a result of physical exercise, (i.e. blood flow) may also play a role in platelet response (Ruggeri, 1993, Kroll et al., 1996).

The stimulation of cells, including endothelial cells, by shear stress of exercise can also lead to the phosphorylation and activation of Akt (Dimmeler et al., 1999). Insulin signalling in skeletal muscle is associated with signal transduction at the level of insulin receptor substrate 1 (IRS-1) and phosphatidylinositol (PI) 3-kinase (Bjornholm et al., 1997, Cusi et al., 2000, Kim et al., 1999, Krook et al., 2000) and glucose transport (Krook et al., 2000, Bjornholm et al., 1997, Andréasson et al., 1991). Akt is a downstream effector of PI 3-kinase that is directly linked to the regulation of glucose transport (Jiang et al., 2003).

The signalling pathways that mediate insulin’s many actions remain incompletely understood, but the following sequence has been well characterized: insulin binds to its receptor, leading to receptor autophosphorylation and activation of receptor tyrosine kinase, which in turn results in tyrosine phosphorylation of endogenous
substrates including insulin receptor substrate proteins. These docking proteins engage downstream signalling molecules such as phosphatidylinositol (PI) 3-kinase (Bergstrom and Hultman, 1966, Taniguchi et al., 2006, Zierath et al., 2000). PI 3-kinase catalyses the phosphorylation of phosphatidylinositol 4,5-bisphosphate on the D3 position of inositol, and the resultant PI 3,4,5-trisphosphate binds and activates more distal signalling proteins, including phosphoinositide-dependent kinase-1 and Akt, a serine/threonine kinase (Figure 1.11). Akt has been implicated as a key signalling protein for several of insulin’s actions, including activation of glycogen synthesis, protein synthesis, and GLUT4 translocation to the cell surface, thereby increasing glucose transport.

Recently an Akt substrate with molecular weight of 160 kDa (AS160) and a molecular signature of a Rab-GAP has been identified as an important regulator of GLUT4 traffic (Figure 1.11). Insulin stimulation of skeletal muscle leads to phosphorylation of AS160, a process dependent on Akt (Bruss et al., 2005, Bouzakri et al., 2006). AS160 is also phosphorylated in response to exercise in human skeletal muscle (Deshmukh et al., 2006) and after in vitro contraction in rodent skeletal muscle (Bruss et al., 2005, Kramer et al., 2006). Thus, AS160 may be critical point of convergence for insulin and exercise mediated glucose uptake in skeletal muscle.

Recently, a novel 160-kDa substrate of Akt has been identified in 3T3L1 adipocytes as AS160, a protein containing a Rab GTPase-activating protein (GAP) domain (Kane et al., 2002) (Figure 1.11). Phosphorylation of AS160 is required for the insulin-induced translocation of GLUT4 to the plasma membrane in 3T3L1 adipocytes (Sano et al., 2003).
Figure 1.11 Insulin signalling that leads to GLUT 4 translocation and glucose uptake. IRS, IR substrate; PI3K, phosphatidylinositol-3-kinase; PDK, phosphatidylinositol-dependent protein kinase; Akt, protein kinase B; AS160, Akt substrate of 160 kDa; GLUT, glucose transporter. Adapted from (Hawley and Lessard, 2008).

Shear stress stimulates the phosphorylation of eNOS on its Akt-related phosphorylation site Ser\textsuperscript{1177} in humans and Ser\textsuperscript{1179} in bovines independently of an increase in intracellular calcium (Dimmeler et al., 1999). The phosphatidyl-inositol-3-kinase (PI3K) and Akt pathway, which causes intracellular calcium-independent eNOS phosphorylation and activation, is involved in a shear stress-activated signal transduction cascade.

Increased consumption of oxygen during exercise raises free radical production. Endogenous antioxidants respond to this state. However, imbalance in limited antioxidants and raised oxidative stress leads to endothelial dysfunction and other tissue damage (Figure 1.12).
It is thought that regular aerobic exercise increases NO production with up-regulation of eNOS gene expression and vascular endothelial growth factor (VEGF)-induced angiogenesis and decreases NO inactivation with augmented antioxidant system, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), and attenuation of nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate (NADH/NADPH) oxidase activity, leading to an increase in NO bioavailability (Higashi and Yoshizumi, 2004).

1.2.4 Human natural defences against deleterious effects of oxidative stress
To minimize free radical damage, there is a complex endogenous antioxidant defence system, including antioxidants within cells, cell membranes and extracellular fluids (Jenkins, 1988, Powers and Lennon, 1999, Halliwell and Gutteridge, 1990, Sen, 1995). To maintain redox balance against oxidant conditions, blood has a central role as it transports and redistributes antioxidants to every part of the body. Deleterious effects of physiologically produced free radicals are neutralised by antioxidants in the body (Figure 1.12). To cope with an excess of free radicals...
produced, humans have developed sophisticated mechanisms to maintain redox homeostasis.

These protective mechanisms, scavenge or detoxify ROS, block their production, or sequester transition metals that are the source of free radicals. They include enzymatic and nonenzymatic antioxidant defences produced in the body, namely, endogenous defences (Hayes and McLellan, 1999, Sies, 1999), and others supplied with the diet, namely, exogenous defences (Benzie, 1999, Yao et al., 2004, Porrini et al., 2005).

Several antioxidant enzymes exist that convert ROS into less noxious compounds, for example, SOD, catalase, thioredoxin reductase, peroxiredoxin and GPx (Hayes and McLellan, 1999, Talalay, 2000, Arnér and Holmgren, 2000) (Matés et al., 1999, Chaudière and Ferrari-Iliou, 1999). Collectively, these enzymes provide a first line of defence against superoxide and hydrogen peroxides. They are of enormous importance in limiting ROS-mediated damage to biological macromolecules, but they are not able to be 100% effective, because certain compounds generated by the interaction of ROS with macromolecules, are highly reactive. It is then mandatory to detoxify these secondary products in order to prevent further intracellular damage, degradation of cell components and eventual cell death.

This second line of defence against ROS is provided by enzymes such as GPx, glutathione S-transferase (GST), aldo-keto reductase and aldehyde dehydrogenase (Armstrong, 1997, Brigelius-Flohé, 1999, Kuhn and Borchert, 2002). The detoxified metabolites produced by these enzymes are eliminated from the cell, by efflux pumps such as the glutathione S-conjugate transporter (Akerboom and Sies, 1989). Thus,
the central role of reduced GSH appears clear in intracellular endogenous antioxidant defences, as it is involved in all the lines of protection against ROS (Selheim et al., 2000).

1.3 Free radicals and antioxidants

Free radicals are electrically charged molecules, which seek out and capture electrons from other substances, to finally neutralise themselves. Although the initial attack causes the free radical to become neutralised, another free radical is formed in the process, resulting in a chain reaction. Until subsequent free radicals are deactivated, thousands of free radical reactions may occur within only a few seconds. The term “antioxidant” refers to any molecule capable of stabilizing or deactivating free radicals before they attack cells. These are the primary antioxidants. There are also molecules deserving the “antioxidant” term, because they act as chelating agents binding metal ions (redox activity).

Free radical formation is associated with the normal natural metabolism of aerobic cells. The oxygen consumption inherent in cell growth leads to the generation of a series of oxygen free radicals. The interaction of these species with molecules of a lipidic nature, produces new radicals: hydroperoxides and different peroxides (Aust and Sringen, 1982, Pryor et al., 1982). This group of radicals (superoxide, hydroxyl and lipid peroxides) may interact with biological systems in a clearly cytotoxic manner. In this respect, flavonoids, phenols and oleuropeosides have been shown to posses an important antioxidant activity towards these radicals, which is principally based on the redox properties of their phenolic hydroxyl groups and the structural relationships between different parts of their chemical structure (Bors et al., 1990b, Visioli et al., 1998a, Bors et al., 1990a, Visioli et al., 1998c) flavonoids, three structural groups are important to determine their radical scavenging and/or
antioxidative capacity: the o-dihydroxy (catechol) structure in the B-ring, which confers greater stability to aroxyl radicals; the 2,3-double bond conjugated with a 4-oxo function, responsible for electron delocation from the B-ring and the presence of both 3- and 5-hydroxyl groups for maximal radical-scavenging capacity and strongest radical absorption (Bors et al., 1990b, Bors et al., 1990a). For oleuropeosides and the other phenols present in olive leaves, it is mainly the o-dihydroxy (catechol) structure present in their moieties, which confers antioxidant properties to these compounds.

Obviously, the antioxidant capacity of any phenolic compound will be determined by a combination of these structural elements. However, this capacity will not be similar or show the same degree of effectiveness towards each of the above mentioned radicals, but will depend on the different action of mechanisms which take place in each particular case. These mechanisms are influenced by structural factors other than those described, such as the presence or absence of glycosidic moieties in the polyphenol, the glycosylation site, number and position of the free and sterified hydroxyls.

1.3.1 Polyphenols

Polyphenols are wide variety of compounds that occur in fruits and vegetables, wine, tea, extra virgin olive oil, chocolate and other cocoa products. Several polyphenols have been demonstrated to have clear antioxidant properties in vitro, and many of their biological actions have been attributed to their intrinsic reducing capabilities. Polyphenols are commonly found in plants and are comprised of numerous subclasses (Kaur and Kapoor, 2001). Important dietary flavonoid subclasses include the flavones, flavanones, flavonols, flavanols and anthocyanins, with flavanols being
the second most abundant (Hertog et al., 1993, Prior and Cao, 2000, Scalbert and Williamson, 2000, Nijveldt et al., 2001). The results of several epidemiological studies support an inverse relation between fruit and vegetable intake, as well as the consumption of flavonoid-rich beverages and a reduction in heart disease mortality, cerebrovascular diseases, common cancers, degenerative diseases and ageing (Pignatelli et al., 2000, Prior and Cao, 2000, Rein et al., 2000b, Schramm et al., 2001).

Flavanols exert their actions via the following mechanisms: 1) Free radical scavenging and chelation of metal ions by virtue of AOX activity, 2) Reduction in platelet activation and aggregation, 3) Inhibition of lipoxygenase (LPO), cyclooxygenase (Casey et al., 2004), phospholipase A₂ (PLA₂) or stimulation of nitric oxide synthase (NOS), 4) Enhancement of prostacyclin (PGI₂) release, 5) Anti-inflammatory action and interaction with biomembranes (de Whalley et al., 1990, Terao et al., 1994, Saija et al., 1995, Rice-Evans et al., 1996, Lotito and Fraga, 2000, Schewe et al., 2002, Schramm et al., 2001).

Flavanols vary in the number and arrangement of hydroxyl group, degree of acylation or glycosylation (Rice-Evans et al., 1996, Bravo, 1998, Hammerstone et al., 2000). The chemical structure of polyphenols governs their absorption, metabolism, bioavailability, AOX activity and specific interactions with cellular receptors and enzymes (Scalbert and Williamson, 2000). Correlation between structure and activity is far from clear, because data on their absorption, metabolism and bioavailability are scarce and fragmentary (Terao et al., 1994, Saija et al., 1995, Nijveldt et al., 2001). Before flavanols can excise their bioactivity, they must first be absorbed, metabolised and made bioavailable in vivo. Absorption and metabolism of flavanols largely
depends on their structural characteristics, molecular size, solubility and the dosage administered. Data available on absorption concentration and metabolism of individual polyphenols studied as part of this thesis have been discussed later. Many epidemiological studies assessing flavonoid activity are carried out in vitro. This cannot be fully extrapolated to the in vivo status of the parent molecule as animal models demonstrate the presence of conjugated metabolites in the plasma, raising the possibility that some bioactivity may be attributable to their metabolites (Piskula and Terao, 1998, Ross and Kasum, 2002). Bioavailability studies investigating tea and cocoa polyphenols reveal that relatively low plasma concentrations are achievable. However, there is evidence to suggest that high flavonoid concentrations may be attained via repeated ingestion (van Het Hof et al., 1999, Scalbert and Williamson, 2000).

1.3.2 Structure-Activity Relationship of Polyphenols

Flavonoids are classified based on the degree of oxidation of the oxygen heterocycle (Scalbert and Williamson, 2000). They also differ in hydroxylation on the A and C rings (C3, C5, C7), and also on the B ring (C3’, C4’, C5’) (Lairon and Amiot, 1999).

Figure 1.13 Basic diphenylpropane (C6-C3-C6) structure of flavanols. Adapted from (Hollenberg et al., 2004).
Flavanols have the diphenylpropane (C6-C3-C6) backbone structure (Figure 1.13) varying in the number and arrangement of hydroxyl groups, degree of acylation or glycosylation (Rice-Evans et al., 1996, Hammerstone et al., 2000). Basic diphenylpropane (C6-C3-C6) "flavan nucleus," is the foundation structure upon which flavonoids are constructed.

1.3.3 Cocoa polyphenols (Catechins, epicatechins and procyanidins)
Whilst most research has concentrated on the polyphenols in tea and red wine, there has been increasing interest in cocoa because it is a polyphenol-rich food, containing flavan-3-ols such as (-)-epicatechin, (+)-catechin, and their oligomeric counterparts, the procyanidins and to a lesser extent, anthocyanins as well as quercetin and its glycosides (Fraga et al., 2001, Scalbert and Williamson, 2000, Schewe et al., 2002). The discovery that cocoa contained four times more flavonoids than found in green/black tea, fuelled even more research as this suggested that cocoa may possess greater AOX potential (Arts et al., 1999).

The touted heart-healthy claims of drinking red wine appear to be overstated, as it appears to be a poor source of bioavailable flavonols, and there are dangers associated with alcohol consumption (Prior and Cao, 2000, de Vries et al., 2001).

Cocoa contains monomeric flavanols (epicatechin and catechin) and oligomeric procyanidins (Hammerstone et al., 1999). Procyanidins, the major polyphenol in unfermented cocoa (Figure 1.14), are composed of flavan-3-ol monomers and their respective oligomers (Porter et al., 1991).

The results of animal studies show that orally ingested (-)-epicatechin and (+)-catechin were rapidly absorbed by the intestinal mucosa, conjugated with glucuronic
acid by uridine 5'-diphosphoglucuronosyltransferase (UGT), followed by conjugates entering the liver, via the portal vein, whereby they are sulfated and methylated by phenolsulfotransferase and catechol-O-methyltransferase respectively.

**Figure 1.14** Structure of the flavan-3-ols and their oligomeric counterparts, the procyanidins. Proanthocyanidin is composed of three linked subunits. The shaded area represents one subunit, which is a flavanol known as Catechin. Epicatechin differs from Catechin only in the spatial orientation of its -OH group at position ‘3’ of ‘C’ ring. Adapted from (Hollenberg et al., 2004).

In the kidney, they may also undergo methylation. Finally, these metabolites are either excreted in the bile or urine (Piskula and Terao, 1998, Bravo, 1998, Rein et al., 2000a, Baba et al., 2001, Donovan et al., 2001). Compared with (+)-catechin, (-)-epicatechin appeared to be absorbed more efficiently as there were more urinary metabolites. When put together, they were competitively absorbed (Piskula and Terao, 1998).

