THE EFFECT OF NATURAL DIETARY ANTIOXIDANTS ON LOW DENSITY LIPOPROTEIN OXIDATION AND ATHEROSCLEROSIS

Nicole Louise Kerry, B.Sc. (Hons)

A thesis submitted to the University of Adelaide

for the degree of Doctor of Philosophy

Department of Clinical and Experimental Pharmacology

University of Adelaide

South Australia

September 1997

TABLE OF CONTENTS

CHAPTER 4

THE ISOFLAVONE GENISTEIN INHIBITS LDL OXIDATION.

CHAPTER 5

THE EFFECT OF RED WINE ON PLASMA LIPIDS, LIPOPROTEINS AND PHYTOSTEROLS IN CHOLESTEROL-FED RABBITS.

ABSTRACT

The objectives of the present studies were to investigate the *in vitro* antioxidant properties of red wine containing polyphenols and the isoflavone genistein. Subsequently, the effect of red wine on LDL oxidation and fatty streak lesion development in cholesterol-fed rabbits was examined. Since LDL oxidation is generally thought to promote the development of atherosclerosis, the hypothesis of the present study was that dietary intervention with antioxidants could inhibit LDL oxidation and slow atheroma formation.

In vitro experiments demonstrated that both red wine and genistein were effective inhibitors of copper and peroxy radical-catalysed LDL oxidation. Red wine containing 0.025 to 20 mg/L gallic acid equivalents (GAE) and genistein at concentrations of 0.2 to 200 μmol/L increased the lag time of conjugated diene formation, inhibited the generation of thiobarbituric acid reactive substances and decreased the relative electrophoretic mobility of LDL in a concentration-dependent manner. These changes were not apparent in LDL incubated with ethanol or red wine stripped of phenols. Red wine polyphenols have the ability to be incorporated into LDL during an *in vitro* incubation with plasma, as evident by the 60% increase in lag time following copper-mediated oxidation of isolated LDL which also resulted in 3-fold lower uptake of this LDL by macrophages compared to control LDL. Genistein did not prevent the oxidation of LDL in this model due to its poor incorporation into LDL following plasma incubations.

Red wine was fractionated into phenolic acids (fraction 1), catechins and monomeric anthocyanidins (fraction 2), flavonols (fraction 3) and polymeric anthocyanidins (fraction 4) and the antioxidant properties of each fraction was determined. All red wine fractions increased the lag time of copper-mediated LDL oxidation compared to control with the order of potency being fraction 2 (92% increase in lag time) > fraction 1 (65%) > fraction 4 (42%) > fraction 3 (37%). Similarly, malondialdehyde concentrations following azo-initiated oxidation of LDL were inhibited by 12 to 40% in the presence of red wine fractions and followed the same order of potency.

In a dietary intervention study, male New Zealand White rabbits were grouped into one of four treatments. One group were fed a normal commerically available rabbit diet (n=6). The remaining 18 rabbits were fed a diet containing cholesterol (0.25 to 0.5% wgt/wgt) alone (n=6), or in combination with red wine (n=6) or ethanol (n=6). Rabbits consumed approximately 22.5 ml of red wine/d for 12 weeks which was equivalent to 36.3 mg GAE/d. Due to the unpalatability of ethanol, rabbits consumed less ethanol, approximately 0.9 g ethanol/d in this treatment compared to red wine-treated rabbits which consumed 1.7 g ethanol/d.

Plasma cholesterol, VLDL + IDL, LDL and HDL-cholesterol levels were significantly elevated in cholesterol-fed rabbits. There were no differences in plasma lipids or lipid and protein compositions in any lipoprotein fractions following dietary intervention with red wine or ethanol. The oxidisability of LDL was determined as the lag time for conjugated diene formation following copper-mediated oxidation. LDL isolated from cholesterol + red wine-treated rabbits displayed a significantly shorter lag time (112.1 ± 1.1 min, $P<0.05$) compared to rabbits fed cholesterol alone (160.8 ± 16.8 min). The lag time of LDL oxidation in ethanol-treated rabbits (124.4 ± 9.6 min) was not significantly different to that measured in rabbits fed cholesterol alone or in combination with red wine. There were no significant differences in oxidation rate, maximum conjugated diene concentration and malondialdehyde formation between rabbit treatment groups. Despite the difference in lag time there were no differences in α-tocopherol or the fatty acid composition in LDL isolated from rabbit plasma.

At completion of the dietary intervention anaesthetised rabbits were killed by exsanguination from the abdominal aorta. Atherosclerosis in aortic arch segments from rabbits was assessed by lipophilic staining using oil red O and quantified using image analysis. The % lipophilic stain in the aortic arches from rabbits were not different in cholesterol-fed rabbits 19.3 ± 7.0, cholesterol + red wine rabbits 21.1 ± 4.1 and cholesterol + ethanol rabbits 15.9 ± 6.1, although the fatty lesions in these rabbits were significantly greater than in control rabbits 0.06 ± 0.04. Rabbits fed cholesterol in combination with red wine displayed significantly

greater cholesterol deposition in segments of the descending thoracic aorta 1.13 ± 0.16 μg cholesterol/mg wt wgt compared to rabbits fed cholesterol alone (0.65 ± 0.03, $P<0.01$), but this was not different to rabbits consuming cholesterol + ethanol (1.11 ± 0.2).

For experiments investigating vasorelaxation, thoracic aortic rings with intact endothelium were mounted in organ bath chambers and smooth muscle contractions to potassium and phenylephrine were measured. Cumulative dose-response curves to acetylcholine, calcium ionophore (A23187) and sodium nitroprusside were performed in precontracted aorta. Although there was an impaired aortic relaxation to acetylcholine in cholesterol-fed rabbits compared to normocholesterolemic rabbits, the response of aorta to acetylcholine was the same in rabbits fed cholesterol alone or in combination with red wine or ethanol.

Catechins (140 mg/L) and procyanidins (polymeric catechins, 400 mg/L) are abundant polyphenols in red wine and were used as markers for red wine bioavailability in this rabbit study. Monomeric catechins could not be detected by high performance liquid chromatography with electrochemical detection in rabbit plasma nor could we detect levels of monomeric and polymeric catechins using a sensitive colorimetric assay.

In summary, red wine was an effective antioxidant *in vitro* protecting against LDL oxidation. In a dietary intervention study red wine and ethanol had no effect on plasma lipids and lipoprotein levels nor endothelium-dependent relaxation of aortic preparations in cholesterol-fed rabbits. Red wine supplementation in conjunction with cholesterol feeding increased LDL oxidisability and thoracic aorta cholesterol concentrations compared to rabbits fed cholesterol alone. There were no significant differences between red wine and ethanol supplemented rabbits. The low plasma levels of red wine polyphenols and the increased ethanol consumption in red wine treated rabbits could contribute to the apparent proatherogenic effects of red wine observed in cholesterol-fed rabbits.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution, and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give my consent to this copy of my thesis, when deposited in the University Library, being available for photocopy or loan.

Date

10/9/97

Nicole L Kerry

ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisor, Dr Mavis Abbey and gratefully acknowledge her guidance, encouragement and expertise throughout.

I wish to thank all at the CSIRO Division of Human Nutrition in which all my laboratory studies and general business were conducted. In particular I would like to thank all those who provided the necessary resources such as the library, computer and animal facilities and the ever helpful support and scientific staff.

I wish to thank Thelma Bridle for her excellent technical assistance and acknowledge her contribution to much of the aortic dissection, fixing and image analysis. I also acknowledge Drs Pat Williams and Peng from the Australian Wine Research Institute for providing the red wine analysis and Dr Graham Jones and Michael Kerrigan from the Department of Horticulture, Viticulture and Oenology, The University of Adelaide for providing the method used to fractionate red wine. I would also like to thank the animal staff, Jim Greenfield, David Courage and Vanessa Courage, who carried out the daily care and dietary protocol required for the rabbit study. I am grateful to the contribution, collaboration and assistance of all these people.

I appreciate the excellent technical support and the enjoyable friendships offered by all who worked in the laboratory or research group, Alice Owen, Cherie Keatch, Sotiria Bexis, Simon LeForgia, Karen Kind, Simone Burghardt, Christina Bursill, Alison Morris and Caroline Bignell. I would like to especially thank Alice for her great companionship, her support and good humour. Thanks also to Drs Peter Clifton, Paul Roach and Mahinda Abeywardena for their excellent scientific contributions, patience and advice in all things.

I would like to thank the Department of Clinical and Experimental Pharmacology at the University of Adelaide for endorsing and supporting my candidature. Particular thanks must

go to Dr. Anne Tonkin in her role as postgraduate coordinate, Prof Felix Bochner and all the staff in the department.

I acknowledge the National Heart Foundation of Australia and The Australian Atherosclerosis Society for providing my postgraduate scholarship and additional funding.

On a personal note, thank you to all my family and friends. Thanks Thomas for your extraordinary patience, perserverance and faith in me. To my parents, Margaret and David, thank you for all the opportunities and encouragement which have allowed me to pursue my ambitions.

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AcLDL	acetylated low density lipoprotein
Apo B	apolipoprotein B-100
CAD	coronary artery disease
CE	cholesteryl ester
d	density
DMEM	dulbecco's modified eagles media
ECD	electrochemical detection
EDRF	endothelium-dependent relaxing factor
EDTA	ethylenediaminetetra-acetic acid disodium salt
g	grams
GAE	gallic acid equivalents
HDL	high density lipoprotein
HPLC	high performance liquid chromatography
IDL	intermediate density lipoprotein
KPSS	potassium chloride physiological salt solution
LDL	low density lipoprotein
MDA	malondialdehyde
PBS	phosphate buffered saline
PUFA	polyunsaturated fatty acid
TBARS	thiobarbituric acid reactive substance
UV	ultraviolet
v	volume
VLDL	very low density lipoprotein
wgt	weight
WHHL	Watanabe heritable hyperlipidemic rabbit

PUBLICATIONS ARISING FROM THIS THESIS

Full Publications

N Kerry, M Abbey. Red wine and fractionated phenolic compounds prepared from red wine inhibit low density lipoprotein oxidation *in vitro*. *Atherosclerosis* 1997; 135: 93 - 102.

N Kerry, M Abbey. The isoflavone genistein inhibits copper and peroxy radical mediated low density lipoprotein oxidation *in vitro*. *Atherosclerosis (Submitted)* 1997.

Abstracts

NL Kerry, M Abbey. Red wine phenols inhibit low density lipoprotein (LDL) oxidation macrophage uptake *in vitro*. *Australian Atherosclerosis Society Inc 20th Annual Scientific Meeting SA, Australia , Sept 1994.*

NL Kerry, M Abbey. Red wine phenolics inhibit low density lipoprotein (LDL) oxidation and subsequent uptake of LDL into Macrophages. *The Royal Australian Chemical Institute's 10th National Convention, SA, Sept 1995.*

M Abbey, N Kerry. Total and fractionated phenolic compounds of red wine inhibit low density lipoprotein oxidation. In: Medically, is wine just another alcoholic beverage. Stockley CS (ed), The Wolf Blass Foundation Inc (Publ). *Proceedings of the Wolf Blass Foundation. International Wine and Health Conference pp 32-36, 1996.*

CHAPTER 1

INTRODUCTION

There is currently great interest in the role of antioxidants as possible protective agents against the development of coronary artery disease (CAD). A number of human studies have demonstrated a link between intake of antioxidant vitamins and a reduction in the incidence of CAD (Stampfer *et al.* 1993, Rimm *et al.* 1993, Gey *et al.* 1991). Animal studies have shown that supplementation with vitamin E, a potent antioxidant, inhibits the development of atherosclerotic plaques (Williams *et al.* 1992). One possible mechanism that could lead to atherosclerosis is through the oxidation of lipids in low density lipoproteins (LDL), which are the major cholesterol carrying lipoproteins in blood. Oxidised LDL undergo chemical and physical modifications that could contribute to the atherogenic process in humans (reviewed by Steinberg *et al.* 1989). Antioxidants have the potential to inhibit LDL oxidation and in so doing could prevent the progression of atherosclerosis and CAD. This chapter will provide an overview of the role of oxidised LDL in atherosclerosis and the protective effects of antioxidants against lipid peroxidation, vascular function and atherogenesis.

1.1 PLASMA LIPIDS AND ATHEROSCLEROSIS

Lipids (cholesterol, triglycerides and phospholipids) are transported in blood as lipid- protein complexes termed lipoproteins. One of the major lipoprotein fractions in blood is LDL. The lipid phase of LDL is composed of approximately 50% cholesterol which is present predominantly in the esterified form (cholesteryl ester). The protein moiety of LDL is composed of a single molecule of apoplipoprotein B-100 (apo B) which is important for the solubility of the LDL particle in blood and is the ligand for the LDL receptor which facilitates the removal of LDL from the circulation.

High levels of plasma cholesterol (hypercholesterolemia) are positively associated with high levels of LDL cholesterol since LDL is the major transporter of cholesterol *in vivo*. Hypercholesterolemia and elevated levels of LDL cholesterol are major risk factors for the development of atherosclerosis. One of the foremost lines of evidence for LDL-cholesterol being atherogenic is observed in patients with Familial Hypercholesterolemia (FH) (Goldstein *et al.* 1975). These patients have a defect in the gene encoding for LDL receptor resulting in dysfunctional receptors. LDL cannot be effectively cleared from the blood

leading to elevated levels of both plasma and LDL-cholesterol and the development of atherosclerosis and coronary artery disease (CAD) at a young age (Fredrickson *et al.* 1978).

Atherosclerosis is an occlusion of the arteries particularly at sites of bifurcation of the aorta, coronary, carotid, basilar and femoral arteries (Simionescu *et al.* 1993). Its pathology is characterised by deposition of cholesteryl ester both intracellularly and extracellularly, endothelial damage, proliferation of smooth muscle cells, platelet aggregation and thrombotic formation which may eventually lead to CAD.

At a cellular level atherogenesis involves a complicated pattern of inflammatory events including adhesion of ligands to endothelium, chemotaxis of blood monocytes from lumen to subendothelium and release of cytokines which stimulate smooth muscle proliferation and the formation of connective tissue. It is generally accepted that an initiating event in the development of atherosclerosis is the formation of foam cells in the artery wall. Microscopic studies reveal that foam cells contain massive intracellular accumulations of cholesteryl ester (Newman *et al.* 1962). These arterial foam cells have distinct morphological and histochemical features of macrophages (Fowler *et al.* 1979, Schaffner *et al.* 1980, Gerrity *et al.* 1981a, 1981b).

1.2 LDL METABOLISM

The major pathway of LDL metabolism in healthy subjects is by hepatic LDL receptors (Goldstein *et al.* 1977). The apo B moiety of LDL binds to the LDL receptor on the cell surface. The LDL receptor-ligand complex is internalised and degraded. LDL-cholesteryl ester is hydrolysed to free cholesterol in cellular lysosomes and is utilised by the cell. Regulatory mechanisms normally exist within the cell which prevent the accumulation of intracellular cholesterol. These are down-regulation of LDL receptor expression, decreased cholesterol synthesis and increased cholesterol esterification. As previously mentioned, patients with FH have a deficiency in this LDL receptor pathway. Despite possessing non-functional LDL receptors, FH sufferers still accumulate cholesteryl ester in macrophages leading to the formation of atheromas and xanthomas. This observation indicates that

cholesterol enters cells via a mechanism distinct from the LDL receptors.

To investigate this uptake of cholesterol by macrophages, Goldstein *et al.* (1979) performed experiments in culture whereby acetylated-LDL (Ac-LDL) was incubated with mouse peritoneal macrophages. These researchers demonstrated an increased degradation and accumulation of LDL-cholesteryl ester in macrophages characteristic of that seen in foam cells *in vivo*. This uptake of Ac-LDL by macrophages was not via the classical LDL receptor as shown by competition binding studies. In contrast to classical LDL receptor function, the uptake of chemically modified LDL by macrophages was not dependent on calcium and was unsaturable. Other studies have presented similar results demonstrating cholesterol accumulation in monocyte/macrophages following incubation with LDL which were chemically modified by maleylation (Goldstein *et al.* 1979), malondialdehyde and succinylation (Fogelman *et al.* 1980, Shechter *et al.* 1981 and Haberland *et al.* 1982, 1984).

A common feature of these chemical alterations to LDL is the loss of positive charges on the lysine and cysteine amino acid residues on the apo B molecule in LDL (Mahley *et al.* 1979, Haberland *et al.* 1982, 1984). These alterations to the LDL binding domain result in a decreased ability to bind to the classical LDL receptor. This chemically modified, strongly anionic LDL particle is converted to a ligand that is recognised by another binding site on macrophages termed the acetylated-LDL receptor or, as it is more commonly referred to, the macrophage scavenger receptor (Brown and Goldstein 1983). However an endogenous ligand for the scavenger receptors on macrophages was yet to be identified. It was hypothesised by Fogelman *et al.* (1980) that malondialdehyde, a product of lipid peroxidation *in vivo*, could cause chemical alterations to LDL in plasma that result in the formation of modified LDL which could bind to scavenger receptors on macrophages.