### 1.3.4 Olive polyphenols (Oleuropein)

Antioxidant properties of virgin olive oil are not related to their phenolic content but only to the presence of catecholic compounds such as oleuropein and hydroxytyrosol.
Visioli et al., 2002a). Olive leaf extract is known to contain a mixture of polyphenolic compounds, among them oleuropein and hydroxytyrosol, both of which are readily absorbed and bioavailable. The biological activities of olive leaf extract are mainly derived from these compounds (Visioli et al., 2002b, Visioli and Galli, 2002). Oleuropein is the bitter component of olives and is found in olive oil and leaves, mainly as its aglycone form. It is a complex phenol belonging to the secoiridoid class of phenolic compounds and constitutes up to 14% in the unripe olives but during maturation undergoes hydrolysis to yield simpler molecules including hydroxytyrosol (Visioli et al., 1998b).

![Figure 1.15 Structure of oleuropein. Adapted from (Konno et al., 1999).](image)

Oleuropein was discovered in 1908 by Bourquelot and Vintilesco, and its structure (Figure 1.15) was specified as being that of a heterosidic ester of β-glucosylated elenolic acid and 2(3,4,-dihydroxyphenyl) ethanol (Pannizzi et al., 1960).

The antioxidant activity of this compound is attributed to its dihydroxyphenol moiety while its secoiridoid glycoside moiety might aid in its transport across the intestine, via the paracellular route (Edgecombe et al., 2000, Stupans et al., 2002). Upon acid hydrolysis, oleuropein is cleaved at the site indicated to yield hydroxytyrosol (the dihydroxyphenol moiety) (Visioli et al., 1994).
The antioxidant activity of oleuropein is due mainly to the hydroxytyrosol moiety in its structure (Benavente-Garcia et al., 2000). Hydroxytyrosol (Figure 1.16) has been shown to exhibit a greater antioxidant potential than oleuropein (de Whalley et al., 1990, Visioli et al., 1998a, Visioli et al., 1995, Visioli and Galli, 2002, McDonald et al., 2001).

![Figure 1.16](image)

**Figure 1.16** The structures of hydroxytyrosol, tyrosol, and caffeic acid which can be extracted from the polar fraction of virgin olive oil. Adapted from (de la Puerta et al., 1999).

In contrast, investigations by Saija et al. showed that oleuropein was more effective than hydroxytyrosol in protecting against ROS damage in aqueous phase (Saijia et al., 1998). It was hypothesized that hydroxytyrosol was only capable of scavenging peroxyl radicals near the cell membrane surface, whereas oleuropein could also act as a scavenger within membranes.

### 1.3.4.1 Olive leaf extract versus olives or olive oil

Olive leaves have been used as medicine in form of boiled tea leaves for about 5000 years but there is very little scientific evidence relating to the effects of the compounds on platelet function and/or thrombosis. Olive leaves stay green all year and may be gathered at any time. Olive leaf extract is concentrated liquid made by soaking chopped or mashed leaves in a liquid such as alcohol and straining out the solid parts. Olive oil is produced in mesocarp cells of fruit and stored in particular type
of vacuoles called lipovacuole. Olives are ground to paste and allowed to stand for some time for oil drops to agglomerate. Industrial decantation of the paste facilitates the process of extraction. This is very expensive and time consuming process, taking a long time from harvest to extraction of oil with waiting periods which can expose the olive paste to oxygen and light. Oxidation begins immediately upon harvesting and at this time fruit’s enzymes are very active, increasingly degrading the endogenous oil and increasing acidity with bitterness. Oleuropein is abundant in developing fruit but its concentration sharply declines when fruits begin to mature (Malik and Bradford, 2006). Thus olive oil which is pressed from mature fruits contains very small amounts of oleuropein (Bernes-Balbuena et al., 1992, Brenes et al., 2000, Romero et al., 2002, Servili et al., 1999). On the other hand, oleuropein is the most abundant polyphenol in olive leaves (Braun, 2005, Malik and Bradford, 2006, Benavente-Garcia, 2000). Olive leaf extract and not olive oil was used in this study due to cost and time effectiveness. Leaves are inexpensive due to larger quantities available throughout the year.

1.3.5 Gamma tocopherol polyphenols

Vitamin E exists in 8 isoforms as 4 tocopherols and 4 tocotrienols, all of which are potent membrane-soluble antioxidants. It is a lipid-soluble antioxidant, which mainly accumulates in cell membranes, where it efficiently scavenges superoxide radicals and also regenerates other oxidized antioxidants such as dehydroascorbic acid (Wang and Jiao, 2000, Ozawa, 1990). Alapha and gamma tocopherols are the strongest antioxidants. The antioxidant activity of tocopherols is rooted in their ability to donate phenolic hydrogens (electrons) to lipid radicals.
Vitamin E (Tocopherols)

Indispensable member of the body’s antioxidant system.

![Vitamin E Structure](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>R¹</th>
<th>R²</th>
<th>R³</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Tocopherol</td>
<td>CH₃</td>
<td>CH₃</td>
<td>CH₃</td>
</tr>
<tr>
<td>β-Tocopherol</td>
<td>CH₃</td>
<td>H</td>
<td>CH₃</td>
</tr>
<tr>
<td>γ-Tocopherol</td>
<td>H</td>
<td>CH₃</td>
<td>CH₃</td>
</tr>
<tr>
<td>δ-Tocopherol</td>
<td>H</td>
<td>H</td>
<td>CH₃</td>
</tr>
</tbody>
</table>

*Tocotrienols have three double bonds in the phytol side chain*

**Figure 1.17** Vitamin E structure. Adapted from (Jiang et al., 2001).

Tocopherols have a saturated phytol side chain with 3 chiral centers that are in an *R* configuration (designated as R¹, R² & R³ in Figure 1.17) in the naturally occurring forms. Tocopherols differ in the number of methyl groups they have at the 5- and 7-positions of the chromanol ring.

The biological disposition and retention of γ-tocopherol appear to be regulated by a metabolism that is quite different from that of α-tocopherol (Figure 1.18). Chylomicron-associated tissue uptake of vitamin E, which occurs before liver metabolism, is possibly important for the accumulation of γ-tocopherol in skin, adipose, and muscle tissue. This could explain the strong correlation in humans between dietary γ-tocopherol uptake and γ-tocopherol concentrations in these tissues (Burton et al., 1998).
Figure 1.18 Absorption, transport, and metabolism of α-tocopherol (α-T) and γ-tocopherol (γ-T) in peripheral tissues (e.g., muscle and adipose). Both α-T and γ-T are similarly absorbed by the intestine along with dietary fat and are secreted into chylomicron particles. Some of the chylomicron-bound vitamin E is transported to peripheral tissues with the aid of lipoprotein lipase. The resulting chylomicron remnants are subsequently taken up by the liver. In the liver, most of the remaining α-T but only a small fraction of γ-T is reincorporated into nascent VLDLs by α-tocopherol transfer protein (α-TTP). Substantial amounts of γ-T are probably degraded by a cytochrome P450–mediated reaction to 2,7,8-trimethyl-2-(ß-carboxyethyl)-6-hydroxychroman (γ-CEHC). Plasma vitamin E is further delivered to tissues by LDL and HDL. γ-CEHC is excreted into urine. Adapted from (Jiang et al., 2001)

Hepatic catabolism of γ-tocopherol appears however, to be responsible for the relatively low preservation of γ-tocopherol in plasma and tissues, whereas α-TTP-mediated α-tocopherol transfer plays a key role in the preferential enrichment of α-tocopherol in most tissues. It is possible that α-TTP maintains the α-tocopherol concentration not only by facilitating its reincorporation into nascent VLDLs but also by preventing it from being catabolised (Jiang et al., 2001). This is in contrast with γ-tocopherol, which appears to be largely degraded by cytochrome P450 once it enters the liver. Evidence supporting this possibility includes the findings that both α- and γ-tocopherol are similarly degraded by cytochrome P450–mediated catabolism in cultured hepatocytes (Parker et al., 2000) and that patients with an α-TTP defect
have substantially lower plasma concentrations of α-tocopherol than do individuals with no such defect.

1.4 Antioxidants and Cardiovascular Diseases

1.4.1 Dietary antioxidants and vascular endothelial dysfunction
The vascular endothelium plays a key role in the regulation of vascular tone by the release of vasodilator substances, mainly NO and prostacyclin, and vasoconstrictor substances, mainly TxA2, free radicals and endothelin. In particular, in normal endothelium, factors such as NO mediate vasodilation, platelet desegregation and antiproliferation, and inhibit inflammation; lipoprotein lipase induces lipolysis, and tissue plasminogen activator (tPA), von Willebrand factor (vWF), and protein C act as thrombolytic agents. In endothelial dysfunction, vasoconstriction, inflammation and proliferation mediated by several growth factors (i.e. bFGF and PDGF), as well as platelet aggregation mediated by adhesion molecules, such as selectins, and thrombosis, result in detrimental functional consequences and adverse long-term effects, including vascular remodelling (Fenster et al., 2003). In CVD, endothelium dysfunction of the coronary arteries may cause or contribute to myocardial ischemia. Numerous clinical trials have been carried out to evaluate dietary interventions, which improve endothelial dysfunction, amongst which many look at the potential of the antioxidants derived from the diet. The degree of the dysfunction correlates with elevated LDL oxidation, dyslipidemias, mainly elevated LDL and decreased high-density lipoprotein levels, hypertension and diabetes.

1.4.2 Dietary antioxidants and Thrombosis
Removal of excess ROS or suppression of oxygen free radical generation by antioxidants may be effective in preventing oxidative cell damage (Beckman and Ames, 1997, Henle and Linn, 1997). A prominent feature of both abnormal platelet
function and dysfunctional endothelium-dependent vasodilation in the setting of cardiovascular and thrombotic disease is oxidative stress. Evidence also suggests that oxidative stress normally accompanies platelet activation. Platelet aggregation is associated with a burst of oxygen consumption (Bressler et al., 1979) and a marked rise in glutathione disulfide (Burch and Burch, 1990). While dramatic changes in platelet redox status occur during normal aggregation, conditions that provoke oxidative stress without inducing a florid aggregation response, have also been shown to be prothrombotic. Reactive oxygen species contribute causally to many pathophysiologic conditions, and superoxide, in particular, is known to augment platelet aggregation responses (Handin et al., 1987). Superoxide and NO readily combine to form OONO−, thereby inactivating NO.

Antioxidants may indirectly inhibit platelets through their interference in the metabolism of reactive oxygen species, many of which alter platelet function. Hydroperoxides produced by activated platelets (prostaglandin G2, 12-hydroperoxy-eicosatetraenic acid, and phospholipid hydroperoxides) are metabolized by the cytosolic selenium-dependent enzyme cellular glutathione peroxidase. Cellular glutathione peroxidase (cGPx) is tightly coupled to the hexose monophosphate shunt through reduced nicotinamide adenine dinucleotide phosphate (NADPH), which maintains the obligate cosubstrate of cGPx reduced glutathione (GSH) and re-establishes the platelet thiol redox state via glutathione reductase. Glutathione depletion in platelets leads to attenuated cGPx activity and increased lipid peroxidation (Calzada et al., 1991). Increased lipid peroxides, in turn, lead to an increased likelihood of lipid peroxyl radical formation (LOO), which can react with and inactivate NO by forming lipid peroxynitrites (LOONO). Cellular GPx potentiates the inhibition of platelet function by NO by reducing both LOOH and derivative LOONOs.
1.4.3 Why study Antioxidants?
In summary, previous studies have clearly shown a role for ROS in the progression of vascular disease. Some human antioxidant trials have been promising. However, overwhelmingly, clinical trials have failed to show a protective effect of antioxidants in humans. A meta-analysis of seven randomized vitamin E trials does not support a protective effect of vitamin E supplementation on the progression of cardiovascular disease or on clinical events in patients at high risk or with established disease (Vivekananthan et al., 2003). The reasons for the failure of these antioxidant trials is likely multifactorial. Firstly all of these studies used alpha tocopherol form of vitamin E. It is still very difficult to identify the subjects who have an imbalance between ROS production and antioxidant defenses and might benefit from antioxidant therapy. Furthermore, most patients enrolled in clinical trials have established cardiovascular disease, and antioxidant intervention in these cases may be too late to be effective. This suggests that antioxidant therapy may be more beneficial as a preventive measure rather than a therapeutic tool. The lack of knowledge on the optimal dosage for the various antioxidants is also a handicap. More recently, it has become apparent that the bioavailability and distribution of administered antioxidants may limit their usefulness.

Based on this literature review, there is a need to evaluate the efficacy of several classes of antioxidants. Before a potential role for antioxidants in the prevention of CVD is elucidated, more carefully designed studies with classic as well as new antioxidants in well-defined patient populations, are warranted to provide a definitive answer.
Studies conducted for this thesis will examine associations between the intake of various antioxidants, their effect on prevention of thrombotic risk factors and cardiovascular disease.

1.4.4 Aim of the studies undertaken for this thesis

Atherosclerosis, one of the major causative factors of CVD, is thought to be initiated by oxidative stress. Much work has been undertaken to determine the atherogenic effects of oxidative damage on platelet activity, lipid profile and glucose uptake by skeletal muscle myotubes. Current research shows that dietary antioxidant supplementation protects against oxidative stress, and therefore may offer preventative measures and treatments for patients with diseases influenced by oxidative stress. Cocoa, olive and Gamma tocopherol (vitamin E) are reported to have potent antioxidant properties and have been shown to inhibit LDL oxidation in vitro. Although there is strong evidence that supports the correlation between antioxidant consumption, cholesterol reduction and cardiovascular protection, it remains to be elucidated whether these agents confer benefits through mechanisms involving platelet function and glucose uptake. Increased platelet activity and impaired lipid profile have been identified to increase the risk factors for cardiovascular disease. Therefore, the primary aims of the in vitro and intervention studies undertaken for this thesis were to examine if different hydrophilic and lipophilic antioxidants could attenuate platelet activity and glucose uptake by skeletal muscle myotubes in response to induced oxidative stress.

The aim of the first in vitro study described in Chapter Two was to determine the dose-response characteristics of olive leaf extract on platelet activity in healthy human subjects. The phenolic compounds of olives leaves used for centuries in the Mediterranean diet have been associated with a reduced incidence of heart disease.
The active phenolic compounds in olive leaf are known for their capacity to scavenge \( \text{H}_2\text{O}_2 \).

Dietary antioxidant supplements are marketed and used by athletes as a means to counteract the oxidative stress of exercise. Whether strenuous exercise does, in fact, increase the need for additional antioxidants in the diet is not clear. Therefore, the aim of the second study described in Chapter Three was to evaluate effect of exercise, training status and cocoa antioxidant on platelet function. The study compared pre and post exercise and cocoa supplementation, platelet activity in trained and sedentary population.

Results of epidemiological studies demonstrated an inverse relationship between vitamin E intake and coronary disease. The activity of vitamin E is derived primarily from alpha-tocopherol (\( \alpha\)-T) and gamma-tocopherol (\( \gamma\)-T). However, the results of clinical trials using \( \alpha\)-T are equivocal.