1.3 OXIDATION OF LDL

Oxidative modification of LDL involves the oxidation of polyunsaturated side chains within the lipophilic core of LDL. The major lipid present in LDL is esterified cholesterol. LDL-cholesterol is preferentially esterified with polyunsaturated fatty acids (PUFA), in particular

linoleic acid. The polyunsaturated side chains within cholesteryl esters are very susceptible to non-enzymatic oxidation as a result of the carbon double bonds. Compounds with sufficient reactivity such as free radicals (R •) can abstract a hydrogen from a bis-allylic methylene group within esterified PUFA to form a carbon centred radical referred to as an alkyl radical (L • reaction A). This alkyl radical undergoes molecular rearrangement to form a conjugated diene which is a long chain carbon molecule with a pair of covalent double bonds separated by a single covalent bond. Under aerobic conditions conjugated dienes react with molecular oxygen to form a lipid peroxy radical (LOO • reaction B). Lipid peroxy radicals can abstract hydrogen from an adjacent esterified PUFA forming lipid hydroperoxide (LOOH) and another peroxy radical (reaction C). This is the propagation stage of lipid peroxidation (reviewed by Stocker *et al.* 1994).

$$-LH + R\bullet \longrightarrow -L\bullet + RH \quad (A)$$

$$-L\bullet + O_2 \longrightarrow -LOO\bullet \quad (B)$$

$$-LOO\bullet + -LH \longrightarrow -LOOH + -L\bullet \quad (C)$$

Reactive oxygen species (ROS) are constantly being formed in the body. Common sources of ROS *in vivo* are from the respiratory bursts that occurs during the phagocytosis of foreign particles by polymorphonuclear cells. ROS are also formed by enzymatic reactions mediated by lipoxygenase and cyclooxygenase and from the mitochondrial electron transport chain within cells. ROS are implicated in many clinical conditions such as ischaemia, cancer and inflammatory-immune injury such as rheumatoid arthritis and atherosclerosis (Maxwell 1995).

In addition to ROS-mediated LDL oxidation (Morel *et al.* 1983), it soon became apparent that vascular cells such as endothelial cells (Henriksonn *et al.* 1981), smooth muscle cells (Heinecke *et al.* 1984), monocytes (Cathcart *et al.* 1985) and macrophages (Parthasarathy *et al.* 1986a) could modify LDL *in vitro*. These modifications of LDL were characterised by increased uptake into macrophages, increased lipid peroxides, increased relative electrophoretic mobility and cytotoxicity to proliferating cells. Studies by Steinbrecher *et al.*

(1984) and Morel *et al.* (1984) demonstrated that modification of LDL by cells involved free radical oxidation of lipid since the modifications of LDL were inhibited by antioxidants such as butylated hydroxytoluene (BHT), vitamin E and glutathione.

The mechanism for cell-mediated oxidation and generation of ROS within these cell systems are not clear. Some hypotheses for the mechanism that mediates cellular oxidation of LDL include the (i) generation of ROS or enzymatic oxidation by cellular lipoxygenase activity (Parthasarathy *et al.* 1989a, McNally *et al.* 1990), (ii) generation of cellular superoxide anion (Heinecke *et al.* 1986, Hiramatsu *et al.* 1987), (iii) generation of nitric oxide (Darley-Usmar *et al.* 1992), (iv) thiol generation (Sparrow *et al.* 1993), and (v) glucose oxidation (Hunt *et al.* 1990). Other studies have demonstrated that lipid peroxidation can also be achieved in cell-free systems by redox metal ions (Aust *et al.* 1985) and purified enzyme systems containing lipoxygenase and phospholipase A_2 (Sparrow *et al.* 1988).

Some of the physicochemical changes of oxidatively modified LDL are (i) loss of esterified PUFA and cholesteryl ester (ii) increase in hydrated density (iii) increase in PUFA and sterol oxidation products (conjugated dienes, lipid peroxides, carbonyl compounds and oxysterols) (Esterbauer *et al.* 1987, Parthasarathy and Rankin 1992). In addition to modifications in lipid fraction of LDL, there are changes to the protein moiety. As mentioned previously, the carbonyl breakdown products of lipid peroxidation, in particular malondialdehyde, react with ε-amino groups of lysine residues in apo B causing an increase in negative charge of the lipoprotein and subsequent fragmentation of apo B (Haberland *et al.* 1992, Mahley *et al.* 1979). The fragmentation of apo B is not mediated by proteolytic enzymes but can be inhibited by antioxidants or metal chelators (Fong *et al.* 1987). The changes to the protein molecule in LDL, mediated by oxidative processes, leads to the formation of a modified LDL particle that has been identified as an endogenous ligand for macrophage scavenger receptors (Parthasarathy *et al.* 1987).

1.4 MACROPHAGE SCAVENGER RECEPTORS

Following the initial characterisations of oxidised LDL and scavenger receptors there have been reports of additional receptors present on macrophages that also bind oxidised LDL. Sparrow *et al.* (1989) described a separate class of receptors on macrophages that recognised endothelial cell-modified LDL (EC-LDL) but not Ac-LDL. These authors performed a series of competitive binding studies using iodinated and unlabelled LDL. In summary they found that EC-LDL was able to compete with approximately 90% of the binding of labelled Ac-LDL, but in contrast unlabelled Ac-LDL competed for only 25% of the binding sites on macrophages in the presence of labelled EC-LDL. The authors concluded that the nonreciprocal cross-competition results observed for EC-LDL and Ac-LDL indicate that these modified LDL are not identical ligands for the scavenger receptor and that there may be separate classes of scavenger receptors present on macrophages. These observations were confirmed by cross-competition studies of labelled modified LDL by Arai *et al.* (1989) and later by Freeman *et al.* (1991) in Chinese Hamster ovary cells transfected with scavenger receptors.

At present, the observations from competitive uptake studies suggest that not all uptake of oxidised LDL is mediated by macrophage scavenger receptors and may involve a number of other macrophage receptors. Kodama and coworkers isolated and purified bovine scavenger receptors (type I) and later went on to partially characterise the structure of scavenger receptors by determining its amino acid sequence and then used this to clone complimentary DNA (Kodama *et al.* 1988, 1990). When the DNA encoding for scavenger receptors was expressed in COS cells, it formed functional type I and II scavenger receptors (Roher *et al.* 1990). Similarly, two classes of macrophage scavenger receptors have been cloned from murine, human and rabbit cDNA libraries (Freeman *et al.* 1990, Matsumoto *et al.* 1990, Bickel *et al.* 1992).

Other receptors present on macrophages have been identified which bind oxidised LDL but not Ac-LDL. Cells transfected with either Fcγ-RII-B2 DNA (Stanton *et al.* 1992) or CD36

DNA (Endemann *et al.* 1993) were able to bind and degrade oxidised LDL but not Ac-LDL. The Fcγ-RII-B2 receptor belongs to a family of receptors involved in the uptake of immune complexes via recognition of the Fc region of IgG. Stanton and coworkers proposed that the Fcγ-RII-B2 receptor could also mediate LDL uptake of immune complexes formed between oxidised LDL and autoantibodies via Fc receptors, since LDL has shown to be immunogenic (Palinski *et al.* 1989). The binding of lipoprotein immune complexes by Fc receptors in cultured monocytes has since been demonstrated by Kiener *et al.* (1995).

Immunohistochemical studies by Matsumto *et al.* (1990) using anti-peptide antibodies recognising scavenger receptors confirmed the presence of these proteins in atherosclerotic lesions. These studies support the role of scavenger receptors in foam cell and atheroma formation. The role of scavenger receptors in xanthoma formation in patients has also recently been investigated. Xanthomas are subcutaneous lipid laden cells. Giry *et al.* (1996) identified subjects with subcutaneous xanthomas despite having normal serum lipid levels. Studies revealed a genetic abnormality in scavenger receptor expression in these subjects since scavenger receptor mRNA levels in their monocytes and macrophages were several fold higher than control subjects. This elevation in mRNA levels was also associated with an increased degradation of labelled LDL by monocytes isolated from these subjects. This study highlights the role scavenger receptors may have in xanthoma formation.

In summary, scavenger receptors type I and II in macrophages have been cloned and have been shown to bind oxidised and chemically altered LDL in culture. Furthermore receptor types that bind specifically oxidised LDL have been identified as Fcγ-RII-B2 and CD36 receptors. Scavenger receptors display a very broad ligand specificity that includes recognition of many polyanionic compounds. The precise role that scavenger receptors perform *in vivo* is not fully understood but is likely to involve foam cell formation in atherosclerosis and host defense functions particularly in the removal of bacterial endotoxins (reviewed by Krieger *et al.* 1993). While there is evidence to support the participation of

macrophage scavenger receptors in foam cell formation and atherosclerosis, the evidence for LDL oxidation *in vivo* has yet to be discussed.

1.5 EVIDENCE FOR *IN VIVO* OXIDATION OF LDL AND ITS ROLE IN ATHEROSCLEROSIS

Initial evidence that lipid peroxidation occurs *in vivo* emerged from studies which used immunostaining techniques to detect the presence of modified LDL in WHHL rabbit atherosclerotic lesions (Haberland *et al.* 1988, Palinski *et al.* 1989, Rosenfeld *et al.* 1990). Oxidised LDL is immunogenic and monoclonal and polyclonal antibodies can be raised to specific epitopes present in oxidised LDL including malondialdehyde-lysine, 4-hydroxynonenal-lysine and mouse monoclonal antibody to protein fragments of copper-oxidised LDL. These antibodies have been used to immunostain histological sections of aorta from WHHL rabbits (Watanabe Heritable Hyperlipidemic rabbits, a rabbit deficient in LDL receptors), and show the presence of oxidised LDL in atherosclerotic lesions. Immunocytochemical studies in apo E deficient transgenic mice, a model of hyperlipidemia and atherosclerosis, have found autoantibodies against epitopes of modified LDL in both atherosclerotic lesions and serum (Palinski *et al.* 1994). In experiments conducted by Yla-Herttuala *et al.* (1989), LDL extracted from human and rabbit atherosclerotic lesions, displayed many of the physicochemical and biological properties of *in vitro* oxidised LDL, such as increased electrophoretic mobility, increase in particle density, fragmentation of apo B, increase chemotaxis for monocytes and increased degradation of LDL by macrophages. Furthermore the apo B from lesion LDL reacted with antisera which recognised modified LDL adducts.

In human clinical studies, there have also been several reports of an increased susceptibility of LDL to copper-mediated oxidation in patients with progressive coronary atherosclerosis or history of coronary disease (Cominacini *et al.* 1993, Regnstrom *et al.* 1992, Liu *et al.* 1992 and De Rijke *et al.* 1992), however no differences in LDL oxidation in patients with peripheral atherosclerosis and matched controls was observed by Zieden *et al.* 1992.

Other investigators have demonstrated an association between oxidised LDL and the progression of atherosclerosis by comparing titres of autoantibodies against epitopes of oxidised LDL in subjects with atherosclerosis. A positive correlation between autoantibodies titres to oxidised LDL and atherosclerosis, as assessed by intimal:medial thickness of the artery measured by ultrasound scanning was demonstrated by Salonen *et al.* (1992). Patients with CAD confirmed by coronary angiography also displayed increased autoantibody titres to oxidised LDL when compared to patients with normal coronary angiograms (Bui *et al.* 1996). Malondialdehyde-modified LDL levels measured by a specific murine monoclonal antibody are increased in patients with myocardial infarction and carotid atherosclerosis (Holvoet *et al.* 1995). A murine model of hyperlipidemia and atherosclerosis used by Palinski *et al.* (1995) provided further evidence of the role of *in vivo* LDL oxidation in atherogenesis by displaying a significant correlation between autoantibody titre and lesion development. However in humans, a 10 year follow up study conducted in non-insulin-dependent diabetes did not find an association between autoantibody titre and intimal:medial thickness of the carotid artery, despite the fact that these patients had a much greater frequency of all cardiovascular events when compared to normal subjects (Uusitupa *et al.* 1996). Although autoantibodies against oxidised LDL were detected in serum, indicating the presence of oxidised LDL *in vivo*, they were not predictive of carotid atherosclerosis in these diabetes patients.

The immunostaining results above confirm the presence of lipid peroxidation products in atherosclerotic lesions and serum but do not specifically indicate the presence of oxidised LDL. This is reinforced by O'Brien *et al.* (1996) who has recently shown that epitopes recognised by autoantibodies to oxidised LDL are also present on non-apo B containing proteins such as human serum albumin and apo A1 (the apolipoprotein present in HDL). These findings indicate that increases in autoantibody titres to malondialdehye-lysine and hydroxynonenal-lysine adducts may not be indicative of increased LDL oxidation epitopes, and equally so, reductions in autoantibody titres may not be due only to a decrease in LDL oxidation.

Other investigators have isolated oxidised free and esterified polyunsaturated fatty acids from atherosclerotic rabbit and human arteries (Wang *et al.* 1991, Kuhn *et al.* 1994, 1992, Carpenter *et al.* 1993, Chisolm *et al.* 1994, Suarna *et al.* 1995). Although there is some concern regarding post-mortem oxidation of lipids in these types of studies, lipid peroxidation products are consistently higher in diseased arteries compared to normal arteries isolated from humans.

In summary, a review of the present literature does provide evidence of *in vivo* LDL oxidation by immunocytochemical techniques and identification of oxidised fatty acid derivatives in atherosclerotic lesions. In patients with coronary disease there is also an increased oxidisability of LDL which suggest that LDL oxidation is related to the development and progression of atherosclerosis.

1.6 ATHEROGENICITY OF OXIDISED LDL: *IN VITRO* EXPERIMENTS

In vitro, oxidised LDL has been shown to exhibit a range of biological properties that may also promote atherogenesis. It has been demonstrated that oxidised LDL can stimulate the recruitment of human monocytes and adhesion to endothelial cells in culture (Quinn *et al.* 1987, Berliner *et al.* 1990). The factor thought to be responsible for monocyte chemotactic activity and adhesion is lysophosphatidylcholine which is present in the lipid phase of oxidised LDL (Quinn *et al.* 1988, Kume *et al.* 1992). Oxidised LDL has also been shown to stimulate the release of monocyte chemotactic protein from endothelial cells *in vitro*, promoting the migration of monocytes (Cushing *et al.* 1990).

In the subendothelial space monocytes undergo phenotypic changes forming macrophages which express increased numbers of scavenger receptors (Fogelman *et al* 1981). In culture, oxidised LDL stimulates the release of factors from endothelial cells which inhibit the mobility of resident macrophages which, in the *in vivo* situation, may lead to the accumulation of macrophages in the intima (Quinn *et al.* 1985). Elevated plasma LDL levels can also lead to an influx of LDL into the artery wall (Nordestgaard *et al.* 1994). Here, LDL can bind to elastin and collagen in the extracellular matrix resulting in an increased residency

time in the intima layer (Podet *et al.* 1991 and Kalant *et al.* 1991). Macrophages, along with endothelial and smooth muscle cells, could promote the oxidative modification of LDL as observed *in vitro*.

Activated macrophages also secrete cytokines (interleukins) and mitogens (platelet derived growth factor and tumour necrosis factor-α). These factors may contribute to the atherogenic process by stimulating smooth muscle cell proliferation (Ku *et al.* 1992). Other studies have reported cytotoxicity of oxidised LDL to endothelial and smooth muscle cells in culture which could also contribute to endothelial damage (Hessler *et al.* 1979, Henriksen *et al.* 1979, Morel *et al.* 1984).

The oxidative modification hypothesis of atherosclerosis proposes that the lipid peroxidation of LDL is an important event in atherogenesis. It has been demonstrated that uptake and degradation of oxidised LDL by macrophages in the intima contributes to foam cell formation and the development of fatty streaks which is an important initiating event in atherosclerosis (Steinberg *et al.* 1989).

In summary, the aetiology of atherosclerosis is a very complicated process involving many cellular interactions with oxidised LDL, cytokines and mitogens. The atherogenicity of all these factors remains to be elucidated. It is recognised however that the formation of oxidised LDL does occur *in vivo* and uptake of oxidised LDL by macrophage scavenger receptors leads to the formation of foam cells which is an important event in the atherogenic process.

Another important occurrence relevant to hypercholesterolemia, oxidised lipoproteins and atherosclerosis in humans and animals is vascular dysfunction. The next section of this chapter introduces endothelium-dependent aortic relaxation and the effect of elevated plasma cholesterol, atherosclerosis and oxidised LDL on vascular function

1.7 ENDOTHELIUM-DEPENDENT RELAXATION

Arteries are composed of 3 concentric layers of tissue which contain varying amounts of smooth muscle cells, connective tissue, elastin and collagen. The first of these layers on the luminal side of the vessel is the tunica intima. Lining the intima is a layer of endothelial cells. The middle layer is termed the tunica media and the outer layer the tunica adventitia.

The endothelial layer is important in regulating vascular tone or smooth muscle contractility. Vascular endothelial cells release a variety of vasoactive substances some of which mediate vessel relaxation termed endothelium-derived relaxation factors (EDRFs) and some which induce endothelium-derived contracting factors (EDCFs). The most common EDRFs are nitric oxide, acetylcholine, prostacyclin (prostaglandin I_2) and endothelium-derived hyperpolarising factor. While the polypeptide, endothelin, and certain eicosanoid metabolites, thromboxane A_2 and prostaglandin H_2, are examples of EDCFs.

1.7.1 Hypercholesterolemia, atherosclerosis and endothelium-dependent relaxation

It is widely documented that in hypercholesterolemic and atherosclerotic conduit vessels there is a decreased responsiveness to endothelium-dependent vasodilators such as acetylcholine (Jayakody *et al.* 1987, Bossaller *et al.* 1987, Verbeuren *et al.* 1986). Normally, acetylcholine acting via muscarinic receptors on endothelial cells, mediates the synthesis and release of endothelium-dependent relaxing factor (EDRF) (Furchgott and Zawadzki 1980) which has been identified as the free radical, nitric oxide (Moncada *et al* 1988). Nitric oxide is synthesised from the amino acid precursor *L*-arginine by an enzyme catalysed reaction. *L*-arginine is converted to a hydroxy intermediate which is oxidised to form *L*-citrulline and nitric oxide (Stuehr *et al.* 1991). The enzyme responsible for this conversion is nitric oxide synthase. There are a number of different isozymes of this enzyme which are classed as either constitutive (endothelial nitric oxide synthase and neuronal nitric oxide synthase) or inducible nitric oxide synthase. The later isozyme is present mainly in macrophages and its activity is calcium independent whereas the constitutive forms of the enzyme are calcium dependent and are activated in response to a receptor agonist interaction

and transduction mechanism (reviewed by Di Rosa *et al.* 1996). Nitric oxide diffuses from the endothelial cells into the smooth muscle layer of the blood vessel where it stimulates soluble guanylate cyclase activity and increases cytoplasmic guanosine 3' 5' monophosphate (cGMP) resulting in vascular smooth muscle relaxation (Lowenstein *et al.* 1994).

In organ bath experiments it has been demonstrated that acetylcholine induces a concentration-dependent relaxation in precontracted aortic segments from healthy animals (Jayakody *et al.* 1987, Bossaller *et al.* 1987, Verbeuren *et al.* 1986). Atropine inhibits acetylcholine mediated relaxation of aortic rings, confirming the involvement of muscarinic receptors (Jayakody *et al.* 1987). This relaxation is endothelium-dependent since relaxation to acetylcholine is absent in vessels that have had the endothelial layer removed. However, in atherosclerotic aortic segments, acetylcholine-mediated relaxation is impaired and cGMP levels are decreased (Jayakody *et al.* 1987, Bossaller *et al.* 1987, Verbeuren *et al.* 1986, Habib *et al.* 1986). This impairment is not due to a loss of endothelial cells as shown by histological techniques but is due to a selective impairment of the nitric oxide pathway.

Studies investigating endothelium-dependent relaxation have used a number of different agents which mediate vasodilatation in an attempt to elucidate the mechanism involved in relaxation. Rabbits fed an atherogenic diet for 10 weeks display a decrease in acetylcholine-mediated relaxation in aortic strips precontracted with phenylephrine (Bossaller *et al.* 1987). Similarly, other endothelium-dependent relaxing agents acting via endothelial surface receptors, such as substance P and histamine, displayed only partial relaxation of aortic strips in hypercholesterolemic rabbits compared to control groups, whilst the calcium ionophore A23187 and the direct acting smooth muscle relaxant nitroglycerin displayed no impairment of the relaxation response (Bossaller *et al.* 1987). Calcium ionophores mediate relaxation by causing an influx of calcium into endothelial cells which activates the constitutive form of the nitric oxide synthase and synthesis and release of EDRF, a process which is independent from endothelial receptors (Dusting *et al.* 1995). Nitrovasodilators are metabolised to nitric oxide in smooth muscle cells and are therefore endothelium-independent relaxation factors.

The impairment of endothelium-dependent relaxation in atherosclerotic aorta has been linked to any number of steps in the nitric oxide pathway including perturbation in the receptor-agonist interaction on endothelial cells, an inhibition of nitric oxide synthase or an increased degradation of nitric oxide (reviewed by Dusting *et al.* 1995). Other authors claim that a lack of endothelium-dependent relaxation is not due to a decrease in nitric oxide synthase activity or nitric oxide formation (Minor *et al.* 1990, Schmidt *et al.* 1991). However, there is general agreement that an impairment in vasorelaxation is not due to changes in contractile properties of smooth muscle since responses to nitrosylated compounds are maintained.

Impaired relaxation to acetylcholine in aortae from hypercholesterolemic and atherosclerotic rabbits has been observed despite an increased release of nitrosylated compounds in these aortas (Minor *et al.* 1990). This finding is consistent with an increased degradation of nitric oxide since the increased nitric oxide production did not mediate relaxation. Superoxide anion is involved in EDRF degradation at least *in vitro* (Gryglewski *et al.* 1986), while *in vivo*, chronic treatment with superoxide dismutase restores endothelium-dependent relaxation (Mugge *et al.* 1991). Hypercholesterolemia and atherosclerosis are associated with increased free radical activity, as indicated by increased levels of oxidised cholesterol and fatty acid derivatives in plasma (Kinter *et al.* 1994) and in aortic lesions from atherosclerotic humans (Kuhn *et al.* 1992, Carpenter *et al.* 1993, Chisolm *et al.* 1994, Suarna *et al.* 1995) and rabbits (Wang *et al.* 1991, Kuhn *et al.* 1994). Therefore, it is possible that free radicals, which are elevated in these disease states, may play a role in nitric oxide degradation. This is supported by the observation that aorta isolated from cholesterol-fed rabbits display an increased endothelial superoxide anion production (Ohara *et al.* 1993) which could inactivate EDRF.

Impairment of endothelium-dependent relaxation may also be due to a defect in the endothelial surface receptors or the signal transduction mechanisms, probably G-proteins, operating within endothelial cells (Michel *et al.* 1993). Another hypothesis is that the lipid-laden intima layer associated with atherosclerotic lesions prevents endothelium-derived nitric oxide from diffusing into the artery wall where it can mediate smooth muscle relaxation

(Flavahan 1992). There have been reports however, that a loss of endothelial-dependent relaxation precedes atheroma development in rabbits. Rabbits fed a cholesterol diet for 2-4 weeks had attenuated endothelium-dependent relaxation despite the absence of atherosclerosis (Osborne *et al.* 1989, Du *et al.* 1992). Despite this, there is a clear relationship between the progression of atherosclerosis and the degree of impairment of vasorelaxation (Bossaller *et al.* 1987, Verbeuren *et al.* 1986).

1.7.2 LDL and Endothelium-dependent relaxation

LDL, in particular oxidised LDL, has been implicated in vascular EDRF dysfunction. Initial studies reported that high concentrations of native LDL inhibited endothelium-dependent relaxation of rabbit aorta *in vitro* (Andrews *et al.* 1987). Chemical modifications to the lysine and arginine residues on the apo B moiety of LDL, did not cause an attenuation of aortic relaxation. The authors therefore concluded that the apo B molecule, the ligand for the LDL receptor, was somehow implicated in the impairment of endothelium-dependent relaxation. In direct contrast, Kugiyama *et al.* (1990) observed no impairment of native LDL or delipidated LDL on acetylcholine-evoked relaxation in aortae isolated from rats, indicating that the EDRF impairment was protein independent. Interestingly, in the experiments conducted by Kugiyama *et al.* (1990), aorta were precontracted with the selective α_1-adrenoceptor agonist phenylephrine prior to the addition of acetylcholine. When Jacobs *et al.* (1990) contracted aortae with serotonin and noradrenaline they observed an impairment of acetylcholine-induced relaxation in the presence of native LDL, despite a normal concentration-dependent relaxation to acetylcholine in phenylephrine contracted vessels. The reason for this is not known.

Oxidised LDL, in particular the lipid fraction of oxidised LDL, is responsible for impaired endothelium-dependent relaxation to acetylcholine (Kugiyama *et al.* 1990). Lysophosphatidylcholine has been identified as the component of LDL which inhibits EDRF (Kugiyama *et al.* 1990, Yokoyama *et al.* 1990). Lysophosphatidylcholine may inhibit EDRF by mediating an increase in superoxide production in aortic tissue (Ohara *et al.* 1994). Other

mechanisms by which LDL could impair acetylcholine-mediated relaxation could be an influence on nitric oxide synthase. Indeed oxidised LDL, but not native LDL, have been reported to inhibit nitric oxide synthase expression and/or activity in endothelial cells, macrophages, neutrophils and platelets in culture (Liao *et al.* 1995, Yang *et al.* 1994, Metha *et al.* 1995, Chen *et al.* 1996). In addition, a number of other alterations have been proposed to contribute to endothelial dysfunction arising from oxidised LDL *in vitro* including changes to the endothelium pertussis toxin-sensitive G protein transduction pathways (Tanner *et al.* 1991), a decreased availability of *L*-arginine. (Cooke *et al.* 1991) or decrease in soluble guanylate cyclase activity (Schmidt *et al.* 1991).

Although the mechanism(s) responsible for the vascular dysfunction arising from hypercholesterolemia, atherosclerosis and oxidised LDL have not been fully elucidated, it seems likely from this review of the current literature that nitric oxide degradation by free radical species is at least part involved in the impairment of endothelium-dependent relaxation. Convincing evidence to support the theory that oxidative mechanisms are responsible for impaired vascular function and atherosclerosis development come from dietary intervention studies with antioxidants in animal models of hypercholesterolemia and atherosclerosis. In these studies antioxidant therapy restores endothelium-dependent relaxation as well as reducing LDL oxidation and atherosclerosis development. The following sections provide an overview of antioxidants and their protective effects against LDL oxidation, atherosclerosis and endothelium dependent relaxation.

1.8 ANTIOXIDANTS

Halliwell (1990) defined an antioxidant as "any substance that when present at low concentration compared to those of the oxidisable substrate, significantly delays or prevents oxidation of that substrate." In the context of the present discussion the oxidisable substrates are the esterified PUFA and lipids in the LDL particle. Oxidation of these compounds leads to the formation of lipid peroxyl radicals, lipid hydroperoxides and oxysterols.

Antioxidants can be classified in terms of their mechanism of action as either scavengers of

ROS (mannitol, superoxide dismutase, catalase and glutathione), chain-breaking antioxidants (eg α-tocopherol, ascorbic acid, probucol) which react with intermediate radicals such as peroxyl radicals or preventative antioxidants (haem, transferrin, albumin and caeruloplasmin) which act by binding metal ions thereby preventing metal ion-catalysed production of ROS. Antioxidants are also distinct in terms of their lipid solubility. LDL particles contain a number of lipophilic antioxidants which are sequestered into the core of the lipoprotein. These antioxidants in circulating LDL include vitamin E isomers (α, λ and β-tocopherol), β-carotene, ubiquinol-10 and lycopene. Other more water-soluble antioxidants like ascorbic acid and uric acid are present in plasma and are capable of preventing the oxidation of circulating LDL (Esterbauer *et al.* 1991). *In vitro* experiments reveal that plasma is a very potent inhibitor of LDL oxidation (Steinberg *et al.* 1989).

It is widely accepted that the accumulation of atherogenic lipoproteins in the artery wall is involved in atherogenesis and that this arterial influx is largely dependent on the concentration of circulating lipoproteins and the permeability of the arterial wall (reviewed by Nordestgaard *et al.* 1994). An elevated plasma and LDL-cholesterol concentration increases the influx of lipoproteins into the arterial wall. Some have proposed that an environment of high oxidant stress, such as the arterial wall, is where the oxidation of LDL is likely to occur (Steinberg *et al.* 1989, Dieber-Rotheneder *et al.* 1991). Consequently, the lipophilic antioxidants incorporated within LDL are probably more important in protecting LDL from lipid peroxidation in the vessel wall. Therefore, an important requisite in the theory that oxidised LDL promotes atherosclerosis, is that the oxidation of LDL occurs in the sub-endothelial space where lipoproteins are sequestered from the water-soluble antioxidants in plasma (Steinberg *et al.* 1989 and Esterbauer *et al.* 1991). Recently Suarna *et al.* (1995) published data revealing that α-tocopherol levels are actually increased in human atherosclerotic arteries despite the increase in oxidised lipids in these tissues. These results indicate that the artery wall does in fact have extensive antioxidant defences, mainly in the form of vitamin E and C. These authors question the notion that oxidation of LDL occurs in

the artery on the basis of the high intimal antioxidant concentrations even in atherosclerotic tissue.

1.8.1 Epidemiological studies: Antioxidants and CAD

Evidence for the protective role of antioxidants towards CAD is extrapolated from large scale epidemiological studies which have demonstrated an inverse relationship between consumption of dietary antioxidants and the incidence of CAD (Stampfer *et al.* 1993 and Rimm *et al.* 1993). In large scale prospective studies, men with a vitamin E intake of > 60 IU/day had a 36% lower relative risk of CAD. Similarly, women grouped into the highest quintile of vitamin E intake (median 208 IU/day) had a 34% reduced risk of coronary disease. In a cross-sectional study of 16 cohorts an inverse relationship between plasma vitamin E levels and ischaemic heart disease was observed (Gey *et al.* 1991) while the Basel Prospective Study (Gey *et al.* 1993) reported a significantly increased risk of ischaemic heart disease and stroke in patients with low plasma concentrations of β-carotene and vitamin C. A smaller epidemiological study in 4 populations did not find a relationship between vitamin E and C intake and incidence of CAD (Riemersma *et al.* 1990).

In addition to antioxidant status, indices of lipid peroxidation have been studied with respect to carotid atherosclerosis (Bonithon-Kopp *et al.* 1997). The Etude sur le Viellisement Arteriel (EVA Study), a 4 year longitudinal study in elderly French subjects, observed a significant inverse relationship between erythrocyte vitamin E levels and atherosclerosis as measured by ultrasound determinations of intimal:medial thickness of carotid arteries. In male subjects with carotid atherosclerosis, plasma levels of lipid peroxidation, as measured by TBARS, were higher than in subjects without carotid plaques although no association between TBARS and intimal:medial thickness was observed overall.

The epidemiological data supporting an inverse relationship between antioxidants and CAD warrants further research into the therapeutic role of dietary antioxidants in CAD protection.

1.8.2 *In vitro* studies: Antioxidants and LDL oxidation

The *in vitro* oxidation of LDL by free radical-mediated techniques has become the standard model for investigating oxidation, atherosclerosis and antioxidants. Pioneering studies by Esterbauer *et al.* (1989a, 1989b and 1991) used the redox active copper to mediate the oxidation of esterified PUFAs in LDL. An early measurement of lipid peroxidation is the formation of conjugated dienes which absorb ultraviolet light at a wavelength of 234 nm. By constructing an absorbance (234 nm) versus time profile of this copper-mediated reaction, Esterbauer and coworkers (1989b) were able to demonstrate lipid peroxidation in LDL. The absorbance profiles were characterised by an initial lag phase in which there was no change in absorbance at 234 nm, followed by a steep increase in absorbance characterised by the formation of conjugated dienes. Esterbauer *et al.* (1989a, 1989b) concluded that the initial lag phase was the period in which antioxidants, in particular α-tocopherol, were protecting LDL from oxidation since studies demonstrated that rapid metal- and cell-mediated oxidation of LDL occurred on depletion of α-tocopherol, lycopene and carotene from LDL (Esterbauer *et al.* 1989a, 1989b, Jessup *et al.* 1990).