Accordingly, the study described in Chapter Five was undertaken to determine the effect of oxidative stress and gamma tocopherol treatment on cultured rat L6 muscle.

In summary, the aim of studies undertaken for this thesis is to provide evidence that antioxidant supplementation may be able to improve cardiac health by maintaining normal platelet function, controlling lipid levels and improving glucose uptake in normal healthy asymptomatic population as well as under induced oxidative stress conditions. Results of these studies may be able to demonstrate that various dietary sources of antioxidants including foods rich in cocoa, olive and gamma tocopherol could be instrumental in combating oxidative stress induced cardiovascular diseases.
Chapter Two: The Effects of Polyphenols in Olive Leaves on Platelet Function

Adapted from *Nutrition, Metabolism & Cardiovascular Diseases* 2008; 18: 127-132.
2.1 Introduction

Olive leaves and olive oil in the Mediterranean diet have been the focus of many epidemiological studies and have been shown to reduce the incidence of heart disease (Keys, 1995, Keys, 1970, Cook and Samman, 1996). Adherence to the traditional Mediterranean diet is associated with a significant reduction in total mortality (Trichopoulou et al., 2003). For example, Estruch et al found that compared with a low-fat diet, Mediterranean diet supplemented with olive oil or nuts have beneficial effects on cardiovascular risk factors (Estruch et al., 2006). The Lyon Diet Heart Study tested a Mediterranean-type diet over long term and suggested that a comprehensive strategy to decrease cardiovascular morbidity and mortality should include primarily a cardio protective diet (de Lorgeril et al., 1999).

The flavonoid polyphenols in olive are natural antioxidants, which have a host of health beneficial effects (Visioli and Galli, 1998). Hydroxytyrosol and tyrosol are some of the many phenol compounds in olives that contribute to bitter taste, astringency, and resistance to oxidation (Visioli et al., 1998b, Visioli and Galli, 2002, McDonald et al., 2001). The active phenolic compounds in the olive leaf extract are part of the secoiridoid family, known for their capacity to scavenge H$_2$O$_2$ (Visioli et al., 1994). Pignatelli et al demonstrated that following stimulation by collagen, there is a burst of H$_2$O$_2$ in the process of platelet activation (Pignatelli et al., 1998). H$_2$O$_2$ activates the enzyme phospholipase C, which brings about arachidonic acid metabolism and platelet aggregation.

There is a growing interest in the use of natural antioxidants as bioactive components in food, and such foods have been termed “functional foods” (Hertog et al., 1993). Due to their ability to scavenge ROS, antioxidants are capable of inhibiting the
process of LDL cholesterol oxidation subsequently decreasing the risk of cardiovascular diseases (Diaz et al., 1997). Although oxidation of LDL can be prevented by the addition of synthetic antioxidants, greater attention is now focused on natural antioxidants because of their better safety compared to that of synthetic compounds (Amro et al., 2002). The protective effect of these diets, which are rich in fruits and vegetables, against coronary heart disease and certain cancers have been attributed partly to the antioxidants found in them, particularly to polyphenols (Hertog et al., 1993).

In the current study, we used extract of Olea europaea L. leaves to determine the effect of olive leaf extract on platelet function in healthy human subjects.

2.2 Methods and Materials

2.2.1 Subjects
Following approval by the RMIT University Human Research Ethics Committee, 11 healthy male volunteers between the ages of 18 to 54 years were recruited for this study. All subjects provided written informed consent to undertake the study.

Criteria for subject recruitment for the study included male (since subject numbers were small only males were recruited to rule out effect of any menstrual cycle related hormonal changes on platelet activity in females), non-smoking, and healthy, with no history of cardiovascular disease or diabetes. Subjects were screened via means of a questionnaire, which requested information regarding level of physical activity, medical history, diet and use of aspirin-type products, non-steroidal anti-inflammatory drugs, blood pressure and other drugs. During the testing phase, subjects were screened for aspirin intake via platelet aggregation in response to the agonist,
Arachidonic Acid. Subjects were requested not to vary their habitual diet for the duration of the study. Subjects were required to complete a food diary by food frequency questionnaire over a 7-day period to monitor the intake of all foods for an estimation of the amount of polyphenol rich food such as omega-3 polyunsaturated fatty acid rich foods, alcohol and cocoa products and to confirm study participants were not consuming antioxidant supplements. Subjects on medication, antioxidant supplements, and who had high dietary intake of alcohol, seafood and cocoa products were also excluded from the study. This study did not involve any additional dietary supplement intake.

2.2.2 Study design
A single blinded study involving 11 healthy male volunteers was conducted in the Division of Laboratory Medicine, School of Medical Sciences, RMIT University. Subjects were overnight fasted before blood collection.

Whole blood, collected on two occasions at weekly interval, was first screened for baseline platelet count (PLT) and mean platelet volume (MPV) before second sample was analysed with and without the in vitro addition of commercially available olive leaf extract (Olive Leaf Australia Pty. Ltd., QLD). The extract was diluted to produce various concentrations, and the amount of platelet aggregation induced at each concentration was investigated. The optimal dose was determined from the dose response curves.

2.2.3 Olive leaf extract
The product Olive Leaf Extract was provided by Olive Leaf Australia Pty. Ltd., QLD. Previous composition investigation studies by the company have shown it to be 98% pure leaf extract of Olea europaea L. leaves. The product was stated to contain 5.40
mg/mL of oleuropein as its active ingredient. The actual product used was dark brown fluid, water-extracted olive leaf extract (from fresh Olea europaea leaves) with no solvents. The product was aqueous and could therefore be added directly to the blood.

2.2.4 Blood collection

A total of 12 mL of venous blood per volunteer was collected by a registered nurse at RMIT Health Services (Bundoora campus) using a tourniquet and 21-gauge Vacutettes© (Greiner bio-one GmbH, Kremsmünster, Austria). Blood was collected into 2mL tri-potassium ethylene diamine tetra-acetic acid (EDTA) (1.8 mg/mL), and 4 mL tri-sodium citrate (3.8%) tubes (Greiner bio-one GmbH, Kremsmünster, Austria). The EDTA tube was collected before the tri-sodium citrate tube to avoid the risk of collecting platelets activated by venipuncture. Care was taken to ensure minimal specimen handling and agitation.

2.2.5 Full blood examination

EDTA anti-coagulated whole blood was analysed for PLT and MPV parameters using the Beckman Coulter A®.T™ 5diff analyser (Coulter Corporation, Miami, FL, USA) within 15-20 minutes of venipuncture. Prior to full blood examination (FBE) each day, the performance of the analyser was validated using Coulter A®.T™ 5Diff Control Plus, low, normal and high controls (Beckman Coulter™, Miami, FL, USA). The analyser was also pre-calibrated with the A®.T™ 5diff Cal Calibrator (Beckman Coulter™, Miami, FL, USA). The PLT and MPV parameters of all subjects were checked to ensure that they fell within a reference range for healthy adults (150-400 x 10^9/L and 6-10 fL respectively).
2.2.6 Sample preparation

Five volume-per-volume (v/v) concentrations of olive leaf extract were added to the blood, i.e. 0.1%, 0.3%, 0.5%, 0.7%, and 1.0% (Table 3.1). The concentrations of oleuropein from the extract were calculated to be 5.4 µg/mL, 16.2 µg/mL, 27.0 µg/mL, 37.8 µg/mL, and 54.0 µg/mL respectively. Whole blood without additives (containing 0% v/v olive extract or 0 µg/mL oleuropein) was used as a control and baseline measurement.

2.2.7 Platelet aggregation

Whole blood platelet aggregation was measured with an impedance aggregometer (Chrono-Log Corp, Philadelphia) equipped with MacLab software (ADInstruments Pty, Ltd, Castle Hill, Australia) for data quantitation and analysis. This method has been described previously (Murphy et al 2003). Calibrations for impedance and ATP release were performed daily before analysing blood samples.

The appropriate volume of olive leaf extract was added to the blood in cuvettes (Chrono-Log Corporation, Philadelphia, PA, USA) and incubated at 37°C ± 1.0 for 30 minutes. Siliconised stir bars (Chrono-Log Corporation, Philadelphia, PA, USA) were then added, and the samples were diluted 1:1 with pre-warmed 0.9% (w/v) saline (NaCl, BDH AnalAr®, MERCK, Pty. Limited, VIC). The samples were then placed inside the heating block testing wells of the aggregometer and the respective electrodes were immersed into samples for 2 minutes at 37°C ± 1.0 to ensure stability before analysis.

After adding 100 ul chronolume reagent, sample was incubated, and mixed with agonists 2 µg collagen/mL [Chrono-Log Corp] [1 mmol arachidonic acid (AA)/L was
used to stimulate platelets for repeating the aggregation as a check for anti-inflammatory intake], and aggregation was recorded for 6 min. ATP release from platelets reacted with luciferin-luciferase in the Chrono-Lume reagent and luminescence was measured, at 650nM by photomultiplier tube (PMT) built in the aggregometer, simultaneously with platelet aggregation. These investigations were carried out within 2 hr post venipuncture.

2.2.8 Statistics
Statistical analyses were performed using Microsoft® Excel 2000 (Microsoft Corporation, Redmond, WA, USA) and SPSS® version 12.0 (SPSS Inc., Chicago, IL, USA); a significance level of P<0.05 was applied. Analysis of variance (ANOVA) adjusted with Sidak simultaneous tests using SPSS® were performed to detect significant differences between the concentrations of olive leaf extract tested. Two-way ANOVA using Microsoft® Excel 2000 was applied to results of ATP release curve.

2.3 Results

2.3.1 Subjects
Baseline FBE results of 11 subjects showed PLT count of 214 ± 34 x 10^9/L and MPV of 8.0 ± 0.8 fl. All parameters fell within normal reference ranges. All subjects had avoided aspirin as normal platelet aggregation curves were obtained using arachidonic acid as an agonist.

2.3.2 Whole blood platelet aggregation
Platelet aggregation, reported as slope (rate of change of impedance in current due to clumping of platelets on the electrode), fell in a dose-dependent fashion with a
steep fall between 0.7% v/v and 1.0% v/v concentrations of olive leaf extract. Sidak 95.0% simultaneous confidence intervals found 1.0% v/v (54.0 µg/mL) olive leaf extract to be significantly different from baseline (Figure 2.1).

![Mean change in slope at increasing concentrations of Olive Leaf Extract](image)

**Figure 2.1** The effect of increasing concentrations of olive leaf extract on platelet aggregation reported as slope-rate of change (Ω/s). Two way ANOVA (Sidak simultaneous tests), significantly inhibited platelet aggregation observed at 1.0% v/v olive leaf extract (adjusted P=0.0001). Results reported as Mean ± SD

### 2.3.3 ATP release

ATP release from platelets was similarly antagonised at the optimal dose of 0.7% v/v and 1.0% v/v concentrations of olive leaf extract, determined from the earlier dose response curves (Figure 2.2).

![Mean of ATP released at each concentration](image)

**Figure 2.2** Mean concentration of ATP released (nM) at each concentration of olive leaf extract (% v/v). Two way ANOVA, significantly inhibited ATP release observed at 1.0% v/v olive leaf extract (P<0.02). Results reported as Mean ± SD.
2.4 Discussion
The findings from this study demonstrate that polyphenols found in olive leaf extract are capable of inhibiting in vitro platelet activation in healthy, non-smoking male individuals. This finding is in agreement with previous work by Karantonis et al reporting that platelet activating factor (PAF) antagonists in olive oil exert significant anti-atherosclerotic activity in rabbits (Karantonis et al., 2006). The study by Karantonis et al used platelet rich plasma, whereas the current investigation used whole blood. Platelet analysis by the whole blood impedance method decreases the processing and handling time of the specimen, thereby preserving the integrity of platelets, and is therefore a better indicator of platelet activity in situ.

Previous studies by (Visioli et al., 1998a) have demonstrated that oleuropein and hydroxytyrosol have capacity to scavenge H$_2$O$_2$ in a dose-dependant fashion. Hence, while the mechanism of action whereby these polyphenols are capable of inhibiting platelet function is not fully understood. It is hypothesized that this is due to their ability to scavenge H$_2$O$_2$ produced during the arachidonic acid metabolism cascade, which leads to platelet aggregation (Pignatelli et al., 1998).

This in vitro study has some limitations. It does not take into account several variables such as absorption and metabolism of the supplement. There is insufficient data in the literature to fully understand the bioavailability of polyphenols such as oleuropein, hydroxytyrosol and tyrosol, which are found in olive leaf extract. It is known that oleuropein is poorly absorbed due to its large size and planar configuration (Edgecombe et al., 2000). It has however been hypothesized that since oleuropein is a glycoside, it could probably access a glucose transporter like a
sodium-dependent glucose transporter (SGLT1) found on the epithelial cells of the small intestine, permitting its entry into the cells. Conversely, Hollman et al. postulated that the absorption of the quercetin glycoside, (a similar polyphenolic), involved active sugar transporters (Hollman et al., 1995).

Although oleuropein is the only active ingredient found in the olive leaf extract used in this study, reports in the literature indicate that oleuropein is not the only phenolic compound found in *Olea europaea* L. leaves. Benavente-García et al. quantified the various polyphenols found in *Olea europaea* L. leaves and reported that oleuropein was found to be the largest fraction. Other polyphenols like hydroxytyrosol, caffeic acid, luteolin and rutin were also isolated from the leaves (Benavente-Garcia et al., 2000). The flava-3-nol catechin was also found in olive leaf extract. Data from our laboratory demonstrated that catechin is capable of inhibiting platelet function because of its antioxidant activity (Murphy et al., 2003). Hydroxytyrosol, caffeic acid, luteolin and rutin are also established to have antioxidant activity and H\textsubscript{2}O\textsubscript{2} scavenging properties (Benavente-Garcia et al., 2002). Polyphenols display a synergic behaviour in mixed form, as occurs in olive leaf extract with a high content of oleuropein and other active polyphenols (Benavente-Garcia et al., 2002, Benavente-Garcia et al., 2000, Benavente-Garcia et al., 1997, Caruso et al., 1999). Therefore, the observed platelet inhibition could be attributed to a synergistic effect of the various polyphenols, as opposed to oleuropein alone. For this reason, oleuropein concentrations were not reported in the dose response curves. The investigations involving this particular olive leaf extract can therefore only be considered a primary but valuable study. Further work is required for a profile on all the polyphenols found in the olive leaf extract. The results of the present study will need to be validated with *in vivo* evaluation of platelet activation such as urinary thromboxane B\textsubscript{2} excretion.
and evaluation of oxidative stress markers such as isoprostanes (derived from the non enzymatic peroxidation of arachidonic acid) to provide insight into the mechanisms responsible for the inhibition of platelet function by polyphenols.

In conclusion, we demonstrated that polyphenols from olive leaf extract significantly inhibited \textit{in vitro} platelet aggregation. This has important benefits for the food industry as the anti-platelet effects in olive leaves may offer a degree of protection from thrombosis and other cardiovascular diseases. Follow up \textit{in vivo} studies will be undertaken to validate the results of current study and to establish the bioavailability of these polyphenols. These polyphenols could also be purified and concentrated for further studies on platelet aggregation to see their pure effect \textit{in vitro} and \textit{in vivo}.