α-Tocopherol has become the subject of much research since it is quantitatively the most important lipophilic antioxidant and is one of the most reactive peroxy radical scavengers in LDL (Bowry *et al.* 1995). The main evidence of α-tocopherol's antioxidant properties emerged from studies in which plasma and LDL were supplemented with vitamin E (Jessup *et al.* 1990, Dieber-Rotheneder *et al.* 1991, Esterbauer *et al.* 1991, Jialal *et al.* 1992, Suzukawa *et al.* 1994). Vitamin E supplementation results in elevated LDL α-tocopherol concentrations which are associated with decreased oxidation of LDL in the presence of prooxidants such as copper or cells. Furthermore, lipid hydroperoxide formation is inhibited during the lag phase of copper-mediated oxidation. This observation led authors to conclude that α-tocopherol is an antioxidant which inhibits lipid peroxidation by trapping lipid peroxy radicals. Despite this observation, α-tocopherol levels in LDL generally do not correlate with

LDL's susceptibility to oxidation (Jessup *et al.* 1990, Esterbauer *et al.* 1992, Kleinveld *et al.* 1992, Croft *et al.* 1995). This observation does not support the theory that α-tocopherol is an antioxidant and protects against LDL oxidation.

In contrast to an antioxidant action, Bowry *et al.* (1992) have proposed a prooxidant action of α-tocopherol during *in vitro* oxidation of LDL with low concentrations of thermolabile azo-compounds which produce slow fluxes of aqueous peroxy radicals. This mechanism is referred to as tocopherol mediated peroxidation (TMP) (reviewed by Bowry and Stocker 1993, Stocker 1994). This TMP mechanism was developed primarily as a model to explain anomalies between concentrations of azo compounds and formation of lipid hydroperoxides. Low concentrations of azo compounds produce slower fluxes of peroxy radicals which result in elevated lipid hydroperoxide formation in the presence of α-tocopherol (Bowry *et al.* 1992). During this azo-initiated oxidation, α-tocopherol is oxidised by peroxy radicals forming α-tocopheroxy radicals within the LDL particle. The α-tocopheroxy radical in this situation can be either reduced back to α-tocopherol by coantioxidants in LDL such as ubiquinol-10, or may react with bisallylic carbons within PUFA forming lipid peroxy radicals which initiate lipid peroxidation. It is via the latter mechanism that α-tocopherol acts as a prooxidant. These authors (Bowry *et al.* 1992, Stocker 1994) suggest that coantioxidants present in LDL such as ubiquinol-10, which act as tocopheroxy reductants, are more important in inhibiting LDL oxidation than the levels of α-tocopherol.

These observations have been supported by Kontush *et al.* (1996a). Under strong oxidative conditions (high concentrations of copper or azo compounds), α-tocopherol supplementation decreased LDL and plasma oxidation. Under mild oxidative conditions an antioxidant effect in α-tocopherol supplemented plasma was observed only in the presence of coantioxidants

such as ascorbate. A prooxidant effect of α-tocopherol in isolated LDL or diluted plasma (low levels of coantioxidants), under mild oxidative conditions was also observed. These observations indicate that the level of oxidative stress and presence of coantioxidants are important determinants of α-tocopherol's antioxidant or prooxidant activity, since physiological levels of coantioxidants are required to reduce α-tocopheroxy radicals.

The discussion on antioxidants so far has focused mainly on the mechanisms involved in *in vitro* oxidation model. Other factors are also important in determining LDL oxidisability, such as the types and concentration of antioxidants and the PUFA content of LDL. There are an overwhelming number of reports in the literature describing antioxidant properties of natural and synthetic compounds. Probucol, BHT and a variety of naturally occurring polyphenols make up part of the ever-growing list of compounds that are capable of inhibiting LDL oxidation *in vitro*. Generally the oxidisability of LDL is tested in a prooxidant system which uses transition metal ions, vascular cells (endothelial cells, smooth muscle cells and macrophages) or azo-initiators to generate peroxy or other free radicals. Although the mechanisms of *in vitro* LDL oxidation and antioxidants activity are not always well understood, the above methods provide a simple and rapid model with which to screen potential antioxidant compounds.

1.8.3 Animal studies: Antioxidants, LDL oxidation and atherosclerosis

The involvement of oxidised LDL in atherogenesis is also supported by data from animal studies where the progression of atherosclerotic lesions in hypercholesterolemic rabbits was reduced when diets were supplemented with vitamin E (Williams *et al.* 1992), BHT (Bjorkhem *et al.* 1991) or probucol (Kita *et al.* 1987, Carew *et al.* 1987, Daugherty *et al.* 1989, Mao *et al.* 1991, Nagano *et al.* 1992). The prevention of atheroma development in a few cases was associated with a mild hypocholesterolemic effect (Williams *et al.* 1992, Morel *et al.* 1994, Kita *et al.* 1987, Mao *et al.* 1991), a reduced susceptibility to copper-mediated LDL oxidation (Mao *et al.* 1991, Kita *et al.* 1987, Williams *et al.* 1992, Wiseman *et*

al. 1995), decreased synthesis of cholesteryl ester in cultured macrophages (Kita *et al.* 1987, Mao *et al.* 1991) and a lower fractional catabolic rate of LDL degradation in aortic atherosclerotic lesions of probucol treated rabbits compared to control (Carew *et al.* 1987). In contrast, the synthetic antioxidant BHT produces a moderate hypercholesterolemic response whilst still decreasing plasma levels of cholesterol autoxidation products (7-ketocholesterol and 5α, 6α-epoxide) and decreasing the progression of atheroma in cholesterol-fed rabbits (Bjorkhem *et al.* 1991). Furthermore, Mao *et al.* (1991) proposed that the anti-atherogenic effect of probucol was independent of any hypercholesterolemic action since studies with a probucol analogue, which exhibited antioxidant activity but had no cholesterol-lowering activity, was effective in reducing the number and severity of atherosclerotic lesions in cholesterol-fed rabbits. Based on these observation and the known ability of vitamin E and probucol to inhibit copper- and cell-mediated LDL oxidation *in vitro* (Steinbrecher *et al.* 1984, Parthasarathy *et al.* 1986b), the anti-atherogenic effects in rabbits was attributed to the antioxidant properties of these compounds.

There are however some reports in the literature that do not find anti-atherosclerotic effects following dietary antioxidant supplementation. Stein *et al.* (1989) reported no anti-atherogenic effect of probucol as measured by the incorporation of tritiated cholesteryl linoleyl ether into the aorta of cholesterol-fed rabbits. In WHHL rabbits, vitamin E supplementation in combination with monounsaturated or polyunsaturated fatty acids (oleic and linoleic), did not attenuate the development of atherosclerosis (Kleinveld *et al.* 1995). This study, and an independent study by Morel *et al.* (1994), demonstrated that vitamin E supplementation produced a decrease in copper-mediated LDL oxidation that was not associated with an anti-atherogenic effect. These results do not support the hypothesis that LDL oxidation promotes atherosclerosis at least in the hypercholesterolemic rabbit. Despite these few reports, an overwhelming number of studies have found a therapeutic role for antioxidants in the prevention of atherosclerosis in animal models of hypercholesterolemia.

1.8.4 Human studies: Antioxidants, LDL oxidation, atherosclerosis and CAD

Supplementation of antioxidant vitamins in human volunteers causes an increase in both plasma and LDL concentrations of these antioxidants, which mediate an inhibitory effect against copper-mediated LDL oxidation *in vitro* (Dieber-Rotheneder *et al.* 1991, Jialal *et al.* 1990 and 1992, Reaven *et al.* 1993, Abbey *et al.* 1993). In addition to inhibiting LDL oxidation, there have been some reports in the literature documenting the effect of antioxidants on atherosclerosis and myocardial infarction. Carotid atherosclerosis regression, determined by changes in ultrasound measurements of carotid stenosis, was evident in seven out of twenty five patients receiving daily supplements of gamma-tocotrienol and α-tocopherol derived from palm oil, while no atherosclerosis regression was observed in untreated subjects (Tomeo *et al.* 1995). Carotid atherosclerosis progression was also reduced in placebo-control subjects participating in the Cholesterol Lowering Atherosclerosis Study (CLAS) following antioxidant vitamin intervention (Azen *et al.* 1996). Dietary intervention with antioxidants either as supplements or antioxidant rich foods such as fruits, vegetables, pulses and nuts to patients with suspected myocardial infarction improved plasma vitamin levels, decreased plasma lipid peroxide and protected against cardiac necrosis (Singh *et al.* 1995, 1996).

Randomised, large, placebo-controlled intervention studies have documented the effect of antioxidants on atherosclerosis and myocardial infarction. A number of these studies include the Atherosclerosis Risk in Communities Study (Kritchevsky *et al.* 1995), the Heart Antioxidant Study (Stephens *et al.* 1996) and the Alpha Tocopherol, Beta Carotene Cancer Prevention Study (Rapola *et al.* 1996). The general outcome of these studies is that antioxidant vitamins are associated with a lower incidence of atherosclerosis and clinical events arising from CAD.

Whilst many intervention studies have been performed with dietary vitamins, studies have also shown beneficial effects of synthetic antioxidants on atherosclerosis and CAD. In FH patients, probucol induced a partial regression of cutaneous and tendon xanthomas

(Yammato *et al.* 1983). Interestingly, this effect was not correlated with the degree of cholesterol-lowering in patients, suggesting that a mechanism distinct from hypocholesterolemia was responsible for the antiatherogenic outcome following probucol treatment. Patients receiving therapeutic doses of probucol have decreased plasma lipid peroxide levels (Paterson *et al.* 1992) and LDL isolated from probucol-treated patients is more resistant to *in vitro* oxidation when compared to normocholesterolemic and hypercholesterolemic control subjects (Parthasarathy *et al.* 1986b, Regnstrom *et al.* 1990).

One of the largest case control studies investigating the effect of antioxidants on atherosclerosis was The Probucol Quantitative Regression Swedish Trial. In this intervention trial the effect of probucol on the development of femoral atherosclerosis was investigated. The outcome of this study was that probucol lowered plasma and LDL-cholesterol, and decreased LDL oxidation but failed to produce an anti-atherogenic effect in hypercholesterolemic patients (Regnstrom *et al.* 1996, Walldius *et al.* 1994). The reasons for this lack of atherogenic effect are not known. It appears that probucol can decrease LDL oxidation but this is not sufficient to bring about a beneficial effects toward atherosclerosis. The authors propose a number of factors to explain why probucol was ineffective in decreasing atherosclerosis which are largely to do with study design, subject selection criteria, coadministration of lipid lowering drugs and lowering of HDL-cholesterol. Despite the lack of effect in patients with peripheral atherosclerosis, probucol was effective in preventing coronary restenosis in angioplasty patients (Lee *et al.* 1996).

1.8.5 Antioxidants and endothelium-dependent relaxation

Endothelium-dependent relaxation can be restored in models of hypercholesterolemia and atherosclerosis by dietary supplementation with L-arginine (Drexler *et al.* 1991), fish oils (Chin *et al.* 1994), cholesterol-lowering therapies (Osborne *et al.* 1989, Leung *et al.* 1993) and antioxidants such as superoxide dismutase (Mugge *et al.* 1991), vitamin E (Stewart-Lee *et al.* 1994, Keaney *et al.* 1993, Matz *et al.* 1994, Klemsdale *et al.* 1994, Andersson *et al.* 1994), β-carotene (Keaney *et al.* 1993) and probucol (Simon *et al.* 1993, Keaney *et al.*

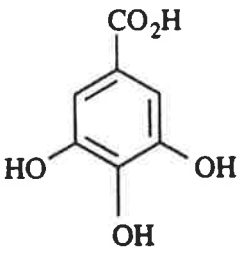
1995). Studies employing *in vivo* photoplethsmography measurements (Klemsdale *et al.* 1994) and *in vitro* organ chamber experiments have demonstrated that dietary antioxidants such as vitamin E can restore endothelium-dependent relaxation in aorta (Keaney *et al.* 1994), coronary circulation (Andersson *et al.* 1994), ear microcirculation (Matz *et al.* 1994) and carotid artery (Stewart-Lee *et al.* 1994) from hypercholesterolemic rabbits. These results were not associated with a hypocholesterolemic effect of vitamin treatment, but were associated with increased plasma vitamin E concentrations and inhibition of VLDL and LDL oxidation. Interestingly the relaxation responses to the calcium ionophore, A23187 were not decreased in cholesterol-fed rabbits. However in one study vitamin E supplementation actually increased relaxation to A23187 (Stewart-Lee *et al.* 1994). Collectively these investigations provide evidence that antioxidants can restore endothelium-dependent relaxation and some authors suggest that this is achieved by inhibiting LDL oxidation or nitric oxide degradation, both of which are free radical/reactive oxygen mediated processes.

1.8.6 Polyphenolic antioxidants

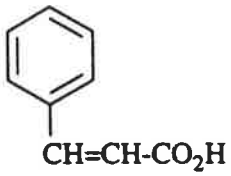
Since it has been recognised that specific dietary components can modify LDL oxidation, the role of antioxidants in the prevention of atherosclerosis and CHD has been investigated. Compounds of interest in this review are the plant-derived phenols. Naturally occurring phenols generally contain more than one hydroxy group and are commonly referred to as polyphenols. Polyphenols can be broadly grouped into two types either flavonoid or non-flavonoid phenols. Flavonoids include a large group of compounds namely flavonols (kaempferol, quercetin and myricetin), flavan-3-ols (catechin, epicatechin) and isoflavones (genistein and daidzein) which are all derived from simple polyphenolic structures with varying degrees of hydroxylation. The general flavonoid structure shown in Figure 1.1 consists of a 3 ring structure with differing types and numbers of substitutions (hydroxylation, hydrogenation, methylation and glycosylation). The main class of non-flavonoid phenolics are the phenolic acids. Some types of phenolic acids are hydroxycinnamic acids and their hydroxylated derivatives (caffeic acid, p-coumaric acid and ferulic acid) and benzoic acids (gallic acid).

Figure 1.1 Chemical structures of non-flavonoid phenolics (phenolic acids) and flavonoids (flavonols, flavan-3-ols, anthocyanidins and isoflavones).

Phenolic acids (non-flavonoid)

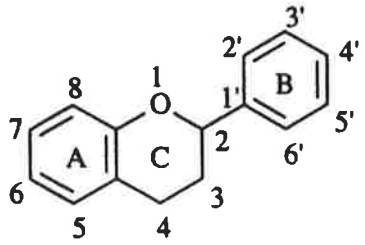


Gallic acid



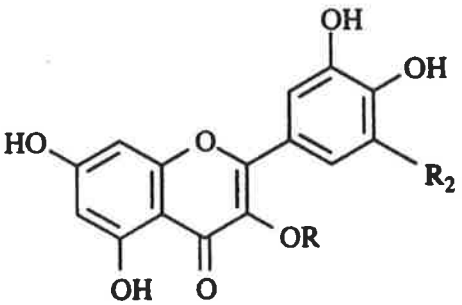
Cinnamic acid

General flavonoid structure

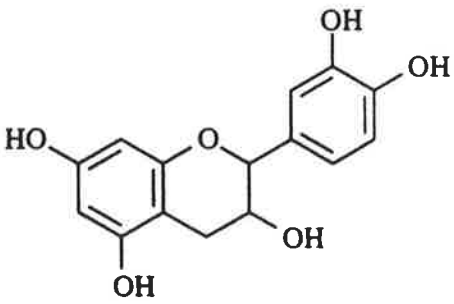


Flavonols

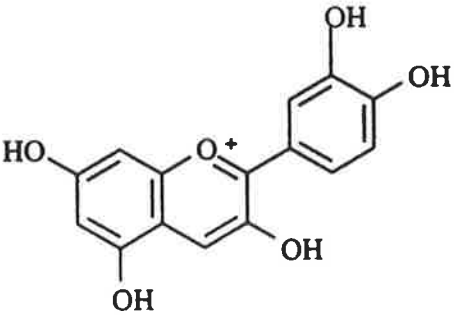
Quercetin	R = H	
Myricetin	R = H, R_2 = OH	
Rutin	R = *D*-Glucose-*L*-Rhamnose	



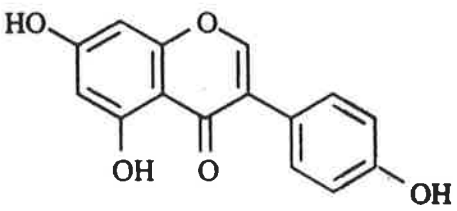
Flavanol-3-ols Catechin



Anthocyanidins



Isoflavones Genistein



A common property of most polyphenolic compounds is their antioxidant activity in a number of prooxidant models. These polyphenolic compounds can scavenge free radicals by donating a phenolic hydrogen group to inactivate free radical species. Oxidation of polyphenols leads to the formation of phenoxide ions that contain an oxygen atoms with unpaired electrons. Delocalisation of the electron to a carbon atom stabilises this compound which may then react with other radicals forming C—C bonds or C—O bonds. In this way oxidation of phenols leads to the formation of polymeric products (Waterman and Mole 1994). Polyphenols also have a strong affinity for divalent ions such as Cu (II) and Zn (II) and may exert antioxidant activity by binding these transitional metal ions which could otherwise initiate metal-catalysed oxidations.