\textbf{Table 2.1} Volumes of olive leaf extract added to whole blood to achieve v/v concentrations of 0.1\%, 0.3\%, 0.5\%, 0.7\% and 1.0\% olive leaf extract concentrations \textit{in vitro}.

<table>
<thead>
<tr>
<th>Volume of Olive Leaf Extract</th>
<th>Volume of whole blood</th>
<th>% concentration of extract (v/v)</th>
<th>Concentration of Oleuropein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µL</td>
<td>1000 µL</td>
<td>0% (Baseline)</td>
<td>0 µg/mL</td>
</tr>
<tr>
<td>1 µL</td>
<td>999 µL</td>
<td>0.1%</td>
<td>5.4 µg/mL</td>
</tr>
<tr>
<td>3 µL</td>
<td>997 µL</td>
<td>0.3%</td>
<td>16.2 µg/mL</td>
</tr>
<tr>
<td>5 µL</td>
<td>995 µL</td>
<td>0.5%</td>
<td>27.0 µg/mL</td>
</tr>
<tr>
<td>7 µL</td>
<td>993 µL</td>
<td>0.7%</td>
<td>37.8 µg/mL</td>
</tr>
<tr>
<td>10 µL</td>
<td>990 µL</td>
<td>1.0%</td>
<td>54.0 µg/mL</td>
</tr>
</tbody>
</table>

Various concentrations of oleuropein prepared by incubation of olive leaf extract with whole blood were used for platelet aggregation.
Chapter Three: The effect of exercise and training status on platelet activation: do cocoa polyphenols play a role?

Adapted from *Platelets* 2006; 17(6): 361–367.
3.1 Introduction

Epidemiological studies have described a complex relation between exercise and coronary ischemic syndromes (Powell et al., 1987). Habitual, sustained exercise has been postulated to reduce the incidence of ischemic heart disease (Morris et al., 1953, Morris et al., 1980, Paffenbarger et al., 1984, Kannel et al., 1986, Paffenbarger et al., 1986, Fletcher et al., 1992). However, acute exertion has also been reported to be a cardiovascular stressor particularly in men who are sedentary (Weaver et al., 1982, Siscovick et al., 1984b). It has been shown that the hemodynamic disturbances and raised shear stress alter the platelet response (Ruggeri, 1994, Kroll et al., 1996). Some studies report that exercise de-sensitises the platelets (Wang et al., 1994), does not affect the platelets (Drygas, 1988, Sakita et al., 1997, El-Sayed et al., 2000), or increases platelet aggregation (Ohri et al., 1983, Ersoz et al., 1997).

We hypothesize that platelets of the subjects who are physically active and those who are sedentary may respond differently to same exercise protocol. Exercise results in an amplified production of free radicals and other reactive oxygen species leading to oxidative stress and cellular damage (Jenkins, 1988). ROS oxidise low-density lipoproteins, promoting foam cell formation thus accelerating the progression of atherosclerosis (Harmut Kuhn, 1997). Excess generation of ROS may overwhelm natural cellular antioxidant defences, more so in sedentary than trained men. Accordingly, antioxidant supplementation may provide a protective mechanism to reduce the harmful effects of ROS (Powers and Hamilton, 1999). We found in our previous study that cocoa supplementation modestly reduced platelet aggregation in normal healthy subjects (Murphy et al., 2003).
The hypothesis of this study was that platelet hyperactivity in response to exercise differs between trained and untrained individuals and short-term cocoa polyphenols may offer protection by reducing the platelet aggregation.

3.2 Methods and Materials
Sixteen healthy, age (23 ± 5 yrs), weight (79 ± 11 Kg) and height (184 ± 6 cm) matched, male volunteers were recruited for this cross over double blinded study, which was approved by the RMIT Human Research Ethics Committee. Twenty volunteers were screened of which eighteen met inclusion criteria (two were taking dietary supplements regularly). Subsequently, two sedentary subjects withdrew because of difficulty performing 1 hour of continuous exercise.

Eight subjects were well trained, as defined by a history of regular (more than 4-5hrs/wk) participation in aerobic exercise (endurance trained cyclists), higher maximum oxygen uptake (VO$_{2\text{max}}$ of 59.53 ± 3.59 mL/kg/min) and mean maximal power of 235 ± 15W. Eight untrained subjects with lower VO$_{2\text{max}}$ of 37.51 ± 3.42 mL/kg/min and mean maximal power of 161 ± 17W, participated in less than 2 hrs/wk of any kind of activity. All subjects were non-smokers with no history of bleeding disorders, diseases of the circulatory system or family history of coronary heart disease, thrombosis or diabetes. Subjects were requested to inform the investigator if they had to take any anti-inflammatory drugs during the study and completed food diary to monitor their intake of polyphenol rich food. During intervention subjects consumed their habitual diet however, subjects were screened for dietary supplements rich in antioxidants such as dietary intake of omega-3 polyunsaturated fatty acid rich foods, alcohol, chocolate and cocoa products. Subjects, who were on
medication, took dietary supplements, and who had high dietary intakes of alcohol, seafood and cocoa and chocolate products were excluded from the study.

3.2.1 Preliminary testing
A blood sample (2 mL) was taken and a full blood examination (FBE) was performed to check basal total platelet count and mean platelet volume (MPV). Subjects with extremely high or low platelet numbers (>400 or <120 x10^9/L) and high MPV (>10fl) were excluded from the study.

All subjects were assessed for VO_{2max} as described by Hawley et al during a progressive incremental exercise test on a cycle ergometer (Groningen, The Netherlands) (Hawley and Noakes, 1992). The test was undertaken under standard laboratory conditions (21–22°C, 40 – 50% room humidity). The maximal test has been described in detail previously (Hawley and Noakes, 1992). Briefly, throughout the maximal test and during portions of the experimental trials subjects breathed through the mouthpiece attached to a Quark b2 metabolic cart (COSMED, Rome, Italy). Expired gas was passed through a flow meter, an O₂ analyser and a CO₂ analyser that were calibrated prior to testing using a 3 L Hans-Rudolph syringe and gases of known concentration (4.00% CO₂ and 16.00% O₂). The flow meter and gas analysers were connected to a computer that calculated minute ventilation (VE), oxygen uptake (VO₂), carbon dioxide production (VCO₂) and respiratory exchange ratio (RER) from conventional equation. A maximal test was terminated at the point of volitional fatigue which coincided with the inability of a subject to maintain a cadence >70 rev.min⁻¹ and/or an RER >1.15. The highest VO₂ for any 60 s was taken as the subject’s VO_{2max}. Peak sustained power output (PPO) was calculated by adding the workload (W) completed on the final (uncompleted) workload to the last successfully completed workload. Based on the results of this maximal test and training history,
subjects were classified as either “sedentary” (\(\text{VO}_{2\max} < 45 \, \text{mL/kg/min}\)) or “trained” (\(\text{VO}_{2\max} > 55 \, \text{mL/kg/min}\)). Subjects with a \(\text{VO}_{2\max}\) of >45 but < 55 mL/kg/min were excluded from the study.

### 3.2.2 Overview of study design
Following preliminary testing, during the first phase, 8 trained and 8 sedentary subjects, as previously determined, were randomly assigned to receive either placebo (color and size matched tablets containing <5mg/day cocoa) or antioxidant enriched cocoa polyphenol (236mg/day) supplements for 7 days. A one week supplementation period was selected to evaluate acute short term supplementation effect. Compliance was excellent as confirmed by exact number of extra tablets returned by all subjects. He subject forgot to bring back the remaining tablets, but claimed he had some leftover tablets at home. Fasting blood samples were taken pre and post 1-hr exercise on 8\(^{th}\) day after seven days supplementation. Following a further 7-day washout period, subjects were swapped to the other treatment and under same protocol; pre and post 1-hr exercise fasting blood samples were collected again on 21\(^{st}\) day. The content of cocoa polyphenols, defined as flavanols and procyanidins in the supplements was determined by HPLC by Mars Technical Division, Ballarat, Victoria, Australia.

### 3.2.3 Experimental trial
On the morning of an experiment subjects reported to the laboratory between 0700-0800 hr after a 12-14 hr overnight fast. After a rest period of 10 min, a venous blood sample (16 mL) was collected. The subjects then commenced 1 hr of sub maximal cycling at 70% of \(\text{VO}_{2\max}\). Subjects were provided with access to water ad libitum throughout the ride. A further blood sample (6 mL) was taken immediately upon
completion of the ride, and analysed for total platelet count, hematocrit, mean platelet volume (MPV), whole blood platelet aggregation, ATP release from platelet granules, and platelet activation by flow cytometry.

3.2.4 Laboratory methods

3.2.4.1 Blood collection

Venous blood was collected per volunteer using a vacutainer adapter and 21 gauge vacuettes (Greiner bio-one GmbH, Kremsmünster, Austria) at two occasions, both pre and post one hour exercise. Blood was collected into 2 mL tri-potassium ethylene-diamine-tetra acetic acid EDTA (1.8 mg/mL), 8 mL tri-sodium citrate (3.8%) and 10 mL SST (serum separator tubes) tubes (Greiner bio-one GmbH, Kremsmünster, Austria). The EDTA tube was used before the tri-sodium citrate tube to avoid collecting platelets activated by venipuncture. Care was taken to ensure minimal specimen handling and agitation. All blood samples collected were tested according to the following protocols.

3.2.4.2 Platelet function tests

Mean platelet volume (MPV) and platelet count were measured by using whole blood collected in EDTA-containing tubes on Beckman Coulter A.C.T™ 5diff analyser (Coulter Corporation, Miami, FL, USA). Prior to FBE examination each day the performance of the analyser was validated using Coulter Calibrator and Controls Plus. Blood collected in citrate-containing tubes was used to determine the extent of platelet aggregation and to measure the release of ATP from platelets. Whole blood platelet aggregation was measured with an impedance aggregometer (Chrono-Log Corp, Philadelphia) equipped with MacLab software (ADIstruments Pty, Ltd, Castle Hill, Australia) for data quantitation and analysis. Detailed method has been
described previously (Murphy et al., 2003). Calibrations for impedance and ATP release were performed daily before analysing blood samples. Briefly citrated whole blood was diluted with saline (1:1), 100 µL chronolume reagent was added, sample was then incubated, and mixed with agonists 2 µg collagen/mL [Chrono-Log Corp] [1 mmol arachidonic acid (AA)/L was used to stimulate platelets for repeating the aggregation as a check for anti-inflammatory intake], and aggregation was recorded for 6 min. ATP release from platelets reacted with luciferin-luciferase in the Chrono-Lume reagent and luminescence was measured, at 650 nM by photomultiplier tube (PMT) built in the aggregometer, simultaneously with platelet aggregation.

Additional aliquots of citrated whole blood were diluted with modified tyrode’s buffer and incubated for 5 minutes in the presence of 2 µg collagen/mL. Subsequently, aliquots of activated blood were incubated in the dark with monoclonal antibodies, phycoerythrin conjugated CD41 (Immunotech, Marseille; which were used to identify platelets because it has specificity for the glycoprotein IIb portion of the glycoprotein IIb-IIIa antigen present on resting and activated platelets), fluorescein isothiocyanate conjugated CD62P (Immunotech; an activation-dependent antibody directed against P selectin, a component of the α-granule membrane of resting platelets that becomes expressed on the platelet surface membrane upon activation), or one of the isotype controls, immunoglobulin G1 (IgG1). Samples were fixed with paraformaldehyde and incubated to prevent further artifactual in vitro platelet activation. Modified tyrode’s buffer was added to terminate the fixation, and samples were analysed on an EPICS Elite flow cytometer (Coulter Electronics) equipped with a 15-mW argon laser, with excitation at 488 nm. The fluorescence of fluorescein isothiocyanate and phycoerythrin was detected by using 525 nM and 575 nM band pass filters, respectively. Single platelets were identified by gating on both phycoerythrin positivity
(CD41 binding) and characteristic light scatter. Because single platelets are smaller and less complex than other blood cells, including aggregated platelets, their forward scatter and side scatter are lower in comparison with other cells. Once identified, the expression of P-selectin was determined by analysing 20000 free platelets, which were collected at a rate between 1300 and 1600 events/s. Activated platelets were defined as CD41-positive events that expressed P-selectin. The data are reported as a proportion of maximum CD62P expression.

3.2.4.3 Serum Total Antioxidant Status (Olympus: AU2700)

Assay was performed using Randox Total Antioxidant Status kit with lot number NX2332. This colorimetric method performs quantitative in vitro determination of total antioxidant status in serum or plasma (serum was used in this study). Metmyoglobin chromogen with ABTS (2,2-Azino-di-[3-ethylben 2 thiazoline sulphonate]) incubated with substrate H$_2$O$_2$ in presence of antioxidants produces suppressed blue green colour measured at 600 nM. Strength of colorimetric wavelength of sample is computed against known standard values to give quantitative measure of antioxidant status. Analyser was validated by calibrating and running controls provided with the Randox kit.

3.2.4.4 Statistical analysis

Statistical analysis was performed using general linear modal (GLM) with three way ANCOVA on Statistical Package for the Social Sciences (SPSS) release 11.5.0, copyright@ SPSS Inc.

There were three factors: training (trained vs. sedentary), post treatment exercise (pre-exercise vs. post-exercise) and treatment (cocoa vs. placebo). Effect of each
factor and combined factors (interaction) on each parameter was analysed. Data is reported as mean ± SD in Table 1.

3.3 Results

3.3.1 Total platelet count
Trained subjects had significantly higher platelet counts compared with the sedentary subjects (235 ± 37 x10⁹/L Vs 208 ± 34 x10⁹/L, p<0.018). Platelet count increased significantly (p<0.005) in all subjects after exercise independent of treatment (Figure 3.1 & Table 3.1).

![Figure 3.1 Total Platelet Count](image)

**Figure 3.1** Total platelet count as mean ± SD in sedentary (first two pairs of columns) and trained groups (last two pairs of columns) before and after exercise both with and without cocoa supplementation. * Significant difference after exercise. ● Significant difference between trained and sedentary (P<0.05).

3.3.2 CD62p expression (P-selectin) as platelet activation marker
Trained subjects showed significantly lower (p<0.05) platelet activation compared with sedentary both before and after exercise in all subjects, whether on placebo or cocoa treatment (Figure 3.2 & Table 3.1).
Platelet activation as mean ± SD in sedentary and trained groups before and after exercise both with or without cocoa supplementation. ● Significant difference between trained and sedentary (P<0.05)

3.3.3 ATP release & Whole blood platelet aggregation

There was an increase in ATP release from platelets in all subjects after exercise (P<0.014). However there was no significant change in aggregation (slope) after exercise between either training or treatment groups (Figure 3.3 & Table 3.1).

ATP release from activated platelets as mean ± SD in sedentary and trained groups before and after exercise both with or without cocoa supplementation. * Significant change post exercise (P<0.05)

3.3.4 Mean platelet volume (MPV) and packed cell volume (Hematocrit)

Mean platelet volume did not change after exercise and was not affected by training status. Hematocrit levels increased in all subjects post exercise but not significantly.
3.3.5 Serum total antioxidant status

The total antioxidant concentration in serum between placebo or cocoa groups after one-week supplementation within trained or sedentary did not show any difference. Though total antioxidant concentration in trained was generally found to be lower than the sedentary however the difference was not significant enough to show any effect (Table 3.1).

Table 3.1 Platelet function test results pre and post one-hr exercise in trained and untrained healthy men after supplementation with ~240 mg/day cocoa antioxidants.

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>EXERCISE</th>
<th>SEDENTARY (N=8)</th>
<th>TRAINED (N=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>Active</td>
<td>Placebo</td>
</tr>
<tr>
<td>Platelet Count (10^9/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre Exercise</td>
<td>211 ± 31</td>
<td>204 ± 37</td>
<td>233 ± 30†</td>
</tr>
<tr>
<td>Post Exercise</td>
<td>263 ± 41*</td>
<td>243 ± 47*</td>
<td>271 ± 32*</td>
</tr>
<tr>
<td>CD62p (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre Exercise</td>
<td>59.3 ± 3.7</td>
<td>57.6 ± 6.1</td>
<td>49.3 ± 6.4†</td>
</tr>
<tr>
<td>Post Exercise</td>
<td>58.4 ± 4.9</td>
<td>55.2 ± 2.6</td>
<td>45.1 ± 7.7†</td>
</tr>
<tr>
<td>ATP Release (µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre Exercise</td>
<td>1.4 ± 0.7</td>
<td>1.5 ± 0.8</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>Post Exercise</td>
<td>1.8 ± 1.4*</td>
<td>2.0 ± 0.9*</td>
<td>2.1 ± 1.3*</td>
</tr>
<tr>
<td>Aggregation/Slope (Ω/sec)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre Exercise</td>
<td>0.20 ± 0.05</td>
<td>0.20 ± 0.07</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td>Post Exercise</td>
<td>0.21 ± 0.04</td>
<td>0.22 ± 0.05</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td>Mean Platelet Volume (fl)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre Exercise</td>
<td>7.9 ± 0.7</td>
<td>7.9 ± 0.8</td>
<td>8.0 ± 0.6</td>
</tr>
<tr>
<td>Post Exercise</td>
<td>7.9 ± 0.6</td>
<td>8.2 ± 0.6</td>
<td>8.1 ± 0.6</td>
</tr>
<tr>
<td>Haematocrit (PCV) (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre Exercise</td>
<td>47 ± 3</td>
<td>46 ± 4</td>
<td>45 ± 2</td>
</tr>
<tr>
<td>Post Exercise</td>
<td>49 ± 4</td>
<td>47 ± 4</td>
<td>46 ± 3</td>
</tr>
<tr>
<td>Total Antioxidant Status (mmol/L)‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre Exercise</td>
<td>1.34 ± 0.33</td>
<td>1.35 ± 0.22</td>
<td>1.25 ± 0.26</td>
</tr>
</tbody>
</table>

Results reported as Mean ± SD
* Significantly different from the pre exercise value, P<0.05
† Significantly different from untrained subjects, P<0.05
‡ Serum for total antioxidants was collected only before exercise
3.4 Discussion
There is growing interest in the utilization of antioxidant-rich plant extracts as dietary food supplements (Packer et al., 1999). However, it has not been clearly established if strenuous exercise increases the need for additional antioxidants in the diet. The practical significance of this study was that many sedentary people engage in occasional strenuous exercise. If the increase in free radicals due to exercise is greater than the ability of antioxidants in the body to neutralize them, the radicals will attack cellular components like lipids, initiating lipid peroxidation, which may lead to platelet hyperactivity and generation of more radicals and ROS that can harm other cellular components. This may predispose them to risk of heart disease due to raised oxidative stress and platelet activity in response to exercise.

The results of the present study demonstrated that: {1} platelet activation was higher in sedentary compared with trained subjects, {2} trained had a higher number of platelets compared with sedentary subjects {3} ATP released from platelets (a measure of platelet activation via dense granular release from activated platelets) was higher after exercise than before in all subjects and {4} platelet count increased after 60 min of exercise at a workload of approximately 70% VO$_{2\text{max}}$ independent of prior training status. However this study did not show that increased platelet activity in response to exercise could be normalised with oral supplementation of 236 mg/day of cocoa polyphenols given for one week. Previous studies by our group have shown that consumption of 236 mg/day for 28 days of cocoa polyphenols, defined as flavanols and procyanadins decreased platelet reactivity in healthy subjects (Murphy et al., 2003). Other studies with the same cocoa compounds have also shown decreased platelet activation and aggregation (ex vivo), but with a higher
concentration of 900 mg/d (Rein et al., 2000b, Holt et al., 2002) and decreased primary haemostasis 2 hr after consumption of 220 mg/d (Holt et al., 2002).

The results of previous studies have suggested that the risk of primary cardiac arrest is transiently increased during vigorous exercise, whereas habitual physical exercise is associated with an overall decreased risk of primary cardiac arrest (Siscovick et al., 1984a, Siscovick et al., 1984b). It has also been shown that platelet response to exercise depends on several factors such as the relative intensity of exercise, mode of exercise and physical fitness. Mild exercise does not alter and may even suppress platelet activation while strenuous exercise causes intensity-dependent platelet activation (Wang et al., 1994, Weiss et al., 1998). Our results clearly show that same intensity of exercise has different effect on platelet activity in trained and sedentary subjects. Sedentary subjects starting the intense exercise show higher platelet activation compared to trained subjects under same exercise protocol.

The results of the current investigation demonstrate that even though trained male subjects had higher numbers of platelets than untrained males, the platelet activation was lower in the trained compared with untrained subjects during aerobic exercise undertaken at the same relative intensity. Since trained males exercise on a regular basis their platelet count increased accordingly and the overall higher platelet count and lower platelet activation in trained men may be due to physiological adaptations in response to exercise. It has been shown aerobically fit (high VO$_{2\text{max}}$) compared to sedentary man of same age have a natural up regulation in endogenous antioxidant defences and more efficient mitochondria functioning (Wilson and Tanaka, 2000, Ji, 2001). It has been shown that reduced glutathione (GSH) protects against ROS-induced damage. Moderate to intensive exercise can decrease GSH in trained
individuals (Sacheck and Blumberg, 2001). GSH peroxidase is a compensatory response to and a sensitive marker of oxidative stress induced by exercise.

An increased rate of oxygen free radical production during exercise may cause variations in the platelet reactivity by inducing prostacyclin (PGI$_2$)/thromboxane (TxA$_2$) imbalance and alteration in antioxidant status. Exercise affects platelet function by increasing total platelet count (Ohri et al., 1983, Ricci et al., 1991) and plasma catecholamines (Ohri et al., 1983, Ikarugi et al., 1997), changing plasma lipoprotein profile (Agren et al., 1991, Baumstark et al., 1993), and performance of alpha-adrenergic receptors (Goto et al., 1996), impairing sensitivity of platelets to PGI$_2$ and nitric oxide (Drygas, 1988, Sakita et al., 1997, Sinzinger and Fitscha, 1986, Kishi et al., 1992). Free radicals increase as oxidative phosphorylation and catecholamines release increases in response to exercise.

Chen et al reported that long term training elevated PGI$_2$ level [an inhibitor of aggregation] and reduced TxA$_2$ levels [aggregating agent] (Chen et al., 1993). Moncada and Vane (1979) suggested that the ratio of PGI$_2$/TxA$_2$ plays an important role in determining the extent of platelet aggregation. This could explain why trained subjects might be less sensitive to the physiological stimuli compared to sedentary subjects when exercising at the same relative intensity. Several other possible mechanisms may explain why exercise training reduced the extent of platelet activation. First, exercise training may decrease resting plasma catecholamine levels (Mazzeo, 1991) and down-regulate the performance of platelet alpha2-adrenergic receptors (Lehmann et al., 1986), thus reducing vWF-platelet interaction and platelet P-selectin expression. Second, exercise training enhances substantial release of nitric oxide from platelet and plasma (Wang et al., 1997, Wang et al., 2000b) as well as expression of endothelial nitric oxide synthases (Sessa et al., 1994, Hambrecht et
al., 2003). Nitric oxide inhibits the formation of thrombus under high shear flow (Kaul et al., 1996) and attenuates agonist-induced up regulation of P-selectin and the binding of vWF to platelets.

Kestin et al. also found the platelet activation in trained subjects did not always occur after exhaustive exercise, however they did not find difference in baseline results between trained and sedentary subjects (Kestin et al., 1993). It is important to appreciate effect of exercise is dependent on the duration, intensity, frequency, volume and type of exercise, and the training status of subjects participating. These factors may be the reason of conflicting baseline results obtained by various studies between trained and sedentary subjects.

The different effects on platelet activation between trained and untrained men in the current study may also be partly explained by results of study by Cadroy et al. in which it was suggested that strenuous exercise increases thrombin generation from the vessel wall in sedentary (untrained) subjects which acts as a trigger for platelet activation (Cadroy et al., 2002).

We found raised ATP release, without an increase in platelet aggregation post exercise in this study. This discrepancy between collagen-induced platelet aggregation and release may be because of the possibility that the signalling pathways in response to exercise do not have similar sensitivities. Ficicilar et al. also demonstrated similar findings (Ficicilar et al., 2003).

There was an increase in hematocrit (packed cell volume) levels post exercise in all the subjects however the increase was not significant enough to shift plasma volume to the extent of dehydration that would affect the platelet function.
This study aimed to determine whether one week supplementation of cocoa polyphenols provided any protection by preventing platelet activation following exercise in trained or sedentary subjects. The dose of cocoa supplement (~240 mg/day) given to the subjects in this study was based on the previous studies conducted on normal subjects, where a significant decrease was observed in aggregation of platelets stimulated with agonists ADP and collagen (p-value <0.02) after four weeks of supplementation (Murphy et al., 2003). However, in the current study, no significant change was found in the total antioxidant levels in serum and therefore no effect on platelet activation was found post exercise as a result of oral consumption of cocoa antioxidants over a week. A dose sufficient to decrease platelet activity of resting platelets may not be enough to decrease hyperactivity of platelets in response to exercise. Further studies need to be conducted over a longer period of time and using a dose-response study to determine if it is possible counteract exercise effect using cocoa polyphenols.

In conclusion, the results from the present study demonstrate that exercise does increase the total platelet count and the amount of ATP released from the platelets. There is an inverse relationship between platelet numbers and activation status between trained and sedentary, suggesting that for optimal platelet function, moderate regular training is better than the sudden exhaustive exercise by the sedentary men. Oxidative stress due to increased O$_2$ uptake during exercise does play a role in platelet activation; however short-term antioxidants (cocoa polyphenol) supplementation did not provide the protection in this study. Future investigations should determine the optimal dose and duration of the time required for cocoa polyphenols to beneficially effect platelet function following exercise.
Chapter Four: Effects of Gamma-Tocopherol Supplementation on Thrombotic Risk Factors

Adapted from Asia Pacific J Clin Nutr 2007; 16 (3):422-428
4.1 Introduction

Vitamin E is an essential nutrient, the main lipid soluble antioxidant, and plays a significant role in protecting biological membranes and lipoproteins from oxidative damage caused by free radicals (Esterbauer et al., 1991, Patel et al., 1991, Brigelius-Flohe and Traber, 1999). Vitamin E is not a single compound with at least four tocopherols (α, β, δ and γ-tocopherol) and four tocotrienols (α, β, δ and γ-tocotrienol) known (Regina et al., 2002). The antioxidant activity of vitamin E is derived primarily from α-tocopherol and γ-tocopherol, of which α-tocopherol is most biologically active and the predominant form found in blood. In contrast, the predominant form of vitamin E found in food is γ–tocopherol (Wagner et al., 2004). The dietary intake of γ-tocopherol is at least two times that of α-tocopherol in Western diets, while the concentrations of α-tocopherol in human blood are generally four times higher than those of γ-tocopherol (Wagner et al., 2004).

An inverse relationship has been found between acute coronary events and antioxidant vitamin E intake. For example, epidemiological studies have shown that vitamin E (α-tocopherol and γ-tocopherol) is associated with reduced number of ischemic cardiac events in patients with documented coronary artery disease (Gey et al., 1991). Tocotrienols have also been shown to be beneficial by attenuating the formation of atherosclerotic lesions and decreasing serum cholesterol effects in animal and in vitro studies but results in humans have been inconclusive (Hosomi et al., 1997, Black et al., 2000, Mustad et al., 2002). The results of large clinical trials examining effect of Vitamin E (tocopherols as well as tocotrienols) on cardiovascular diseases have been equivocal (Devaraj and Traber, 2003). Supplementation with large amounts of α-tocopherol (1,200 IU per day) has been shown to decrease blood concentrations of γ-tocopherol (Handelman et al., 1985). Gamma tocopherol has
been found to be more effective than α-tocopherol in protecting against certain specific types of oxidative damage.

Saldeen et al showed an antithrombotic effect of vitamin E (γ-tocopherol and α-tocopherol) on thrombus formation, with more pronounced effect with gamma-tocopherol (Saldeen et al., 1999, Liu et al., 2003). These workers also demonstrated that platelet aggregation was inhibited more potently with a mixed tocopherol preparation (100 mg γ-tocopherol, 40 mg δ-tocopherol, and 20 mg α-tocopherol) than with α-tocopherol alone and attributed this observation to increased NO release, endothelial constitutive nitric-oxide synthase (ecNOS) activation, and superoxide dismutase protein content in platelets in response to mixed tocopherol (Saldeen et al., 1999).

In a placebo controlled double-blind study, we determined the effect of 5 wk of supplementation with γ-tocopherol at different doses (100 mg/d or 200 mg/d) on thrombotic risk factors, including platelet aggregation and activation. The effect of supplementation on plasma lipids and inflammation marker CRP was also investigated.

4.2 Methods and Materials

4.2.1 Subjects and dietary intake
After obtaining approval from RMIT Human Research Ethics Committee, 42 healthy volunteers were recruited by newspaper advertising and then randomly assigned to one of the three groups. Sample size and supplementation period was based on a similar study (Liu et al., 2003). All the subjects were between 20 to 40 years old with no known medical history. None were on any form of medication for the duration of
the study. Exclusion criteria included heavy drinking, smoking, and taking anti-inflammatory medication and antioxidant supplements or antioxidant rich food interfering with platelet function in the 2 wk prior to study entry. None of the subjects had a history of bleeding disorders, diseases of the circulatory system or diabetes. Two subjects withdrew from the study due to time constraints while one subject was withdrawn due to difficulty in phlebotomy. No subjects used any platelet inhibitors during study.