A substantial amount of literature details the antioxidant activity of individual polyphenols in scavenging reactive oxygen species such as superoxide anion (Takahama *et al.* 1985, Robak *et al.* 1988), hydroxy radicals (Husain *et al.* 1987), peroxy radicals (Torel *et al.* 1986), inhibiting lipooxygenase-dependent linoleic acid peroxidation and inhibiting lipid peroxidation in human erythrocytes, rat liver microsomes and mitochondria (Afanas'ev *et al.* 1989). Hanasaki *et al.* (1994) found a good correlation between reactive oxygen scavenging (hydroxy radical and superoxide anion) and antioxidant effects toward peroxidation of methyl linoleate by some flavonoids. Interestingly other flavonoids (quercetin, morin and myricetin) increased hydroxy radical production in the Fenton system and exhibited only weak superoxide scavenging effects but displayed a strong antioxidant effect on lipid oxidation. These results indicate that polyphenolic flavonoids are capable of scavenging free radicals in *in vitro* systems but the mechanism of antioxidant activity is not fully understood.

Comprehensive reviews have elucidated the structure-antioxidant activity relationship of flavonoid polyphenolics (Rice-Evans *et al.* 1996, van Acker *et al.* 1996). Antioxidant activity is attributed to a few critical components in the chemistry of flavonoid structure. Hydroxylation at position 3 of the C ring, C=C bonds between positions 2 and 3 of the C ring, C=O (carbonyl group) on position 4 of the C ring and the number and pattern of hydroxylation on the A and B rings confers antioxidant activity (Figure 1.1) (Rice-Evans *et*

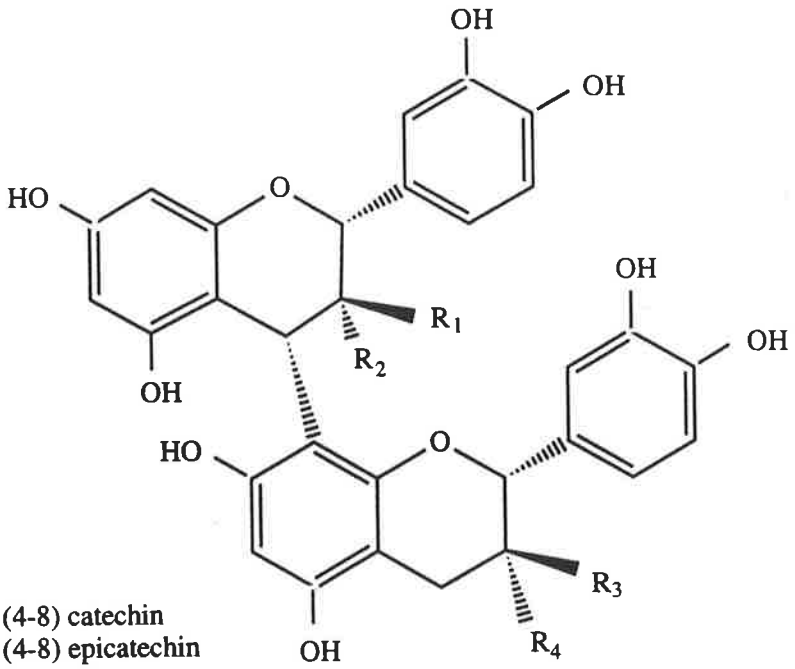
al. 1996). The presence of C4 carbonyl group and hydroxy groups at positions 3 and 5 of the C ring increase the ability of polyphenols to sequester metal ions. Whereas the presence of sugar moieties impede or sterically hinder adjacent hydroxy groups and therefore decrease the compounds antioxidant activity.

Studies by DeWhalley *et al.* (1990) and Mangiapane *et al.* (1992) have investigated the antioxidant activity of flavonoids towards LDL oxidation. Flavonoids (1 to 30 μmol/L) inhibited copper- and cell-mediated LDL oxidation as measured by decreases in lipid hydroperoxide and degradation of labelled LDL by macrophages. These authors proposed that flavonoids may be useful natural anti-atherosclerotic components of the diet.

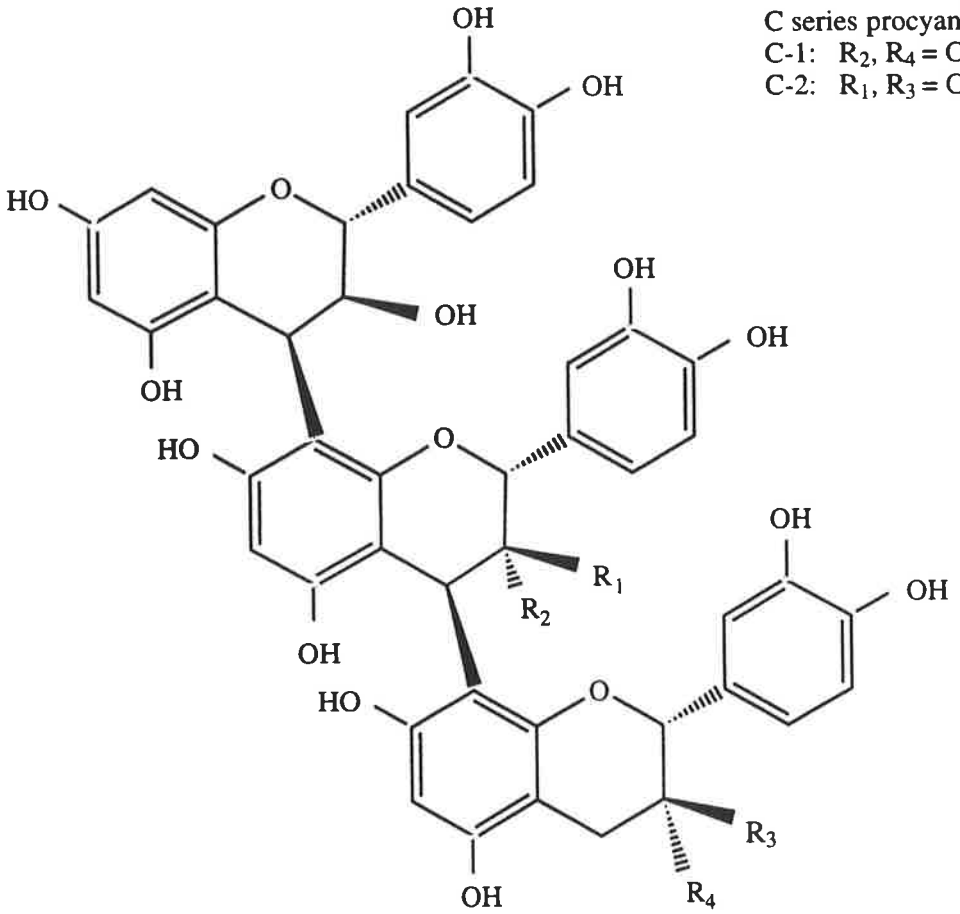
1.9 RED WINE POLYPHENOLICS

Red wine is an abundant source of dietary polyphenols. Red wine contains approximately 1 to 3 g phenols/L which are extracted from *Vitis vinifera* grapes during wine preparation (Singleton 1988). Grapes contain approximately 4 g phenols/kg, depending on the variety and cultivar, and are found mainly in the seeds and skins of grapes. The polyphenolic compounds present in red wine are derived almost exclusively from grapes since very little phenolic material is synthesised by yeast and fermenting bacteria although aging wine in oak has been reported to be a likely source of phenolic lignins. The principle types of polyphenolics in grapes and red wines are phenolic acids, flavonols, flavanols, monomeric catechins, anthocyanidins and procyanidins (see Figure 1.1 and 1.2) (Singleton 1988, Kinsella *et al.* 1993). There are five main types of anthocyanidins in red wine (cyanidin, peonidin, delphinidin, petunidin and malvidin) which contain between 4 to 6 OH groups. Anthocyanidins are present predominantly as glycosides in grapes and red wine and may also be acylated by acetic acid, p-coumaric or caffeic acid. The anthocyanidins are responsible for the colour of red wine and are not found in white wine. Catechins are present in wines either as monomers or polymers of repeating catechin units. These polymeric catechin compounds are referred to as procyanidins or condensed tannins. The simplest procyanidins are dimers (B series) and trimers (C series) which are usually formed by covalent bonds between the 4 carbon on the C ring and 8 carbon on the A ring (Figure 1.2).

Figure 1.2 Structure of procyanidins (polymeric catechins)



B series procyanidins
B-3: R_1, R_3 = OH, catechin (4-8) catechin
B-4: R_1, R_4 = OH, catechin (4-8) epicatechin



C series procyanidins
C-1: R_2, R_4 = OH
C-2: R_1, R_3 = OH,

1.9.1 Epidemiological studies: Red wine and CAD

Favourable epidemiological relationships between red wine or flavonoids, and coronary heart disease mortality have been observed (St Leger *et al.* 1979, Renaud and de Logeril 1992a, Hertog *et al.* 1993, Criqui and Ringel 1994, Grønbaek *et al.* 1995). A study by Renaud *et al.* (1992a) claimed that wine consumption in 17 countries taking part in the MONICA project (a worldwide monitoring system for cardiovascular disease) was the only dietary factor inversely associated with mortality rate from CAD. Although risk factors for CAD such as saturated fat intake, plasma cholesterol levels, smoking and blood pressure were similar between French and American cohorts, the French had a much lower incidence of CAD. By performing multivariate analysis of dietary risk factors, the authors identified red wine as a cardioprotective agent. This apparent anomaly between risk factors and CAD mortality was termed the French Paradox.

Most cross cultural epidemiological studies have shown that red wine is effective in reducing CAD mortality. An example of this is the study by St Leger *et al.* (1979) in which a strong inverse association between CAD mortality and wine consumption in 18 countries was reported. Similarly, Criqui *et al.* (1994) demonstrated that wine ethanol intake consistently produced an inverse relationship with the incidence of CAD, although the authors where mindful of the fact that wine consumption did not benefit total mortality. Many epidemiological studies have been designed in order to evaluate the effect of different types of alcoholic beverages on the relative risk of CAD. A comprehensive review of epidemiological studies investigating the relationship between alcoholic beverages and CAD has been published by Rimm *et al.* (1996). The emerging data from prospective cohort studies are inconsistent with some studies showing a benefit of red wine above all other alcoholic beverages while other studies find a more beneficial effect of beer or spirits (reviewed by Rimm *et al.* 1996).

In summary, there is a curious U-shaped relationship that exists between alcohol consumption and CHD mortality which asserts that moderate drinkers have a lower risk of CAD mortality when compared to abstainers and heavy drinkers. Several epidemiological

studies (Hegsted *et al.* 1988, Rimm *et al.* 1991) have suggested that moderate alcohol intake of 20 to 30 g/day can reduce the risk of CAD, an effect which some hypothesise may be due to the apparent protective actions of alcohol by elevating HDL levels and decreasing thrombotic activity (Kannel *et al.* 1988, Colsher *et al.* 1989, Gaziano *et al.* 1993, Seigneur *et al.* 1990). Despite the lack of conclusive evidence to indicate that red wine ethanol has a greater cardioprotective effect compared to other types of ethanol, researchers are focusing on other components of red wine that may influence cardiovascular function such as polyphenolics.

1.9.2 *In vitro* studies: Red wine and LDL oxidation

Red wine is recently being considered as a dietary antioxidant due to its ability to inhibit LDL oxidation *in vitro*. Initially Frankel *et al.* (1993a) reported on the antioxidant properties of red wine in transition metal-dependent oxidation of LDL. He demonstrated that red wine distilled of ethanol could inhibit copper-mediated LDL oxidation. The susceptibility of human LDL to oxidation was investigated by measuring the hexanal (derived from oxidation of PUFA) and conjugated diene formation. When red wine was diluted to 10 μmol/L of polyphenols, hexanal and conjugated diene formation was completely inhibited, which was similar to the antioxidant potency of the flavonoid, quercetin and more potent then α-tocopherol in this particular model of LDL oxidation. A more recent study by Kanner *et al.* (1994) has outlined the antioxidant capacity of red wine polyphenols under a number of pro-oxidant conditions including the myoglobin, cytochrome c and iron-ascorbate. Many of the polyphenols in red wine, phenolic acids (Nardini *et al.* 1995, Abu-Amsha *et al.* 1996), quercetin (Manach *et al.* 1995), resveratrol (Frankel *et al.* 1993b), catechins (DeWhalley *et al.* 1990, Mangiapane *et al.* 1992) and grape extract itself (Lanninghamfoster *et al.* 1995) have also been shown to inhibit *in vitro* oxidation of LDL in concentration ranges considerably lower than that found in red wine (Singleton 1988).

1.9.3 Human intervention studies: Red wine and LDL oxidation

Many human dietary studies (Fuhrman *et al.* 1995, Simonetti *et al.* 1995, Struck *et al.* 1994,

Maxwell *et al.* 1994, Kondo *et al.* 1994, Whitehead *et al.* 1995), but not all (de Rijke *et al.* 1996), have claimed beneficial effects on parameters of lipid peroxidation and plasma antioxidant levels following red wine consumption. In separate studies Maxwell *et al.* (1994) and Whitehead *et al.* (1995) found an increase in antioxidant capacity as assessed by a chemiluminescence assay in serum from healthy volunteers following the consumption of approximately 300 to 400 ml of red wine. The antioxidant activity in serum increased to a maximum level at 90 min following red wine ingestion and was maintained at elevated levels for up to 4 hours when compared to control subjects (Maxwell *et al.* 1994). Furthermore, the *in vitro* antioxidant capacity of red wine was greater than that of white wine, which was in turn greater than the antioxidant capacity of grape juice when added directly to the luminescent reaction (Whitehead *et al.* 1995). These authors did not measure any specific indices of LDL oxidation nor did they quantitate levels of polyphenols in red wine or serum. Kondo *et al.* (1994) demonstrated an increased lag time before the onset of oxidation of LDL *ex vivo* following red wine ingestion at a dose of 0.8 g ethanol/kg body weight/ day for 14 days. A similar dose of ethanol in the form of vodka did not alter the susceptibility of peroxy radical-initiated LDL oxidation. In contrast, deRijke *et al.* (1996) failed to find an antioxidant effect of red wine on LDL oxidation in normolipidemic subjects. The plasma concentrations of antioxidants (α-tocopherol, ubiquinol-10, vitamin C and urate) were also unchanged following red wine intervention.

There is very little data available regarding the absorption and metabolism of polyphenolic antioxidants present in red wine. Fuhrman *et al.* (1995) measured polyphenols in plasma and LDL following the supplementation of 400 ml of wine/day for 4 weeks in healthy subjects. These authors reported a decrease in LDL and plasma oxidation which was associated with a 4-fold increase in LDL polyphenol levels. The polyphenols in this study were measured by spectrophotometry using the assay described by Singleton (1988). This is a colorimetric assay based on the reaction of phenols with phosphomolybdic phosphotungstic acid and is commonly used to quantitate polyphenols in wine. The application of this assay in biological fluids is not routinely performed and may be problematic due to the lack of sensitivity and

specificity of this assay, especially since plasma proteins absorb ultraviolet light at the wavelength used to detect phenolic compounds. Further studies are needed to determine whether the red wine polyphenols can be absorbed after oral administration and whether they play a role in protecting against LDL oxidation and the prevention of atherosclerosis.

1.9.4 Animal studies: Red wine and atherosclerosis

To our knowledge only one study has investigated the effect of red wine on atherosclerosis. Klurfeld *et al.* (1981) studied the effect of alcohol consumption and atherogenesis in cholesterol/casein fed rabbits. Rabbits were maintained on the atherogenic diet for 3 months and were supplemented with either 12.5% glucose-water, red wine, white wine, whisky, beer or 9.5% ethanol. Rabbits administered wine, whisky or ethanol drank almost half the volume of liquid compared to control rabbits (33 to 46 ml/day compared to 73 ml/day). The extent of atherosclerotic lesions in the aortic arch, thoracic aorta and small myocardial arteries were decreased in rabbits supplemented with red wine only. The authors did not examine the effect of red wine on lipid peroxidation in these rabbits nor did they quantitate the absorption of any red wine polyphenols. They could not attribute the antiatherogenic effect of red wine to ethanol since the other alcoholic beverages had no effect on atherosclerosis progression. It was suggested that components in red wine other than ethanol may be responsible for the observed antiatherogenic effect. This findings have not been confirmed by other researchers. In light of the emerging evidence that red wine possesses antioxidant properties, it would be meaningful to conduct a similar study investigating the effect of red wine consumption on LDL oxidation and atherosclerosis *in vivo*.