Dietary γ-tocopherol supplementation was achieved through consumption of tablets after signed informed consent was obtained from all participants. Fourteen healthy subjects (7 male and 7 female) consumed 100 mg/day of γ-tocopherol and 13 (7 male and 6 female) consumed 200 mg/d of γ-tocopherol while 12 (5 male and 7 female) received colour and flavour-matched placebo (soybean capsules with less than 5 mg/d γ-tocopherol) for 5 wks. Tama Biochemical Company Ltd Japan supplied all γ-tocopherol and placebo capsules.

Subjects were required to complete a food frequency questionnaire over a 7-day period before commencing dietary supplementation, to confirm they were not consuming antioxidant rich foods or antioxidant supplements. If volunteers were on antioxidant supplements or were regularly taking food very rich in antioxidants were not included to participate in the study. Baseline alpha and gammatocopherol levels were analysed to confirm that concentrations of serum tocopherols were not significantly different between subjects.
Fasting blood samples were collected for baseline assessment prior to the study and after 5 wks of supplementation. All the procedures were followed in accordance with RMIT HREC guidelines.

4.2.2 Experimental Trial
On the morning of an experiment subjects reported to the laboratory between 0700-0800 h after a 12-14 h overnight fast. A resting blood sample was obtained for total platelet count, whole blood platelet aggregation, ATP release from platelet granules, mean platelet volume (MPV), flow cytometry for platelet activation marker, total cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol and triacylglycerols. CRP and serum α- & γ-tocopherol concentrations were also evaluated.

4.2.3 Laboratory methods
4.2.3.1 Blood collection
A total of 20 mL of venous blood was collected using a vacutainer adapter and 21 gauge vacuettes (Greiner bio-one GmbH, Kremsmünster, Austria) on two occasions. Blood was collected into 2 mL tri-potassium ethylene-diamine-tetra acetic acid EDTA (1.8 mg/mL), 8 mL tri-sodium citrate (3.8%) and 10 mL SST (serum separator tubes) tubes (Greiner bio-one GmbH, Kremsmünster, Austria). The EDTA tube was collected before the tri-sodium citrate tube to avoid collecting platelets activated by venipuncture. Care was taken to ensure minimal specimen handling and agitation. All blood samples collected were tested according to the protocols described subsequently.
4.2.3.2 Platelet function tests

Platelet count and MPV were measured by using whole blood collected in EDTA-containing tubes with the use of a Beckman Coulter A\textsuperscript{5}.T 5diff CP analyser (Coulter Corporation, Miami, FL, USA). Performance of the analyser was validated using Coulter Calibrator and Controls Plus.

Whole blood platelet aggregation was measured with an impedance aggregometer (Chrono-Log Corp, Philadelphia) equipped with MacLab software (ADInstruments Pty, Ltd, Castle Hill, Australia) for data quantitation and analysis. This method has been described previously (Murphy et al., 2003). Calibrations for impedance and ATP release were performed daily before analysing blood samples. Briefly citrated whole blood was diluted with saline (1:1), 100 uL chronolume reagent was added, sample was then incubated, and mixed with agonists 2 µg collagen/mL [Chrono-Log Corp] [1 mmol arachidonic acid (AA)/L was used to stimulate platelets for repeating the aggregation as a check for anti-inflammatory intake], and aggregation was recorded for 6 min. ATP released from activated platelets reacted with luciferin-luciferase in the Chrono-Lume reagent added to diluted blood sample. This reaction increased luminescence, which was measured, at 650 nM by photomultiplier tube (PMT) built in the aggregometer.

Additional aliquots of citrated whole blood diluted in modified tyrode's buffer were activated with 2 µg/mL collagen, and then incubated in the dark with monoclonal antibodies, phycoerythrin conjugated CD41 (Immunotech, Marseille; which were used to identify platelets because it has specificity for the glycoprotein IIb portion of the glycoprotein IIb-IIIa antigen present on resting and activated platelets), fluorescein isothiocyanate conjugated CD62p (Immunotech; an activation-dependent antibody directed against P-selectin, a component of the \(\alpha\)-granule membrane of resting
platelets that becomes expressed on the platelet surface membrane upon activation), or one of the isotype controls, immunoglobulin G\(_1\) (IgG\(_1\)). Samples were fixed with paraformaldehyde to prevent artifactual in vitro platelet activation. Modified tyrode's buffer terminated the fixation, and samples were analysed on an EPICS Elite flow cytometer (Coulter Electronics) equipped with a 15-mW argon laser, at an excitation of 488 nm. The fluorescence of fluorescein isothiocyanate and phycoerythrin was detected by using 525 nm and 575 nm band pass filters respectively. Activated platelets were defined as CD41-positive events that expressed P-selectin. The data are reported as a proportion of maximum CD62P expression.

### 4.2.3.3 Lipid Screening and CRP Analysis

Lipids and CRP were analysed on the automated Olympus AU2700 biochemical analyser. The instrument was validated by calibrating and running controls for each parameter. Enzymatic colorimetric methods were used for measuring total cholesterol (based on cholesterol oxidase method), HDL cholesterol (determined using anti human-beta-lipoprotein antibody binding to lipoproteins other than HDL) and triacylglycerols (based on a method with glycerol blank).

LDL cholesterol was calculated, using following Friedwald formula (Friedwald et al., 1972): 

\[
\text{LDL Cholesterol} = \text{Total cholesterol} - [\text{HDL Cholesterol} + \text{VLDL}], \quad \text{Where,} \\
\text{VLDL} = \frac{\text{triacylglycerols}}{5}
\]

CRP was measured using immuno-turbidimetric method using anti-human CRP antibodies to form insoluble aggregates.

### 4.2.3.4 Serum Alpha and Gamma-tocopherol Concentration
The Shimadzu HPLC system (Kyoto, Japan) used in this study consisted of a chromatograph LC-10AD and spectrofluorophotometer detector (RF-551) equipped with a 12 µl LC flow cell. \( \gamma \)-tocopherol, \( dl-\alpha \)-tocopherol and \( dl-\alpha \)-tocopheryl acetate were detected at excitation \( \lambda = 298 \) nm, emission \( \lambda = 325 \) nm, recorded by a CR6A Chromatopac Recorded /Integrator.

For the analysis a reversed-phase C-18 column, with a mobile phase of Acetonitrile: Dichloromethane: Methanol (7:2:1), at a flow rate of 1.0 mL/min was employed. Quantitation of these compounds was achieved using linear calibration curves constructed from the peak area versus the concentration of the standard compound. Before starting a sample run, external standards, followed by an internal QC (plasma frozen and allocated into multiple vials on day one from a normal subject) were checked.

**4.2.3.5 Statistical analysis**

Data are presented as means ± SDs. The data analyses were performed using a SPSS version 11.5 program (SPSS Corporation, Chicago IL, USA). Effects of different Vitamin E (\( \gamma \)-tocopherol) doses (dose effect) on the parameters were analysed using GLM repeated measures of ANOVA adjusted for baseline values. Changes between pre and post treatment in same dose (treatment effect) were assessed using paired t-test. \( p \) values <0.05 were considered significant.

**4.3 Results**

Compliance was good, as assessed by the number of pills returned and measure of serum \( \alpha \) and \( \gamma \)-tocopherol concentrations. All the subjects returned all of the extra tablets (three extra doses were given to all subjects on placebo and 100mg/day \( \gamma \)-
tocopherol and two extra doses was given to subjects on 200mg/day γ-tocopherol). Two subjects on placebo and one subject on 100mg/day γ-tocopherol missed one or two doses as they returned more tablets than expected. No side effects from the active or placebo tablets were reported.

Baseline results for all groups were comparable. Five week of supplementation with 100 mg and 200 mg γ-tocopherol resulted in increased concentrations of serum γ-tocopherol (p<0.05). The serum level of α-tocopherol did not change after intervention in any group (Table 4.1).

There was a significant effect of γ-tocopherol treatment on platelet activation: p-selectin (CD62p%) decreased significantly after both doses (p<0.05), with little change after placebo (Table 4.2).

MPV decreased significantly with 100 mg γ-tocopherol treatment (p<0.05) but the decrease was not statistically significant for the higher dose. Platelet aggregation also decreased significantly with 100 mg γ-tocopherol (p<0.05) though little effect was seen with 200 mg γ-tocopherol or placebo (Table 4.2).

Gamma-tocopherol treatment resulted in a significant effect on LDL cholesterol. LDL cholesterol was significantly decreased following 100 mg/d of γ-tocopherol supplementation (p<0.05) (Table 4.3).

HDL cholesterol increased significantly with 100 mg γ-tocopherol and placebo. However a similar increase was not found with 200 mg γ-tocopherol suggesting the increase is random and unlikely due to γ-tocopherol treatment (Table 4.3).
Inflammation marker CRP also decreased though not significantly after supplementation with 200 mg γ-tocopherol. There were no significant changes in the serum levels of triacylglycerol, or in the whole blood platelet count and ATP release.

4.4 Discussion
The potentially beneficial effects of vitamin E on cardiovascular disease have been intensively investigated in many interventional and epidemiological studies. However, most of these studies focused exclusively on α-tocopherol and the protective effects of α-tocopherol supplementation on CVD is equivocal (Jha et al., 1995, Marchioli, 1999). Accordingly, we evaluated the effect of pure γ-tocopherol on platelet function, lipid concentrations and inflammation marker in blood. To the best of our knowledge, this is the first study that provides in vivo relationship between pure γ-tocopherol and thrombotic risk factors.

Specifically, the present study determined the effect of two doses of γ-tocopherol on platelet function, lipid profile and inflammation marker CRP in human subjects. We observed a significant increase in serum γ-tocopherol concentrations after both doses. A significant decrease in platelet activity post 200 mg and 100 mg γ-tocopherol dose was also observed, though the changes between groups were not statistically significant. There was also a significant reduction in MPV, platelet aggregation and LDL cholesterol post 100 mg γ-tocopherol supplementation only.

Serum γ-tocopherol concentrations increased significantly in all subjects in accordance with the concentration of dose, while no change was observed in serum α-tocopherol concentrations. This suggests that any changes observed in this study
in the platelet function and lipid profile would have been due to γ-tocopherol ingestion.

Several independent investigations have demonstrated that the blood concentration of γ-tocopherol, not α-tocopherol, was negatively correlated to the incidence of coronary heart disease (Kontush et al., 1999, Ohrvall et al., 1996). Supplementation with large amounts of α-tocopherol was shown to increase the breakdown and decrease blood concentrations of γ-tocopherol, (Morinobu et al., 2003, Handelman et al., 1985) as a result of the function of the hepatic α-tocopherol transfer protein (α-TTP), which preferentially incorporates α-tocopherol into the plasma (Devaraj and Traber, 2003). Hosomi found that the biological activity of vitamin E analogs correlates to their affinity for α-TTP (Hosomi et al., 1997). Chopra and Bhagavan showed that bioavailability of both natural and synthetic α-tocopherol significantly suppresses the serum γ-tocopherol to the same extent (Chopra and Bhagavan, 1999).

Our results could explain the findings of previous epidemiological studies showing inverse correlation between vitamin E intake and incidence of CVD. It has been shown dietary source of vitamin E is much higher in γ-tocopherol (Wagner et al., 2004) compared to α-tocopherol, but all the investigational studies used very high concentration of α-tocopherol, which could have broken down the γ-tocopherol (Handelman et al., 1985) and nullified the effect of vitamin E seen by previous studies. High dose of α-tocopherol reduces intestinal absorption, cell membrane transport and utilisation of other forms of vitamin E, especially γ-tocopherol (Handelman et al., 1994, Handelman et al., 1985, Friedrich, 2004). The hepatic α-TTP has the greatest affinity for α-tocopherol compared to γ-tocopherol and is crucial for the relative percentage of transport of the various forms of vitamin E in the plasma.
lipoproteins (Friedrich, 2004, Hosomi et al., 1997, Wagner et al., 2004). This imbalance of α-tocopherol / γ-tocopherol levels in plasma may have significant health consequences (Ohrvall et al., 1996, Hosomi et al., 1997).

Liu et al have shown in various animal and human studies that mixed tocopherols are more potent in preventing platelet aggregation and have stronger inhibitory effect on lipid peroxidation than α-tocopherol alone (Liu et al., 2002, Liu et al., 2003, Li et al., 1999, Saldeen et al., 1999). We further narrowed down the mix tocopherol to γ-tocopherol as it is a rich source of dietary antioxidant. In the current study we used a pure form of γ-tocopherol and our results suggest that γ-tocopherol supplementation had a direct positive effect on platelet activity and LDL cholesterol concentrations in blood of normal healthy people.

Circulating activated platelets are useful markers of local thrombotic events occurring in cardiovascular diseases. We used flow cytometry to detect circulating activated platelets through expression of platelet surface glycoprotein, p-selectin, also known as CD62p. The p-selectin, is rapidly translocated from α-granules inside the platelets to the cell surface on stimulation by physiological agonists. During agonist-induced platelet activation, energy-dependent fusion of both alpha and dense granules with the plasma membrane permits expression of p-selectin, which promote and propagate platelet adhesion to endothelial cells, neutrophils and monocytes (Hsu-Lin et al., 1984).

One possible mechanism by which γ-tocopherol might have reduced platelet activity (i.e. the reduced expression of p-selectin through decreased CD62p binding on platelet surface) could be related to platelet derived NO bioactivity. Impaired platelet NO production and decreased bioavailability of NO have been associated with
coronary disease states (Freedman et al., 1998). Platelet-derived NO has been found to inhibit platelet aggregation and reduce platelet recruitment to a growing thrombus (Williams and Nollert, 2004). α-tocopherol might increase platelet NO release by its free radical scavenging activity and by preventing it’s quenching by peroxyl radicals (Huie and Padmaja, 1993, Freedman et al., 2000). One compensatory mechanism is nitration of γ-tocopherol, through available nitration-prone positions on the chromanol ring of γ-tocopherol (Christen et al., 1997, Rubbo et al., 1994) and thus depleting NO by reacting with it, which leads to an up-regulation of NO synthesis (Williams and Nollert, 2004). Nitration of gamma tocopherol uses up nitric oxide and thus by feedback mechanism NO synthesis might be upregulated. Freedman suggested α-tocopherol might also be responsible in balancing NO and SOD in human platelets (Freedman et al., 2000). Saldeen et al suggested up-regulation of SOD by both α-tocopherol and γ-tocopherol might be important mechanism for the effect of γ-tocopherol on platelet aggregation, but they found γ-tocopherol to be more potent than α-tocopherol in these effects (Saldeen et al., 1999).

A decrease in platelet activation could possibly be explained in terms of antioxidant effect of γ-tocopherol. Jiang et al demonstrated cyclooxygenase-2 (COX-2) catalyses the synthesis of prostaglandins through oxidation of arachidonic acid, which are important elements within the inflammatory process as well as platelet activation. They found COX-2 activity is inhibited by γ-tocopherol but not by α-tocopherol (Jiang and Ames, 2003, Jiang et al., 2001, Jiang et al., 2000).