1.9.5 *In vitro* studies: Red wine and Endothelium-dependent relaxation

Red wine, grape seed extract and isolated polyphenolic compounds (quercetin and tannic acid) can produce vasorelaxation in precontracted rat aorta *in vitro* (Fitzpatrick *et al.* 1993). This relaxation was characteristic of an acetylcholine-mediated response in that it was endothelium-dependent, produced a concentration dependent increase in aortic cGMP levels, was inhibited by nitric oxide synthase inhibitors and restored by *L*-arginine. These results provide the first evidence that red wine containing polyphenols can mediate agonist-like

endothelium-dependent relaxation. As well as mediating relaxation, grape seed extract also decreased the contractile response of aorta to phenylephrine as shown by an increase in agonist concentration required to produce 50% contraction (EC_{50}) and a decrease in maximum contraction. These observations provide evidence of an *in vitro* protective effect of red wine and polyphenols on endothelial relaxation. To our knowledge no one has investigated the endothelium-dependent relaxation in aorta from hypercholesterolemic animals supplemented with red wine. If red wine polyphenols can exert these vasorelaxant effects *in vivo* they may offer some protection against atherosclerosis and coronary heart disease.

1.10 GENISTEIN

A naturally occurring polyphenolic compound known to have antioxidant properties is the isoflavone genistein (5,7,4 trihydroxyisoflavone, see Figure 1.1). Genistein is present in soybeans and soy products including, miso, tofu and soy milk. Much of the research impetus into genistein has focused on its potential anti-cancer properties since it has been shown to possess antiestrogenic effects (Setchell *et al.* 1984), inhibit growth of cancer cells (Akiyama *et al.* 1991), inhibit cellular signal transduction systems (Akiyama *et al.* 1987) and inhibit angiogenesis (Fotsis *et al.* 1993). Several investigators have proposed that soy consumption is one dietary component that may contribute to the lower rates of some cancers in Asian countries (reviewed by Messina *et al.* 1994).

1.10.1 *In vitro* studies: Genistein and LDL oxidation

Genistein exhibits antioxidant effects *in vitro* including the inhibition of ADP and NADPH dependent lipid peroxidation in rat liver microsomes (Jha *et al.* 1985) and inhibition of the coupled oxidation of β-carotene and linoleic acid (Pratt *et al.* 1979). Record *et al.* (1995) have demonstrated genistein's antioxidant activity in liposomes following a number of prooxidant challenges including UV exposure, peroxy radical lipid peroxidation and hydroxy radical generating systems. Hydrogen peroxide and superoxide generation from stimulated polymorphonuclear cells in culture was also inhibited by genistein (Wei *et al.* 1993). These

antioxidant effects of genistein *in vitro* have been proposed to contribute to its anticancer properties since there is evidence that reactive oxygen species can cause protein, RNA or DNA damage that could lead to carcinogenesis (Dreosti 1991).

There have been some reports in the literature concerning genistein's antioxidant properties towards serum and LDL oxidation (Tsai and Chait 1995, Rifici *et al.* 1994, Hodgson *et al.* 1996). Previously Tsai *et al.* (1995) reported that 50 μmol/L of genistein was able to inhibit conjugated diene and TBARS formation following copper-mediated LDL oxidation. These authors performed experiments in which LDL was incubated with genistein and then re-isolated. They found that genistein in this instance did not protect LDL against oxidation, suggesting that genistein was not incorporated into LDL, however genistein concentrations in LDL were not measured.

Genistein's inhibitory properties against LDL oxidation have been documented by Rifici *et al.* (1994). In these experiments, the effects of insulin and related hormones (insulin-like growth factors I and II, glucose and proinsulin) on cell-mediated LDL oxidation *in vitro* was investigated. To elucidate the stimulatory effects of insulin on LDL oxidation, cells were incubated with genistein, a specific inhibitor of insulin receptor tyrosine kinase. A single concentration of genistein (approximately 150 μmol/L) inhibited copper-mediated and macrophage-mediated LDL oxidation by 67% and 77%, respectively. The inhibitory actions of genistein in the absence of cells suggest that the inhibitory effect of the isoflavone was due to mechanisms other than inhibition of insulin activity so the authors suggested a possible antioxidant effect of genistein in these experiments.

1.10.2 *In vivo* studies: Genistein bioavailability

There are an increasing number of studies in the literature focusing on the plasma and urinary concentrations of genistein following the consumption of isoflavone rich diets. The concentration of isoflavones in Japanese subjects consuming a traditional diet rich in soy products was quantitated by Adlercreutz *et al.* (1991 and 1993). In these subjects urinary excretion and plasma concentrations of genistein were approximately 6 μmol/day and 276

nmol/L, respectively. In subjects receiving 80 mg of genistein daily in the form of a soy supplement, their plasma genistein concentrations was approximately 900 nmol/L (Gooderham *et al.* 1996). In premenopausal females consuming a diet supplemented with isoflavones, their urinary excretion of genistein ranges from 0.3 to 8.5 μmol/day (Cassidy *et al.* 1994). Similar studies in post-menopausal women detected plasma concentrations of genistein of 150 ng/ml following supplementation with soy flour or clover sprouts (Morton *et al.* 1994). These studies indicate that genistein can be absorbed and detected in plasma following intake of isoflavone rich diets.

It is possible that genistein is absorbed at significant concentrations following oral administration to exert it's antioxidant properties *in vivo*. If so, genistein may be a useful therapeutic agent in the prevention of LDL oxidation and atherosclerosis.

1.11 OVERALL OBJECTIVES

The aim of this research was to investigate the effect of polyphenolic antioxidants (red wine and genistein) on the oxidation of LDL *in vitro*. The effect of dietary supplementation with red wine on LDL oxidation and fatty streak lesion development in cholesterol-fed rabbits was investigated. Other objectives of the present studies were to determine the oral bioavailability of red wine derived catechins and to examine the endothelium-dependent vasorelaxation in aortic preparations following red wine administration in rabbits. The effects of red wine on plasma lipids and lipoproteins were investigated.

1.12 RESEARCH OUTLINE

The models of oxidation used *in vitro* were copper- and peroxy radical-mediated LDL oxidation. In these experiments both the aqueous and lipid-soluble antioxidant activity of polyphenolic compounds were determined. Polyphenols were added directly to LDL in an aqueous environment and oxidation by copper or azo-initiator performed. Other experiments were conducted in order to determine whether phenols can be incorporated into LDL. To achieve this whole plasma was incubated with the compound of interest prior to isolation and oxidation of LDL. The reason for performing these experiments was based on the hypothesis

that oxidation of LDL *in vivo* occurs in the artery wall, where it is unprotected by hydrophilic plasma antioxidants. Therefore lipophilic antioxidants that are incorporated into the lipophilic core of lipoproteins afford greater protection against LDL oxidation. The ability of some polyphenolic antioxidants to be incorporated in the LDL particle was measured by HPLC.

Studies to investigate the antioxidant, lipid metabolism and antiatherogenic properties of red wine derived polyphenolic compounds were performed in rabbits. New Zealand White rabbits are a good model for hypercholesterolemia and atherosclerosis because they are very responsive to dietary cholesterol and the development of atherosclerosis. Rabbits were fed a cholesterol diet, supplemented with red wine. Following this diet regime the plasma lipid and lipoprotein profiles were measured. The *in vitro* susceptibility of isolated LDL to copper-mediated oxidation was determined. Rabbit arteries were dissected and atherosclerotic plaque formation assessed by lipophilic staining and quantification of artery cholesterol concentrations. The effect of polyphenolic antioxidants on the endothelial dependent relaxation responses in aortic rings prepared from rabbits following dietary supplementation were also investigated. Development of a sensitive assay for the determination of catechins in rabbit plasma, as a marker for red wine bioavailability, was performed.

CHAPTER 2

GENERAL METHODS

2.1 LDL ISOLATION BY SINGLE STEP ULTRACENTRIFUGATION

Blood was collected in tubes containing 1 mg/ml ethylenediaminetetra-acetic acid disodium salt (EDTA). Plasma was isolated from whole blood by centrifugation at 3 000 x *g* for 10 min and stored at -80 °C until use. LDL was isolated from plasma by a single-step ultracentrifugation method as described by Chung *et al.* (1986). Plasma (0.65 ml) was adjusted to density 1.21 g/ml with KBr and overlayed with 1.006 g/ml density solution containing 0.1% (w/v) EDTA in a Beckman 11 x 32 mm (1.5 ml) Quickseal ultracentrifugation tube. The samples were placed in a Beckman TLA 120.2 rotor and centrifuged in an Optima TLX benchtop ultracentrifuge (Beckman Instruments, California, USA) at 435 680 x *g* for 30 min at 4 °C. The LDL is clearly visible as a yellow/orange colour band approximately two-thirds up from the bottom of the tube. The LDL band can be removed from the tube with a needle and syringe. Approximately 0.25 ml of LDL is collected from each tube.

2.2 COPPER-MEDIATED OXIDATION OF LDL

Prior to oxidation experiments, LDL was dialysed against three changes of phosphate-buffered saline (PBS containing 4 mmol/L KH_2PO_4, 16 mmol/L Na_2HPO_4.anhydrous and 150 mmol/L NaCl at pH 7.4) with no EDTA for a total of 20 h. The dialysing buffer was purged with nitrogen to remove oxygen from solution. Following dialysis the protein concentration in the LDL fractions were determined by the Lowry method (Lowry *et al.* 1951) on a Cobas Bio automated centrifugal analyser (F. Hoffmann-La Roche, Basle, Switzerland) using bovine serum albumin (BSA) as standards (Clifton *et al.* 1988). Immediately following isolation and removal of salts, 50 mg protein/L of LDL was incubated with 5 µmol/L cupric sulphate in PBS (final volume of 1.25 ml) at 37 °C maintained by Beckman Temperature Controller. The oxidation of LDL was measured by the formation of conjugated dienes determined as the increase in UV absorbance at 234 nm (Esterbauer *et al.* 1989a). Absorbance was continuously monitored at 2 min intervals for 2 to 3 h at 37 °C using Beckman Kinetics Soft-Pac module and DU-65 spectrophotometer (Beckman

Instruments, California, USA). The parameters of oxidation determined from the LDL absorbance profile were lag time, oxidation rate and maximum conjugated diene concentration. The lag time was determined as the intercept of the baseline and propagation phase of the absorbance curve and was expressed in absolute value (mins) or as a % change from control value. The oxidation rate is equal to the slope of the linear propagation phase and was calculated using the molar extinction coefficient of conjugated dienes (ε_{234} 29 500). LDL oxidation rate was expressed as nmol conjugated diene/mg LDL protein/min. The maximum conjugated diene concentration was determined as the intercept of the propagation and degradation phases. All oxidations were performed in duplicate.

2.3 AZO-INITIATED OXIDATION OF LDL

Prior to azo-initiated oxidation experiments, LDL was dialysed overnight against PBS containing 1 mmol/L EDTA. The oxidation of LDL mediated by an azo compound was determined by incubating 100 mg protein/L of LDL with 5 mmol/L aqueous azo-initiator (2, 2'-Azobis-[2-amidinopropane] hydrochloride) in PBS + 1 mmol/L EDTA for 8 h at 37°C in a shaking waterbath. The azo compound is thermal labile and at 37°C decomposes and produces peroxy radicals. The reactions were stopped by the addition of 50 µmol/L butylated hydroxytoluene (BHT) and storage at 4°C.

2.3.1 Thiobarbituric acid reactive substances (TBARS) assay

Azo-initiated oxidation of LDL was quantified at hourly intervals as the generation of malondialdehyde equivalents (MDA) as measured by the thiobarbituric acid reactive substance method (TBARS) (Buege *et al.* 1978) with the modifications described by Abbey *et al.* (1993). The concentration of MDA was calculated using the molar extinction coefficient of MDA (ε_{535} 1.56 x 10^5).

2.3.2 Electrophoretic mobility of LDL

The electrophoretic mobility of LDL following azo-initiated oxidation was determined using an agarose gel electrophoresis system (Colf and Verheyden 1967). Both native (non-oxidised) and oxidised LDL were run on Paragon 0.5% agarose (Beckman, California, USA) at 100 V for 2 h and then stained with Sudan black. Electrophoretic mobility was determined by scanning laser densitometry (Pharmacia-LKB Ultrascan Densitometer, Uppsala, Sweden) and was expressed relative to the electrophoretic mobility of native LDL.

2.4 LDL-α-TOCOPHEROL MEASUREMENTS BY HPLC

α-Tocopherol was measured in LDL fractions by HPLC with UV detection, using α-tocopherol acetate as the internal standard. The concentration of α-tocopherol standards was determined using its molar extinction coefficient value ε_{292} 75 800 (Yang and Lee 1987). Standard concentrations of α-tocopherol were dissolved in ethanol, dried under nitrogen and stored at -80 °C. The LDL isolated from human plasma was used as the matrix in this assay. The standard curve was prepared by reconstituting α-tocopherol in ethanol and diluting in LDL to achieve final concentrations of 2.2 to 11.0 mg/L. A blank standard of LDL was used in order to subtract background α-tocopherol levels. α-Tocopherol was extracted from LDL with hexane as described by Yang and Lee (1987). Briefly, 200 µl of samples were vortexed for 2 mins with 75 µl of ethanol, internal standard (25 µl of 0.5 g/L α-tocopherol acetate) and 400 µl of hexane. The samples were centrifuged and the hexane layer was removed and evaporated to dryness with nitrogen. The sample was reconstituted in 150 µl of mobile phase for injection onto HPLC.

The mobile phase (22% methanol, 11.5% dichloromethane, 11.5% hexane and 55% water) was pumped through a Supelcosil LC18-DB (25 cm x 4 mm) column at 1 ml/min. α-Tocopherol was detected by measuring absorbance at ultraviolet (UV) wavelength of 292 nm following the injection of 40 to 60 µl of sample. The retention times of α-tocopherol and α-tocopherol acetate were 7.1 and 8.1 mins, respectively.

The HPLC system used was from GBC Scientific Equipment, Dandenong, Victoria, Australia. The system included GBS pump (model LC1120), UV/Vis detector (model LC1210), and autosampler (model LC1610). The analysis of HPLC chromatographs was performed with GBC WinChrom chromatography data management system version 1.

2.5 LDL FATTY ACID MEASUREMENTS

The fatty acid composition of LDL was determined by gas chromatography. An internal standard, triheptadecanoate (0.5 mg/ml), was used in these measurements. Lipids where extracted from 0.2 ml LDL by the Folch method (Folch *et al.* 1957) using 3 ml of chloroform: methanol (2:1, v/v). The chloroform layer was removed and evaporated to dryness with nitrogen. Methyl esters of the fatty acids were prepared by incubating the dried chloroform extract with 1.5 ml of 1% H_2SO_4 in dry methanol at 100 °C for 45 min. After the samples had cooled to room temperature, 3 ml of water were added. The methyl esters were extracted from the aqueous phase with 5 ml petroleum spirit (distilled at 40 to 70 °C) by vigorous shaking for 5 min. The lower organic phase was collected and the aqueous phase was extracted a second time with another 5 ml of petroleum spirit. The organic phase was collected and evaporated to dryness with nitrogen. The methyl esters were reconstituted in 1.5 ml of hexane and loaded unto 20 mm glass column packed with activated magnesium silicate to remove contaminates from the sample. The methyl esters were eluted off the column with 2 ml of 10% diethyl ether in hexane and then dried under nitrogen. The methyl esters were dissolved in 50 µl of 2,2,4-trimethylpentane (isoctane) for GC injection.

The gas chromatograph was Hewlett-Packard 5711 model, the column was 30 mm x 0.53 mm bonded phase vitreous silica, the carrier gas was hydrogen and the make up gas nitrogen and the temperature program incremented from 130 to 230°C at 8°C / min. The peak area ratios of fatty acids to internal standard were determined. The fatty acids in LDL measured were 14:0 (myristic acid), 16:0 (palmitic acid), 16:1 (palmitoleic acid), 18:0 (stearic acid), 18:1 (oleic acid), 18:2 (linoleic acid), 18:3 (α-linolenic acid) and 20:4 (arachidonic acid) and were expressed as the summed percentage of total fatty acids.

2.6 PHENOLIC MEASUREMENTS IN RED WINE BY FOLIN-CIOCALTEU METHOD

The phenolic content in each red wine fraction and total red wine was determined by the Folin-Ciocalteu method using gallic acid as standard equivalents (Singleton 1988). One volume of red wine was diluted in 4 volumes of water. Samples and gallic acid standards (0.1 ml volume) were added to 0.9 ml water and 5 ml of 10% (v/v) Folin-Ciocalteu's phenol reagent and then vortexed. After 10 min, 3.5 ml of 11.5% (v/v) Na_2CO_3 was added to the samples and incubated at room temperature for 2 h. The absorbance of the samples was measured by spectrophotometry at a visible wavelength of 765 nm. The gallic acid standard curves were linear in the concentration used and the coefficients of variation were < 5% at any one concentration.