Significant decrease in LDL cholesterol is indicative of improved lipid profile, which may also explain the reduced platelet activity observed in this study. In recent in vitro studies, γ-tocopherol has been shown to inhibit lipid per oxidative damage (Wolf,
and to trap mutagenic electrophiles (Christen et al., 1997) more efficiently than \( \alpha \)-tocopherol. This finding supports our results demonstrating an improved lipid profile and a trend of reduction in platelet activity post \( \gamma \)-tocopherol supplementation. Since LDL cholesterol is decreased therefore concentration of oxidised LDL should also be low, thus reducing risk of foam cell formation and hence risk of thrombosis.

Contrary to our findings, Jiang and Ames also provided strong evidence that \( \gamma \)-tocopherol shows anti-inflammatory activity \textit{in vivo} in rats (Jiang and Ames, 2003). One of the reasons for that could be that most of baseline results in our study were normal, around 2 mg/L and so there was not much scope for the values to further go down. It will be more realistic in future to evaluate the effect of \( \gamma \)-tocopherol on populations with higher inflammatory conditions.

In conclusion, the results from the current study suggest that the daily consumption of small amounts of \( \gamma \)-tocopherol, in conjunction with usual dietary intake from mixed food sources may provide protection from oxidative damage and prevent thrombosis. Further cellular research would be valuable in helping to understand the mechanisms behind the biological effects of \( \gamma \)-tocopherol in higher risk populations such as diabetic subjects or those with known cardiovascular disease. Potential synergistic effects between \( \gamma \)-tocopherol and other antioxidants and comparison with \( \alpha \)-tocopherol supplementation could also be explored to clarify the role of \( \gamma \)-tocopherol in human health.
Table 4.1: Serum Gamma (γ-) tocopherol and alpha (α-) tocopherol

<table>
<thead>
<tr>
<th>Treatment Dosage</th>
<th>γ-tocopherol (mg/ml)</th>
<th>α-tocopherol (mg/ml)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;5mg G-T</td>
<td>5.27±3.29</td>
<td>26.68±5.56</td>
<td></td>
</tr>
<tr>
<td>(Placebo n=12)</td>
<td>5.43±2.27</td>
<td>26.72±5.04</td>
<td></td>
</tr>
<tr>
<td>100mg G-T</td>
<td>5.34±3.52</td>
<td>24.87±8.21</td>
<td></td>
</tr>
<tr>
<td>(n=14)</td>
<td>16.80±7.23</td>
<td>22.38±5.37</td>
<td></td>
</tr>
<tr>
<td>200mg G-T</td>
<td>5.37±4.14</td>
<td>22.82±7.47</td>
<td></td>
</tr>
<tr>
<td>(n=13)</td>
<td>30.09±20.69</td>
<td>24.05±9.75</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.000</td>
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<tr>
<td></td>
<td></td>
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<td>0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.000</td>
</tr>
</tbody>
</table>

Each parameter was analysed using GLM repeated measures of ANOVA.

a,b: Significant difference within groups (Paired-Samples t-test).
<table>
<thead>
<tr>
<th></th>
<th>&lt;5mg G-T (Placebo n=12)</th>
<th>100mg G-T (n=14)</th>
<th>200mg G-T (n=13)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>P-selectin CD62p (%)</td>
<td>47.47±9.66</td>
<td>45.87±15.54</td>
<td>50.03±4.95a</td>
<td>45.34±7.11b</td>
</tr>
<tr>
<td>Agg/Slope (Ω/Sec)</td>
<td>0.22±0.05</td>
<td>0.21±0.07</td>
<td>0.25±0.08a</td>
<td>0.22±0.06b</td>
</tr>
<tr>
<td>ATP release (um)</td>
<td>2.01±1.28</td>
<td>1.80±1.07</td>
<td>2.24±1.38</td>
<td>2.68±2.25</td>
</tr>
<tr>
<td>Platelet (x10^9/L)</td>
<td>231±47</td>
<td>230±36</td>
<td>237±49</td>
<td>226±48</td>
</tr>
<tr>
<td>MPV (fl)</td>
<td>8.35±0.69</td>
<td>8.09±0.70</td>
<td>8.62±0.95a</td>
<td>8.35±0.75b</td>
</tr>
</tbody>
</table>

Each parameter was analysed using GLM repeated measures of ANOVA.

a,b: Significant difference within groups (Paired-Samples t-test).
### Table 4.3: Lipids and inflammation marker

<table>
<thead>
<tr>
<th>Parameter</th>
<th>&lt;5mg G-T (Placebo n=12)</th>
<th>100mg G-T (n=14)</th>
<th>200mg G-T (n=13)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>4.87±0.87</td>
<td>5.12±1.03</td>
<td>4.59±0.82</td>
<td>5.11±1.20</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.79±0.63</td>
<td>2.90±0.73</td>
<td>3.03±0.97</td>
<td>2.62±0.83</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.46±0.39 b</td>
<td>1.70±0.54 a</td>
<td>1.33±0.39 b</td>
<td>1.49±0.42 a</td>
</tr>
<tr>
<td>Triacylglycerols (mmol/L)</td>
<td>1.36±0.88</td>
<td>1.13±0.56</td>
<td>1.11±0.32</td>
<td>1.04±0.44</td>
</tr>
<tr>
<td>C-reactive Protein (mg/L)</td>
<td>2.08±3.48</td>
<td>1.75±2.67</td>
<td>2.64±3.02</td>
<td>3.14±4.46</td>
</tr>
</tbody>
</table>

Each parameter was analysed using GLM repeated measures of ANOVA. a,b: Significant difference within groups (Paired-Samples t-test).
Chapter Five: Oxidative stress-induced insulin resistance in skeletal muscle cells is ameliorated by gamma-tocopherol treatment.

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5.1 Introduction

There is accumulating evidence that the generation of reactive oxygen species leads to increased oxidative stress in a number of insulin sensitive tissues resulting in a variety of conditions including type 2 diabetes and cardiovascular disease (West, 2000). Antioxidant agents have been proposed to arrest some of the deleterious effects of ROS (Chan, 1998) either by donating hydrogen to free radicals or by accepting free radicals and therefore neutralising their effect. Gamma tocopherol, an isoform of Vitamin E present in food, has been shown to have potent antioxidant effects (Wolf, 1997). It has been shown to inhibit platelet aggregation more potently in platelets isolated from humans supplemented with a mixed tocopherol preparation than in platelets isolated from humans supplemented with alpha-tocopherol alone (Liu et al., 2003). In addition, we have previously demonstrated that pure gamma tocopherol inhibited platelet aggregation and improved lipid profile in normal healthy subjects, thereby playing a role in prevention of thrombosis (Singh et al., 2007).

Type 2 diabetes mellitus (T2DM) is characterized by insulin resistance in various tissues and in particular, skeletal muscle, the primary site for insulin-stimulated glucose disposal (DeFronzo et al., 1985). While there is evidence that oxidative stress leads to insulin resistance (Evans et al., 2003), tissue damage and pathogenesis of late diabetic complications (Rosen et al., 2001), the mechanisms responsible remain unclear. In skeletal muscle cells, uptake of glucose depends on the insulin stimulated translocation of the glucose transporter GLUT4 to cell surface (Houseknecht and Kahn, 1997). Signalling to GLUT4 in skeletal muscle requires tyrosine phosphorylation of insulin receptor substrate (IRS)-1, which recruits and activates phosphatidylinositol (PI) 3-kinase (Thirone et al., 2006).
Recently an Akt substrate with molecular weight of 160 kDa (AS160) and a molecular signature of a Rab-GAP has been identified as an important regulator of GLUT4 traffic (Kane et al., 2002). Rab-GAP domains modulate the activity of Rab proteins, which are involved in the regulation of several membrane transport steps, including vesicle budding, motility, tethering and fusion (Zerial and McBride, 2001). Insulin stimulation of skeletal muscle leads to phosphorylation of AS160, a process dependent on Akt (Bruss et al., 2005, Bouzakri et al., 2006). Impairments in insulin action on AS160 accompany defects in glucose transport in humans. In skeletal muscle from Akt2 knock-out mice, insulin-mediated AS160 phosphorylation and glucose uptake are severely blunted, which highlights an absolute requirement of Akt for insulin-mediated action to AS160 (Kramer et al., 2006).

The effects of ROS on insulin signalling cascade are equivocal (Hayes and Lockwood, 1987, Heffetz et al., 1990, Hansen et al., 1999, Rudich et al., 1997), but the results of many studies demonstrate that ROS impairs insulin mediated glucose uptake (Hansen et al., 1999, Rudich et al., 1998, West, 2000). While it has been proposed that vitamin E supplementation might prevent ROS-induced impairment of insulin signalling (Faure et al., 1997), studies of the effects of antioxidant treatment on insulin signalling and functional measures of glucose transport are lacking. If ROS are involved in causing damage to cellular signalling machinery, antioxidant treatment may be able to alleviate or abolish that outcome. Accordingly, the primary purpose of the current investigation was to determine whether gamma tocopherol, which we have recently demonstrated to have potent antioxidant properties (Singh et al., 2007), could ameliorate the deleterious effects of oxidative stress on insulin sensitivity. To the best of our knowledge, this is the first study to investigate effect of
gamma tocopherol on glucose uptake and the most distal signalling protein involved in GLUT4 translocation, AS160.

5.2 Methods and Materials

5.2.1 Cell culture
L6 skeletal myoblasts (American Type Cell Collection) were cultured following aseptic techniques in 75cm² flasks containing growth media, which consisted of DMEM supplemented with 10% FBS, 1% penicillin/streptomycin (v:v) at 37°C in 5% CO₂/95%O₂ in humidified air. Media was replaced daily with pre-warmed media (37°C) until the cells were 60–70% confluent. Cells were washed twice with pre-warmed PBS then detached with 2 ml of 0.25% trypsin for 3–4 min with intermittent shaking. Once the cells were completely detached the total number of cells were counted then seeded into six well plates at 7000 cells per cm². Cells were grown to about 70% confluency in α modified essential medium (αMEM) +10% FBS before changing to differentiation media (αMEM +2% FBS) to allow myoblasts to differentiate into myotubes.

Experimental treatments were conducted after two days, by which time nearly all of the myoblasts had fused to form myotubes. Cells were incubated with Gamma tocopherol for 24 hr (with concentrations indicated) and oxidative stress was introduced by incubation with 100mU/mL glucose oxidase for 1 hr.

5.2.2 Deoxyglucose uptake
For determination of 2-deoxy-D-[³H]-glucose uptake, myotubes were incubated in αMEM without glucose with 0.1% FBS for 4 hr. Cells were washed twice with warm PBS (containing 0.1% FBS) and equilibrated in 2mL no Glucose-α-MEM for 30 min
followed by incubation with 100 nM insulin for 30 min or without (basal), before 800 µL of radioactive media (1 µCi/mL 2-deoxy-D(3-H)glucose, 10 µM 2-deoxy-D-glucose in α-MEM, 0.1%FBS) was added to each well. After 30 min media was aspirated and the assay stopped by washing cells twice in ice-cold PBS. Cells were then lysed in 1 mL of 0.3M NaOH and 800 µL of the lysate in 4 mL of scintillation fluid was counted via liquid scintillation.

5.2.3 Determination of protein content in L6 myotubes by Western blotting

L6 myotubes were grown, differentiated and treated as described under cell culture above. Lysis buffer (20 mM HEPES (pH 7.4), 2mM EDTA, 50mM NaF, 5 mM Na₄P₂O₇, 1% NP40 and phosphatase and protease inhibitor cocktails) was added and cells rapidly scraped then transferred to a 1.5ml tube and rapidly frozen in liquid nitrogen for later analysis. Subsequently, cell culture lysates were spun at 16,000 g for 5 minutes, then 5µl of the supernatant taken and diluted 1:25 with water, before the protein concentration was determined using a commercially available kit (BCA™ Protein Assay Kit, Pierce, USA) using absorbance spectrophotometry. Absorbance of standards and samples was determined at 560 nm on a spectrophotometer (Victor 3™ 1420, Multilabel Counter, Wallac) and the protein content calculated from the linear regression.

Proteins were then solubilised in Lamelli’s buffer (4x pH 6.8 consisting of 40% glycerol, 8.2%SDS (Sodium Dodecyl Sulphate), 0.5 M Tris-HCl, 40mM dithiothreitol plus bromophenol blue) was added to 40 µg of sample heated for 5 min at 85°C, and proteins resolved by SDS-PAGE using pre-cast polyacrylamide gradient gels and commercially available electrophoresis reagents (Invitrogen, Australia). A molecular weight protein standard (Precision plus Protein Standards, Biorad), and solubilised
protein samples were loaded into individual wells. The gel was run at 150 V until proteins had sufficiently migrated through the gel.

Separated proteins were then transferred to a PVDF membrane using pre-developed reagents and standard transfer sandwich apparatus (Invitrogen, Victoria, Australia). Proteins were transferred at a constant voltage of 20 V for 100 (Akt) or 180 (AS160) min. After the transfer, the membrane was washed with Tris buffered saline with Tween (TBST; 20 mM Tris, 140 mM Nacl, 0.05% Tween 20, pH 7.6). The membrane was blocked with 5% skim milk powder (w/v) in TBST on a rocker at room temperature for 1 h. After blocking, membranes were washed with TBST and incubated with primary antibodies (diluted 1:1000 in TBST+1%BSA) specific for phospho-Akt-Ser473, β-actin and Phospho-Akt-Substrate (Cell Signalling Technology), overnight on a rocker at 4°C. Membranes were washed three times with TBST then incubated with secondary antibody (1:5000 anti-rabbit antibodies in TBST) for 1 h at room temperature. The membrane was then washed (6x10 min) with TBST and immunoreactive bands detected with 2 ml of chemiluminescence reagent (ECL™ Western Blotting Detection Reagents, Amersham Biosciences, England) and exposed using the Chemidoc EQ system (Biorad). Proteins were quantified using Quantity One software version 9 (Biorad Laboratories, Hercules, CA, USA).

5.2.4 Statistical Analysis

Results are presented as mean ± SEM. Differences were determined using an unpaired t test and P values of <0.05 were considered significant.
5.3 Results

5.3.1 Effect of γ-tocopherol on glucose uptake in L6 myotubes exposed to oxidative stress

One hour treatment with 100 mU/mL glucose oxidase significantly decreased glucose uptake both with and without 100 nM insulin stimulation (Figure 5.1).

Pre-treatment with 100 μM and 200 μM gamma tocopherol partially protected cells from the effect of glucose oxidase, where 200 μM gamma tocopherol restored both basal and insulin stimulated glucose transport to normal levels (Figure 5.1).

Figure 5.1 2-Deoxyglucose uptake in L6 myotubes that were differentiated as stated in methodology. Cells were treated for with 100 or 200 μM gamma-tocopherol (GT) for 24 hr, 100 mU/mL glucose oxidase (GO) for 1 hr and 100 nM insulin for 30 min. * denotes difference between basal and insulin within a treatment, or both basal and insulin when compared to another treatment, P<0.05.
5.3.2 Insulin signalling in L6 myotubes exposed to oxidative stress and gamma tocopherol

**Figure 5.2** Phosphorylation of Akt serine-473 (A) and AS160 by Akt (B) relative to total β-Actin in L6 myotubes that were differentiated as described in Methodology. Cells were treated for with 200 μM γ-tocopherol for 24 hr, 100 mU/ml glucose oxidase for 1 hr and 100 nM insulin for 10 min. PAS: Phospho-Akt substrate. * denotes different to all other conditions, † denotes difference between treatments, P<0.05.
To assess whether the protective effect on basal and insulin-stimulated glucose transport of gamma tocopherol after induction of oxidative stress was due to improved signalling from the insulin receptor to GLUT4 vesicles, the phosphorylation of Akt and AS160 on amino-acid residues that are important for insulin signalling were assessed.