2.7 PLASMA LIPID MEASUREMENTS

Plasma cholesterol and triglyceride were measured on a Cobas Bio automated centrifugal analyser (F. Hoffmann-La Roche, Basle, Switzerland) using commercial available reagent kits (Roche Diagnostic System, F. Hoffmann-La Roche, Basle, Switzerland). Quality control samples for cholesterol and triglyceride determinations were included in each assay (Ciba Corning, Australian Diagnostics).

2.8 ISOLATION OF LIPOPROTEIN FRACTIONS BY SEQUENTIAL ULTRACENTRIFUGATION

Plasma (3 ml) was adjusted to density 1.019 g/ml with KBr and aliquoted into 3 ultracentrifugation tubes (11 x 32 mm Quick Seal tubes, Beckman) and overlayed with 1.019 g/ml density solution. The samples were centrifuged in Beckman TLA 120.2 rotor at 120 K for 2.5 h at 15°C in TLX ultracentrifuge. The top of the tubes containing lipoproteins of d < 1.019 g/ml (chylomicrons + VLDL + IDL) were removed. The bottom fraction of plasma was pooled and adjusted to density 1.063 g/ml. Equal volumes of the d = 1.063 g/ml plasma were added to ultracentrifugation tubes and overlayed with the same density solution. The samples were centrifuged at 120 K for 3 h at 4°C. The top fraction of the tubes containing LDL (d <1.063 g/ml) were collected. The bottom fraction was then adjusted to a density of 1.21 g/ml and centrifuged at 120 K for 6 h to isolate the HDL fraction. The isolated lipoprotein fractions were stored at -80°C for latter determination of lipoprotein composition.

2.9 STATISTICAL ANALYSIS

All data are expressed as mean ± standard error of the mean (sem) unless stated otherwise. The Bartlett's statistical test was performed to test the homogeneity of the data. Significant differences between treatments were determined using either a parametric one-way analysis of variance (ANOVA) with Student-Newman-Keuls multiple comparison unless stated otherwise. Data that were not homogenously distributed, as determined by the Bartlett's statistical test, were logged and parametric ANOVA and post-hoc tests were performed. P-values of <0.05 were considered significant. The Graphpad Instat V2.04 software (Graphpad software, California, USA) was used for all statistical tests.

2.10 CHEMICALS AND COMPOUNDS

Red wine (Cabernet Sauvignon), 12.7% (v/v) ethanol and red wine stripped of phenols by treatment with polyvinyl polypyrrolidone, were kindly supplied by the Australian Wine

Research Institute, Adelaide, SA. All the samples were sealed under nitrogen in glass ampoules and stored at 4°C.

Genistein, (±)-α-tocopherol (vitamin E), catechin, (-)-epicatechin, 5 α-cholestane, 5β-cholestan-3α-ol, bovine albumin (fraction V) and 2-thiobarbituric acid were obtained from Sigma-Aldrich, Castle Hill, Australia. The 2,2'-Azobis-(2-amidinopropane) hydrochloride was purchased from Polysciences Inc, Warrington, PA, USA and Folin-Ciocalteu's phenol reagent from Merck, Darmstadt, Germany. Cholesterol were obtained from Labchem and EDTA-disodium salt was purchased from BDH Chemicals, Kilsyth, Victoria, Australia. All solvents were obtained from BDH Laboratory Supplies, Poole, England and were of analytical or HPLC grade.

CHAPTER 3

RED WINE AND PHENOLIC COMPOUNDS FRACTIONATED FROM RED WINE INHIBIT LDL OXIDATION.

3.1 INTRODUCTION

Low density lipoproteins (LDL) which contain polyunsaturated fatty acids (PUFA) are susceptible to oxidation. The most commonly used method to investigate the susceptibility of LDL to oxidation is to promote oxidation using catalysts such as divalent copper ions. Experimentally LDL is isolated from plasma and incubated with Cu (II) at 37 °C and the formation of conjugated dienes is measured spectrophotometrically (Esterbauer *et al.* 1989a).

LDL is isolated from plasma by density gradient ultracentrifugation. Once LDL is isolated from endogenous antioxidants in plasma, it may undergo auto-oxidation. To prevent or slow this process, plasma and LDL are stored in ethylenediaminetetra-acetic acid disodium salt (EDTA). EDTA acts as a preventative antioxidant by chelating metal ions which may otherwise mediate lipid peroxidation (Halliwell 1990). Prior to *in vitro* oxidation, EDTA must be removed from the LDL sample. To achieve this, LDL is dialysed overnight in EDTA-free phosphate-buffered saline. This preparation of LDL is a lengthy process taking up to 24 h and may therefore increase the possibility of autoxidation and chemical changes in the composition of the lipoproteins.

It has been reported by Scheek *et al.* (1995) that LDL dialysed for 22 or 44 h, with or without EDTA, have significantly lower LDL-antioxidant levels than undialysed LDL and consequently parameters of lipid peroxidation, such as lag time and rate of conjugated diene formation, following copper-mediated LDL oxidation, were affected. These results would suggest that *in vitro* experiments, with LDL desalted by traditional dialysis methods, are not representative of *in vivo* LDL. It was proposed by Scheek *et al.* (1995) that gel filtration chromatography (Puhl *et al.* 1994) would be a much improved method for removing EDTA and salts from LDL, and that LDL treated in this manner may be more representative of *in vivo* LDL oxidisability.

In preliminary experiments the objective was to compare two methods of removing EDTA

and salts from LDL, dialysis or gel filtration, on (i) LDL-α-tocopherol levels and (ii) conjugated diene formation in LDL following copper-mediated oxidation.

It has been proposed that the oxidative modification of LDL is an important initiating event in atherosclerosis. The oxidation of PUFA within LDL particles gives rise to many modifications of the lipoprotein that render it atherogenic. These atherogenic changes, which are well described in the literature and reviewed by Steinberg *et al.* (1989), include the formation of oxidised lipids which act as chemotactic and mitogenic agents and the modification of the charge on the apolipoprotein B-100 (apo B) moiety of LDL creating a ligand for scavenger receptors on macrophages. With increasing evidence that oxidation of LDL occurs *in vivo*, and that lipid peroxidation is associated with the development of atherosclerosis (Yla-Herttuala *et al.* 1989, Palinski *et al.* 1989), it is reasonable to hypothesise that agents which could slow or prevent the oxidative process may be beneficial in lowering the incidence of atherosclerosis and CAD.

The aim of the present study is to examine the antioxidant activity of red wine against LDL oxidation *in vitro* using two models of prooxidant challenge, copper-mediated and peroxy radical-mediated oxidation. Red wine contains polyphenolic compounds that have *in vitro* antioxidant properties (Frankel *et al.* 1993a). The antioxidant compounds present in red wine are derived almost exclusively from grapes and have been identified as phenolic acids, flavonols, monomeric catechins, polymeric catechins (procyanidins), and anthocyanidins (Singleton 1988, Kinsella *et al.* 1993). The identification of the active phenolic compound or class of phenols which are responsible for red wine's antioxidant properties has raised much interest. In previous studies, phenolic compounds such as, phenolic acids, resveratrol, quercetin, rutin and catechins have all been shown to have antioxidant properties *in vitro* (Abu-Amsha *et al.* 1996, Frankel *et al.* 1993b, Manach *et al.* 1995, Salah *et al.* 1995, Mangipane *et al.* 1992). Although these compounds were not derived from red wine in the

cited experiments, the levels of these compounds present in red wine has been estimated to be as low as 1 mg/L resveratrol, 30 mg/L of flavonols (quercetin and kaempferol), 140 mg/L phenolic acids and up to 300 mg/L catechins (Singleton 1988). These concentrations of polyphenolics in red wine are equivalent to the concentrations that inhibit LDL oxidation *in vitro* (Abu-Amsha *et al.* 1996, Frankel *et al.* 1993b, Manach *et al.* 1995, Salah *et al.* 1995, Mangipane *et al.* 1992). The antioxidant potential of the individual classes of phenolic compounds fractionated from red wine, in copper and peroxy radical catalysed LDL oxidation, was investigated.

Another aim of these studies was to examine the antioxidant activity of red wine and its phenolic compounds against lipid peroxidation of LDL both in aqueous and lipid phases. Most publications to date have investigated the antioxidant effect of red wine in aqueous conditions by adding exogenous red wine dilutions to isolated LDL in a buffered solution (Frankel *et al.* 1995, Teissedre *et al.* 1996). The present study aims to investigate whether antioxidant compounds in red wine can be incorporated into lipophilic LDL particles following the incubation of red wine with whole plasma. Subsequent isolation of LDL and pro-oxidant challenge would determine whether red wine can associate with LDL particle and display an antioxidant effect.

3.2 METHODS

3.2.1 Preliminary experiment: Suitability of dialysis or gel filtration for removal of EDTA from LDL prior to use in oxidation studies.

3.2.1.1 LDL isolation and dialysis in phosphate-buffered saline

The methods for isolating LDL by a single step ultracentrifugation and for dialysing LDL are described in Chapter 2.

3.2.1.2 LDL isolation and gel filtration

The LDL isolated from plasma LDL was filtered using PD-10 columns packed with sephadex G-25 M (Pharmacia Biotech) to remove EDTA and salts as described by Puhl *et al.* (1994) The following modifications to this method were employed. The columns were pre-washed with approximately 15 ml phosphate-buffered saline (PBS containing KH_2PO_4 4 mmol/L, Na_2HPO_4.anhydrous 16 mmol/L and NaCl 150 mmol/L at pH 7.4). One ml of LDL was loaded onto the column followed by 2 ml of PBS. A further 2 ml of PBS was used to elute the LDL off the column. This gel filtration procedure was repeated using a second column to ensure complete removal of salts and EDTA.

3.2.1.3 Copper-mediated oxidation of LDL

Following dialysis or gel filtration, the protein concentration in LDL was determined by the Lowry method (Lowry *et al.* 1951) on an automated Cobas Bio using BSA as standards (Clifton *et al.* 1988). The LDL was oxidised in the presence of cupric sulphate as described in Chapter 2.

3.2.1.4 LDL-α-tocopherol measurements by HPLC

Aliquots of unoxidised LDL were stored at -80°C for α-tocopherol determinations (Chapter 2).

3.2.2 Fractionation of red wine into different classes of phenolic compounds

Red wine was fractionated into different classes of phenolic compounds according to their hydrophilic properties as described by Oszmianski *et al.* (1988). Red wine was centrifuged at 1500 x *g* for 5 min and the supernatant adjusted to pH 7 with NaOH. Red wine (0.5 ml) was loaded onto a SEP-PAK C_{18} cartridge (Waters Associates, Millipore, MA, USA) which had been pre-conditioned with 2 ml methanol and 2 ml water. Red wine fractions were then eluted with solvents of increasing hydrophobicity. Fraction 1 containing phenolic acids was

eluted with 2 ml of water, fraction 2 containing monomeric catechins, procyanidins and monomeric anthocyanidin was eluted with 4 ml of 16% aqueous acetonitrile, fraction 3 containing flavonols was eluted with 10 ml of ethyl acetate and fraction 4 containing polymeric anthocyanidin was eluted with 2.5 ml of methanol. The composition of each red wine phenolic fraction has been previously characterised by other authors (Jaworski *et al.* 1986, Oszmianski *et al.* 1988, Kerrigan 1995).

Red wine fractions 3 and 4 were dried under a stream of nitrogen and fractions 1 and 2 were freeze dried by centrifugation (Savant SC100) connected to a vacuum pump and a refrigerated condensation trap (Savant RT490) set at -80°C. Each fraction was then reconstituted in 12% (v/v) aqueous ethanol. This fractionation and quantification of red wine was performed 7 to 8 times.

3.2.3 Copper-mediated oxidation of LDL in the presence of red wine

Red wine phenolic content was measured by the Folin-Ciocalteu method (see Chapter 2). To determine the effect of red wine phenols on copper-mediated LDL oxidation, aliquots of red wine containing 0.025 to 0.3 mg/L GAE (final concentration) were added to the incubation. Preliminary experiments also investigated the effects of ethanol and red wine stripped of phenols on copper-mediated LDL oxidation.

Copper-mediated LDL oxidation were also performed in the presence of different classes of red wine phenols that were fractionated from whole red wine. Fractions 1 to 4 where used at a final concentration of 0.2 mg/L GAE. Oxidations performed with whole red wine (final concentration 0.2 mg/L GAE) and with 12% (v/v) aqueous ethanol served as the control samples in these series of experiments.

3.2.4 Incorporation of red wine-derived antioxidants into LDL

For experiments investigating the incorporation of red wine phenolics into LDL, plasma was incubated with (i) red wine containing 75 mg/L GAE, (ii) 0.6% (v/v) ethanol (equivalent to

the ethanol concentration in red wine) or (iii) red wine stripped of phenolic compounds (0.6%, v/v ethanol), for 3 h in a shaking waterbath set at 37°C, prior to the isolation of LDL. The LDL was then dialysed and oxidised in the presence of copper.

3.2.5 Preparation of [^{3}H]cholesteryl ester (CE)-LDL

The LDL was labelled with [^{3}H]cholesterol as described by the method of Barter and Jones (1979). Briefly, 50 µl of [^{3}H]cholesterol was dried under nitrogen and dissolved in 10 µl ethanol then incubated with 1 ml plasma at 37 °C for 24 h. At 21 h incubation time, red wine (75 mg/L GAE, final concentration), ethanol (0.6% v/v, final concentration) or water were added to the incubation mixture to allow incorporation of these components into lipoproteins. Plasma was then washed three times with red blood cells which had been washed with 0.15 M NaCl. The [^{3}H] CE-LDL was isolated, dialysed in PBS and sterilised by filtration. Tritiated LDL was oxidised in the presence of Cu (II) for 2 h at 37 °C under sterile conditions. The reaction was stopped by the addition of 100 µmol/L EDTA.

3.2.6 Macrophage uptake of LDL

The cellular uptake of LDL was measured by incubating J774 macrophages with either native (non-oxidised) or copper-oxidised LDL based on the methods of Suzukawa *et al.* (1994). Macrophages were cultured in Dulbecco's modified Eagles media (DMEM) containing 10% (v/v) heat inactivated foetal calf serum (FCS), penicillin (50, 000 U/ml), streptomycin (50 mg/ml) and L-glutamine (200 mmol/L) at 37°C in an atmosphere containing 5% CO_2 in air. For uptake experiments macrophages were trypsinised, resuspended in DMEM + 10% FCS and plated into 6 well culture plates containing 5 x 10^5 cells/well. After 2 days of culture, the macrophage monolayers were washed twice with PBS and incubated with 10 µg (protein) of either native [^{3}H]CE-LDL or copper-oxidised [^{3}H]CE-LDL for 6 h at 37 °C in DMEM + 10% lipoprotein deficient serum. The media was removed and monolayers of macrophages were washed with 2 mg/ml BSA in PBS and three times with PBS alone. Macrophages where incubated for 30 min in 1 ml of hexane: isopropanol (3:2) to extract cellular lipids. The

organic solvent was collected and aliquoted into scintillation vials containing 5 ml of scintillation cocktail (OptiPhase Hisafe, Wallac Scintillation products, Finland) to measure [^{3}H]CE-LDL. The macrophage monolayers were incubated for a further 30 min with 1 ml of 0.1M NaOH. Cellular protein was determined by Cobas Bio using BSA standards. The uptake of [^{3}H]CE-LDL by macrophages was determined as the radioactivity of the sample divided by the specific activity of [^{3}H]CE-LDL and expressed per mg of cellular protein. The copper oxidation and macrophage uptake experiment was repeated and was measured in a total of 4 wells for each experiment.

3.2.7 Azo-initiated oxidation of LDL in the presence of red wine

For experiments investigating the effect of red wine on azo-initiated LDL oxidation (see Chapter 2), 2.5 to 20 mg/L GAE of red wine (final concentration) and controls containing either ethanol or red wine stripped of phenols were added to the incubation mixture. The malondialdehyde (MDA) concentrations and relative electrophoretic mobility (REM) of azo-oxidised LDL were determined as described in Chapter 2.

Azo-initiated LDL oxidation were performed in the presence of different classes of red wine phenols that were fractionated from whole wine. Each red wine fraction and whole red wine were diluted to a final concentration of 2.5 mg/L (final concentration) GAE for azo-initiated oxidation experiments.

3.2.8 Statistical Analysis

All values are expressed as mean ± standard deviation. Unless indicated otherwise, all experiments were performed three times. One-way analysis of variance (ANOVA) with Tukey Kramer multiple comparisons test was performed on data using Graphpad Instat V2.04 software (Graphpad software, California, USA).