Glucose oxidase did not impair basal or insulin stimulated phosphorylation of Akt or AS160, but 200 µM gamma tocopherol did improve insulin-stimulated phosphorylation of both Akt and AS160 and might partly explain the restoration of glucose transport in this condition (Figure 5.2).

5.4 Discussion

In the present study, we investigated the effects of gamma tocopherol on basal and insulin stimulated glucose transport and insulin signalling in cultured rat L6 myotubes exposed to a H₂O₂ generating system. When the cells were exposed to oxidative stress using 100mU/ml glucose oxidase, glucose transport was impaired under both basal and insulin stimulated conditions. Pre-treatment with gamma tocopherol for 24 h prevented this decrease in glucose uptake. In addition, we found that high (200 µM) but not low (100 µM) doses of gamma tocopherol restored glucose transport to normal levels; it also appeared to impact basal transport through mechanisms other than augmentation of insulin stimulated Akt or AS160 phosphorylation, because basal transport with 200 µM gamma tocopherol alone was not different from that when cells were treated with gamma tocopherol as well as stimulated with insulin.
Furthermore, neither basal Akt nor AS160 were impacted upon by 200 µM gamma tocopherol treatment.

Our finding that H$_2$O$_2$ inhibits insulin-mediated glucose uptake is in agreement with some (Maddux et al., 2001, Hansen et al., 1999, Rudich et al., 1997) but not all (Kozlovsky et al., 1997, Fischer et al., 1993, Evans et al., 2005, Timar et al., 1997). Discrepancies in result between the various studies can, in part, be explained by the differences in the experimental design, such as in vivo versus in vitro models and dose and duration of exposure to the compounds under investigation. Various levels of oxidative stress have been induced between 25-100 mU/mL glucose oxidase for periods varying between 5 min to 24hr. We found that the induction of H$_2$O$_2$ via 100 mU/mL glucose oxidase for 1hr in L6 myotubes impaired insulin-stimulated glucose transport, in line with previous studies (Hansen et al., 1999, Rudich et al., 1997), but not the phosphorylation of Akt and AS160. Thus the precise mechanism by which ROS impairs glucose uptake has yet to be determined. Nevertheless improved glucose uptake and insulin signalling by pre-treatment with gamma tocopherol suggests this antioxidant plays some role in counteracting effect of oxidative stress.

Disconnection between glucose transport and insulin signalling (i.e. Akt and AS160 phosphorylation) in the current study strongly suggest that there are other mechanisms by which gamma tocopherol improved glucose transport. Given that the pre-treatment with gamma tocopherol was more prolonged than the glucose oxidase exposure, it is possible that reduced lipid peroxidation affords some improvement in the membrane dynamics and interaction of transporters such as GLUT4, or some other factor in the vicinity. The role of free radical attack in diabetes and in the
cardiovascular complications of the disease has been documented largely through
the effects of free radicals on lipids and proteins (Giugliano et al., 1996, Oberley,
1988, Ozdemirler et al., 1995). Gamma tocopherol has been shown to act on cell
surface by preventing oxidation of LDL in cell membrane (Hodis et al., 2002).
However, there is no evidence to suggest such mechanism exists to prevent ROS-
induced insulin resistance, and this is an area that requires further study.

The mechanisms by which H$_2$O$_2$ and other mediators of oxidative stress cause
insulin resistance are largely unknown. A possible explanation for the inhibitory effect
of H$_2$O$_2$ on insulin action is that it triggers an alteration in cellular redox balance due
to prolonged exposure. Stress inducers, including H$_2$O$_2$, can function as signalling
molecules to activate a number of stress sensitive serine/threonine kinase pathways
linked to insulin resistance (Evans et al., 2003). Insulin signalling enzymes offer a
number of potential substrates for these activated serine kinases including the insulin
receptor and the family of IRS proteins. Increased serine phosphorylation of IRS-1
decreases the extent of insulin stimulated tyrosine phosphorylation and attenuates
insulin action (Evans et al., 2002). Increased serine kinase activity that
phosphorylates IRS-1 was identified in vitro and in vivo insulin resistance (Keren et
al., 1997). Numerous agents that induce insulin resistance, such as TNF-α and other
cytokines, platelet-derived growth factor, angiotensin, hyperinsulinemia, all increase
IRS-1 serine phosphorylation (Sykiotis and Papavassiou, 2001) through induction of
ROS (Nishikawa et al., 2007), which might explain their insulin de-sensitizing effects.
The ability of gamma tocopherol to preserve the intracellular redox balance and
prevent the activation of stress sensitive serine kinase cascades maybe instrumental
in preventing the serine phosphorylation of IRS-1. Additional research which links the
activation of serine kinase cascades in oxidative stress induced insulin resistance is clearly warranted to firmly establish this link.

In conclusion, we found that acute oxidative stress impaired glucose transport in Rat L6 myotubes. Pre-treatment with gamma tocopherol reversed the effect of oxidative stress and improved glucose uptake both in the absence and presence of insulin. However, disconnection between glucose uptake and the phosphorylation state of key proteins in the insulin signalling cascade suggest that gamma tocopherol improves glucose transport via insulin dependent and independent mechanisms. Our results suggest gamma tocopherol may play a preventive role in counteracting effect of oxidative stress on glucose uptake by skeletal muscle cells. Identification of the molecular basis for this observation might lead to the discovery of pharmacological targets for novel therapies to prevent, reverse or delay the onset of insulin resistance and resultant pathogenesis.
Chapter Six: Summary and Conclusions
The primary aim of the experiments undertaken in this thesis was to evaluate the efficacy by which various antioxidants may attenuate risk factors leading to thrombosis, atherosclerosis and other cardiovascular diseases under conditions of increased oxidative stress and the concomitant free radical production such as acute exercise, or hyperglycemia. Some of the most common risk factors of thrombosis leading to other cardiovascular disease are platelet hyperactivity and stickiness (Elwood et al., 1991), impaired lipid profile (Steinberg and Witzum, 1990), decreased glucose uptake and metabolism. All of these factors decrease available energy required for normal body functioning and contributes to disturbing redox balance between reactive oxygen species and antioxidants (Smith and Muscat, 2005). Many in vitro studies have shown that diet rich in fruits and vegetables act as effective antioxidant in biological systems such as blood cells, lipoproteins and cultured cells by inhibiting lipid oxidation in human plasma exposed to various patho or physiologically relevant types of oxidative stress. For that reason different antioxidants commonly consumed as part of a regular diet, including olive leaf by Mediterranean population in particular, cocoa and gamma tocopherol (one of the vitamin E isoforms) were examined with respect to their potential to prevent thrombosis by improving platelet function, lipid profile or glucose transport in two intervention and two in vitro studies undertaken for this thesis.

A direct link has been demonstrated between Mediterranean diet rich in olives and olive oil and lower incidence of cardiovascular diseases. Although olive oil is rich in antioxidants, olive leaf extract has been shown to have even more potent antioxidant properties. Olive leaves have been traditionally used to make tea in routine for medicinal purposes in some Mediterranean countries for centuries. The first study, presented in chapter two, determined that 54 µg/mL olive leaf extract was effective in
improving platelet function by reducing platelet aggregation and ATP released from activated platelets. Lower ATP release by platelets in response to physiological agonist stimulation suggests olive leaf extract may be instrumental in attenuating platelet activity and thus preventing risk of thrombosis, atherosclerosis and other cardiovascular disease. These results provide novel information with respect to a potential mechanism by which olive leaf extracts reduces risk of cardiovascular diseases. The results of this study provide the first evidence that olive leaf extract treatment might be able to improve platelet function in normal healthy population.

In light of these findings, the next study, described in chapter three, was designed to evaluate platelet activity in response to increased oxidative stress and to examine the effect of antioxidant supplementation on markers of platelet function. Platelet activity has been shown to increase in response to exercise by various studies. This has been attributed to increased production of reactive oxygen species and other free radicals during exercise. It has also been suggested that sudden acute exercise poses increased risk of CVD (Wang, 2006). On the other hand chronic exercise is considered therapeutic in prevention of CVD. It seemed likely that the platelet response to acute exercise would differ in the trained versus sedentary individuals and that antioxidant supplementation may be able to attenuate the increase in platelet activity induced by exercise.

Accordingly, the study presented in chapter three compared platelet response to an acute bout of submaximal exercise between trained and sedentary subjects both before and after short term cocoa polyphenol supplementation. The results of this study demonstrated that regular training increased platelet number but resulted in lower stimulated platelet activity and aggregation compared to sedentary subjects in
response to standardised exercise undertaken before and after 1 wk period of cocoa supplementation. Higher platelet number has been linked to an increased chance of platelet aggregation under shear stress. However, the increased rate of aggregation with lower number of platelets in sedentary subject indicates that a single bout of submaximal exercise may be resulting in increased production of free radicals in sedentary subjects more than in trained individuals, or, conversely, that regular exercise training could improve endogenous antioxidant defence (Wilson and Tanaka, 2000, Ji, 2001). It has been shown that antioxidant enzyme activity is stimulated by acute exercise, which could be part of defence mechanism of cells against oxidative stress (Belviranli and Gokbel, 2006). There is insufficient information about relationship of exercise and antioxidants. However, short term supplementation with cocoa polyphenol was not enough to counteract the effect of acute sudden exercise on platelet activity in sedentary population. The results of this study suggest that the risk of thrombosis is probably greater in untrained subjects participating in a single bout of exercise, and that short term cocoa supplementation was not enough to combat effect of increased reactive oxygen species production in sedentary population. These findings warrant for further studies with long term antioxidant supplementation.

Olive leaf extract and cocoa polyphenols used in the two previous studies are both hydrophilic antioxidants. So the next step was to assess if effect of a lipophilic antioxidant is also equally important in preventing risk of thrombosis. Gamma tocopherol (vitamin E), a lipophilic antioxidant, has been inversely related to risk of cardiovascular disease. Almost all of the trials used alpha tocopherol form of vitamin E but the predominant form of vitamin E found in food is gamma tocopherol. It has been demonstrated that supplementation with alpha tocopherol reduces the effect of
gamma tocopherol. Thus it makes sense to increase gamma tocopherol supplementation or not to disturb the balance between alpha and gamma tocopherol intake as found in natural dietary plant sources.

Based on this fact it seems more likely that gamma tocopherol would be more effective in preventing atherogenesis. One of the primary steps of atherogenesis is endothelial dysfunction which results in reduced nitric oxide generation. At the same time free radicals like superoxide react with nitric oxide producing peroxynitrite, thus reducing bioavailability of nitric oxide. Given the potential of gamma tocopherol in neutralising reactive nitrogen species more potently, the clinical trial described in chapter four supplemented normal healthy subject’s diet with two different doses of gamma tocopherol. The results of this study provide first evidence of the preventive effect of pure gamma tocopherol on risks of thrombosis by improving lipid profile that is less LDL cholesterol for oxidation and less foam cell formation. Decreasing mean platelet volume as well as platelet activity and aggregation on stimulation with agonists can all be instrumental in attenuating risk factors of thrombosis. These results provided evidence for distinct role of gamma tocopherol in preventing risk factors leading to thrombosis and other CVD in normal healthy population.

In light of these findings in vitro cell culture experiments (chapter five) evaluated the effect of gamma tocopherol under conditions of increased oxidative stress. Insulin-stimulated glucose uptake by skeletal muscle under conditions such as hyperglycemia in diabetes is impaired due to the presence and action of free radicals. The results of this study demonstrated that gamma tocopherol reversed the effect of oxidative stress on insulin stimulated glucose uptake and increased expression of Akt (Ser$^{473}$) and AS160 in Rat L6 skeletal muscle myotubes. However,
unlike glucose uptake, the expression of Akt (Ser\textsuperscript{473}) and AS160 did not change when myotubes were exposed only to glucose oxidase-induced oxidative stress. This study provides novel information with respect to a potential mechanism by which glucose transport may be facilitated by gamma tocopherol. Increased expression of Akt (Ser\textsuperscript{473}) and AS160 in conjunction with increased insulin stimulated glucose uptake by skeletal muscle myotubes after treatment with gamma tocopherol demonstrate that gamma tocopherol is instrumental in improving glucose transport under oxidative stress conditions. These results suggest that there may also be some other mechanisms also involved by which gamma tocopherol reverses glucose uptake by oxidised myotubes. Further investigation into the effect of gamma tocopherol and other antioxidants on various signalling proteins of insulin signalling pathway and other mechanisms are needed.

In summary, the results from the studies undertaken for this thesis provide novel information regarding the role of various hydrophilic and lipophilic antioxidant suppletations in potentially preventing cardiovascular disease through mechanisms involved in platelet activation and aggregation and glucose transport under conditions of oxidative stress. Antioxidant supplementation particularly under high oxidative stress conditions is likely to play a preventive role in reducing risk of thrombosis and other related complications leading to CVD. Incorporating a balance of natural antioxidants in the diet and incorporating regular exercise into daily living may be able to improve general public health. Results from these studies support the hypothesis that various dietary sources of antioxidants including foods rich in cocoa, olive and gamma tocopherol are instrumental in combating oxidative stress induced cardiovascular diseases. Antioxidant supplementation may show potential health benefits by reducing risk of thrombosis and thus indirectly improving quality of life,
reducing pain and the potential costs of disease treatment particularly by preventing, life style mediated conditions leading to diabetes, thrombosis and as a consequence other cardiovascular diseases.

Several important questions should be addressed in future work. For example, what are most appropriate doses and durations for antioxidants alone or in combination to be effective in preventing risk of conditions leading to CVD? In addition, future work should determine the mechanisms by which oxidative stress impairs platelet activity, lipid profile and glucose transport. Such information may provide clues for the development of more effective preventive as well as therapeutic antioxidant agents from natural food sources that specifically target platelets, endothelial wall and skeletal muscle maintenance. Further studies should include comparative and synergistic effect of various doses of alpha tocopherol and gamma tocopherol in humans with and without oxidative stress on mechanisms of platelet activity as well as other thrombotic coagulation markers and mechanisms leading to glucose uptake.

Continued research into various antioxidants and the mechanisms by which they act to overcome the impairments of CVD may lead to establishing more effective doses necessary in preventing progress of asymptomatic atherosclerosis to CVD. Given the increasing prevalence of sedentary life style, obesity, diabetes (all of which show increased oxidative stress) and CVD universally, it seems research in this field has a long way to go. One area of interest will be comparing antiplatelet therapies with antioxidant supplementation in normal healthy subjects and diabetic subjects.
Chapter Seven: References