3.3 RESULTS

3.3.1 Preliminary experiment

3.3.1.1 Copper-mediated oxidation of LDL

The lag time (min) prior to the formation of conjugated dienes and oxidation rates (nmol conjugated diene/mg LDL protein/min) of LDL are presented in Table 3.1. There was no oxidation of undialysed LDL following 2 h incubation with copper. There were no differences in the parameters of LDL oxidation for dialysed and gel filtered LDL. The coefficients of variation for lag time determinations in dialysed LDL and gel filtered LDL samples were 9.5% (n=8) and 6.8% (n=6), respectively.

3.3.1.2 LDL α-tocopherol concentrations

Standard curves for α-tocopherol were linear between the concentration range of 2.2 and 11.0 mg/L with correlation coefficients of $r > 0.99$. The coefficients of variation for low (2.2 mg/L), medium (6.6 mg/L) and high (11.0 mg/L) concentrations of α-tocopherol were 11.8%, 3.1% and 2.8% respectively. The recovery of α-tocopherol following hexane extraction was 92.7% with a coefficient of variation of 7.3%. There were no differences in LDL α-tocopherol concentrations between undialysed, dialysed or gel filtered LDL prior to oxidation experiments (Table 3.1).

	LDL		
	undialysed	dialysed	gel filtered
Lag time (min)	nd	47.3 ± 2.5	47.0 ± 3.2
Oxidation rate (nmol diene/mg LDL protein/min)	nd	18.30 ± 0.91	17.05 ± 0.95
LDL α-tocopherol (μg α-tocopherol/mg LDL protein)	6.53 ± 0.27	6.37 ± 0.14	6.54 ± 1.12

Table 3.1

Copper-mediated oxidation and α-tocopherol in LDL isolated and prepared by dialysis or gel filtration.

LDL was isolated from human plasma by single-step ultracentrifugation and EDTA and salts were removed by dialysis against PBS or by gel filtration using SEP-PAK PD-10 columns. The α-tocopherol content of LDL was determined by HPLC. The LDL (50 mg protein/L) was oxidised by incubation with copper (5 μmol/L). Undialysed LDL did not oxidise following incubation with 5 μmol/L copper at 37°C for 2 h and values were not determined (nd). Values in the same row were not significantly different from one another. Values are mean ± standard deviation.

3.3.2 Phenolic concentration of red wine and red wine fractions

The phenolic concentration of red wine was 1688 ± 162 mg/L GAE. The concentrations of phenolic compounds in red wine fractions separated by SEP-PAK C18 column were 494 ± 76 mg/L GAE in fraction 1, 423 ± 64 mg/L GAE in fraction 2, 243 ± 23 mg/L GAE in fraction 3 and 557 ± 46 mg/L GAE in fraction 4.

3.3.3 Copper-mediated oxidation of LDL in the presence of red wine

LDL from a normal healthy volunteer, displayed a lag time of 58.5 ± 1.1 min when subjected to copper-mediated oxidation. Comparison of LDL oxidation in the presence of different components of red wine showed that ethanol and red wine stripped of phenolic compounds did not alter lag time (59.0 ± 0.8 min and 59.3 ± 0.5 min, respectively). The effect of red wine, expressed as a % increase compared to control lag time, on copper-mediated LDL oxidation is shown in Figure 3.1. Red wine containing phenolic compounds (0.025 to 0.3 mg/L GAE) increased the lag time before the formation of conjugated dienes in a concentration-dependent manner. The rate of copper-mediated LDL oxidation was 14.8 ± 0.6 nmol conjugated diene/mg LDL protein/min in the absence of red wine and it did not differ in the presence of red wine at all concentrations tested (results not shown).

The effect of red wine fractions (0.2 mg/L GAE) on the lag time of copper-mediated LDL oxidation is depicted in Figure 3.2. All red wine fractions increased the lag time for the oxidation of LDL. Phenolic fractions 1, 2 and 4 displayed a similar increase in lag time to that seen with total red wine. Only fraction 3 displayed a significantly decreased antioxidant effect compared to whole red wine ($P < 0.05$). Fraction 2 displayed a greater antioxidant capacity when compared to fractions 3 ($P < 0.01$) and 4 ($P < 0.05$).

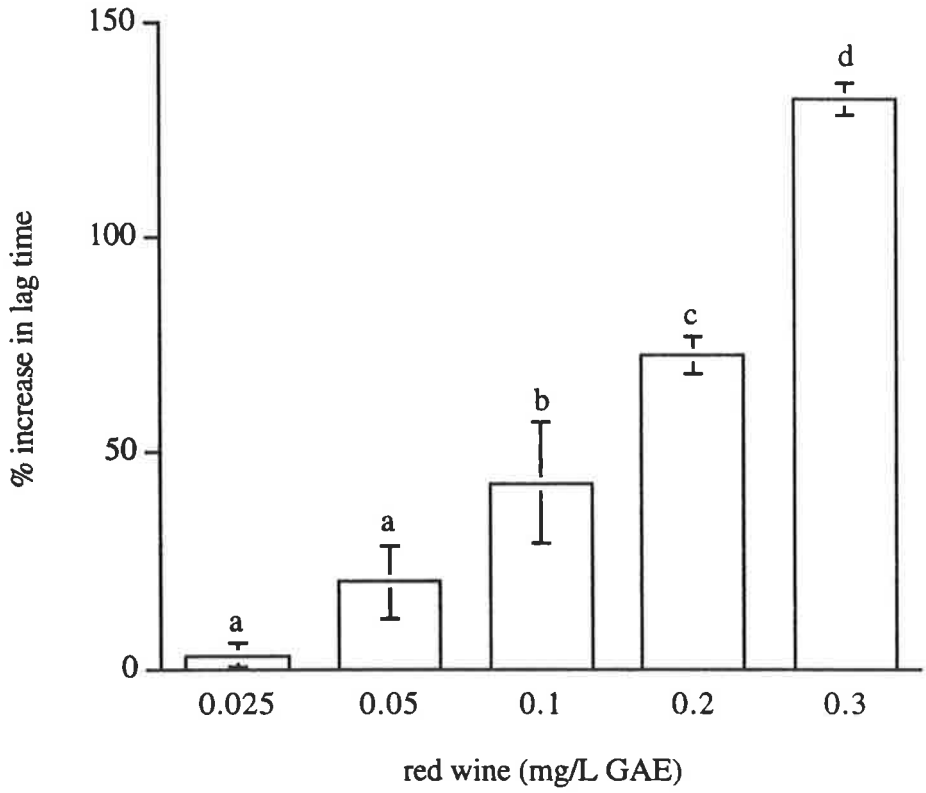


Figure 3.1

The effect of red wine on the lag time of copper-mediated LDL oxidation.

LDL (50 mg protein/L) was oxidised by copper (5 μmol/L) at 37 °C in the presence of red wine containing 0.025 to 0.3 mg/L GAE. Oxidation was determined as the lag time for conjugated diene formation and was expressed as % increase in control LDL lag time. Control LDL was oxidised in the presence of 0.7% ethanol. All oxidations were performed in duplicate and values are mean ± standard deviation (n=3). Columns with differing alphabetic notations are significantly different at $P < 0.05$ as determined by parametric ANOVA with Tukey Kramer multiple comparisons.

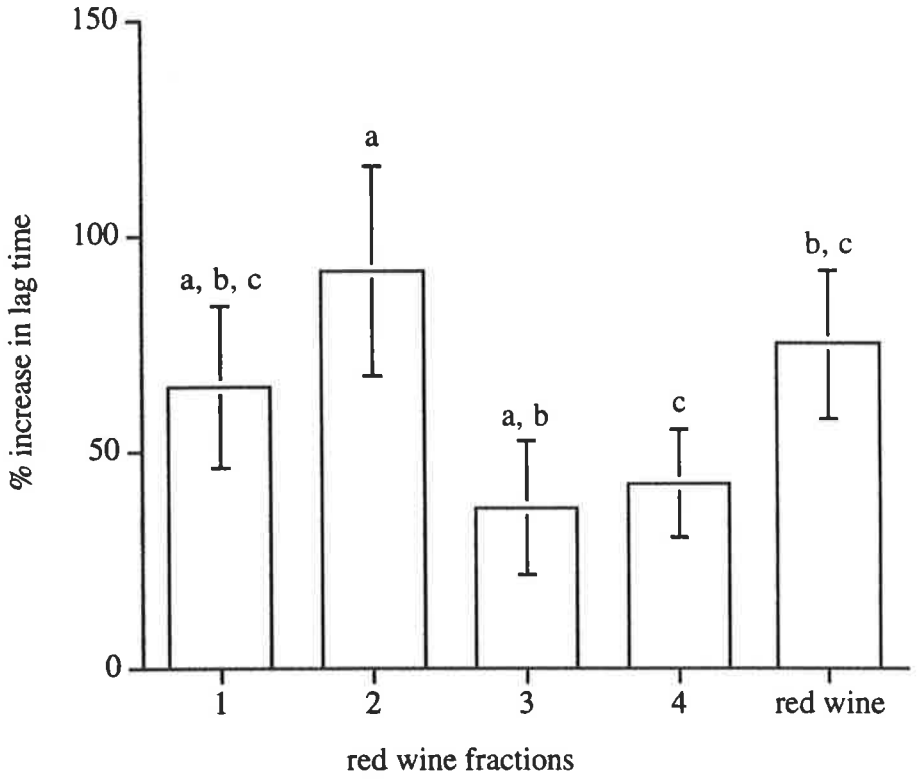


Figure 3.2

The effect of different phenolic fractions (0.2 mg/L GAE) prepared from red wine on the lag time of copper-mediated LDL oxidation.

LDL (50 mg protein/L) was oxidised by copper (5 μmol/L) at 37 °C in the presence of red wine fractions or whole red wine containing 0.2 mg/L GAE. Oxidation was determined as the lag time for conjugated diene formation and was expressed as % increase in control LDL lag time. Control LDL was oxidised in the presence of 0.7% ethanol. All oxidations were performed in duplicate and values are mean ± standard deviation (n=3). Columns with differing alphabetic notations are significantly different at $P < 0.05$ as determined by parametric ANOVA with Tukey Kramer multiple comparisons.

3.3.4 Incorporation of red wine-derived antioxidants into LDL

The oxidation of LDL isolated from plasma after preincubation with red wine, displayed a 60% increase in lag time compared to control ($P < 0.01$), but LDL isolated after preincubation with ethanol or wine stripped of phenolic compounds did not. (Figure 3.3).

3.3.5 Macrophage uptake of LDL

The uptake of native LDL by J774 macrophages was similar for LDL isolated from plasma preincubated with water (control), red wine and ethanol (0.65 ± 0.09, 0.58 ± 0.14 and 0.52 ± 0.16 μg LDL/mg cell protein, respectively). Following the copper-mediated oxidation of control and ethanol [^{3}H]CE-LDL, there was almost a three-fold increase in LDL uptake by macrophages to levels of 1.78 ± 0.38 and 1.71 ± 0.29 μg LDL/mg cell protein, respectively. This intracellular accumulation of [^{3}H]CE-LDL following LDL oxidation was significantly decreased ($P<0.001$) to 0.59 ± 0.16 μg LDL/mg cell protein in LDL isolated from plasma which had been preincubated with red wine when compared to control and ethanol samples.

3.3.6 Azo-initiated oxidation of LDL in the presence of red wine

The generation of MDA during azo-initiated LDL oxidation is displayed in Figure 3.4. Control LDL incubated with azo-initiator displayed a significant increase in MDA formation following 2 h oxidation ($P < 0.01$) compared to MDA levels prior to azo-initiated oxidation. This increase in MDA was significant for up to 8 h ($P < 0.001$) and was maximal following 6 h oxidation (33 nmol MDA/mg LDL protein, $P < 0.001$). The LDL oxidised in the presence of red wine containing phenols displayed a concentration-dependent inhibition in MDA formation over time. There was a significant increase in MDA formation after 5 h and 8 h incubations compared to 0 h, in the presence of red wine containing 2.5 mg/L GAE and 5 mg/L GAE, respectively ($P < 0.05$). Red wine containing phenolic compounds at concentrations of 10 mg/L GAE and 20 mg/L GAE inhibited MDA formation for up to 8 h compared to 0 h incubation. In addition, red wine at phenolic concentrations of 10 mg/L GAE and 20 mg/L GAE completely inhibited LDL oxidation when compared to control

levels ($P < 0.001$) at all time points. Only when LDL was oxidised for 8 h in the presence of red wine containing 2.5 mg/L, did MDA formation reach the same levels as control LDL (31.5 compared to 33.0 nmol MDA/mg LDL protein). The LDL oxidised in the presence of dilutions of red wine stripped of phenolic compounds or ethanol displayed no inhibition of MDA concentrations compared to control LDL (data not shown).

The relative electrophoretic mobility (REM) of LDL before (time 0) and after (time 8 h) azo-initiated oxidation was measured. Control LDL samples oxidised for 8 h in the presence of azo-initiator had a REM of 2.01 ± 0.26. This increase in LDL electrophoretic mobility in control LDL was significantly inhibited ($P < 0.05$) to 1.31 ± 0.24 and 1.22 ± 0.15 in the presence of red wine containing 10 and 20 mg/L GAE, respectively.

Figure 3.5 illustrates MDA formation following azo-initiated LDL oxidation in the presence of the different red wine fractions at concentrations of 2.5 mg/L GAE. Following 6 h incubation of azo-initiator and LDL, red wine and all phenolic fractions, except fraction 3, significantly inhibited MDA formation ($P < 0.05$, at least) compared to the ethanol control. Both fraction 2 ($P < 0.01$) and total red wine ($P < 0.05$) displayed a significantly greater antioxidant effect when compared to fraction 3.

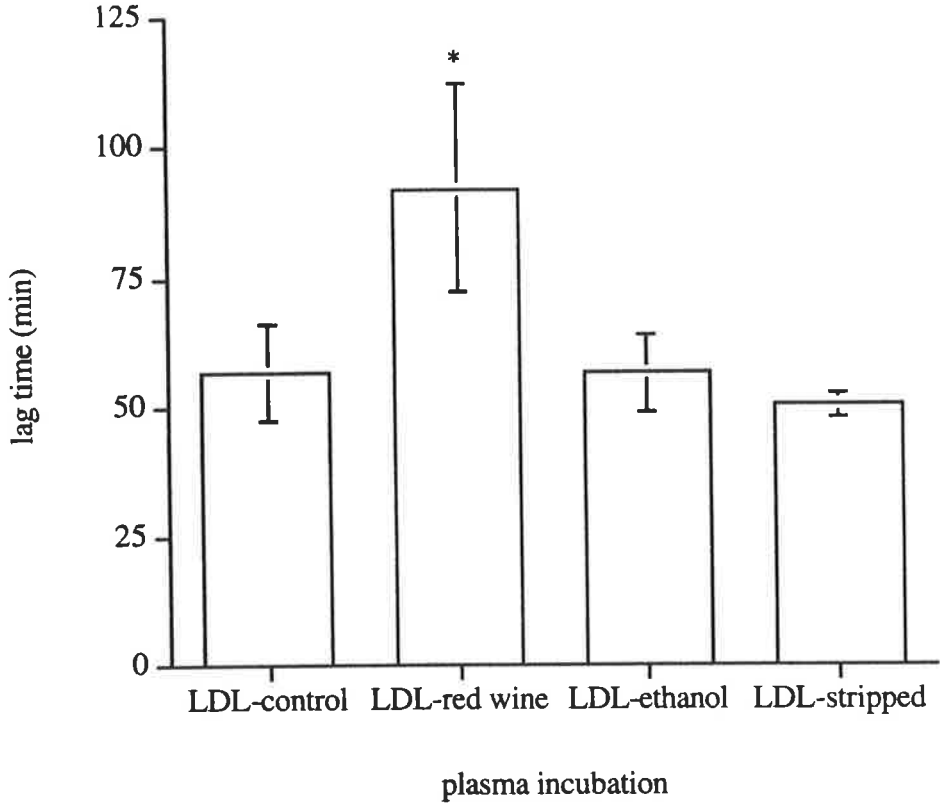


Figure 3.3

The effect of preincubating plasma with red wine, ethanol or red wine stripped of phenols on the lag time of copper-mediated oxidation of isolated LDL.

Plasma was incubated with red wine (75 mg/L GAE), ethanol (0.6%, v/v), wine stripped of phenols (0.6% ethanol, v/v) or water (control) at 37°C for 3 h. LDL was isolated by ultracentrifugation and dialysed prior to copper-mediated oxidation. Oxidation of LDL was measured as lag time for conjugated diene formation. Incubations and oxidation were performed in duplicate and values are mean ± standard deviation (n=3). The (*) denotes significantly to all other groups at $P < 0.05$ as determined by parametric ANOVA with Tukey Kramer multiple comparisons.

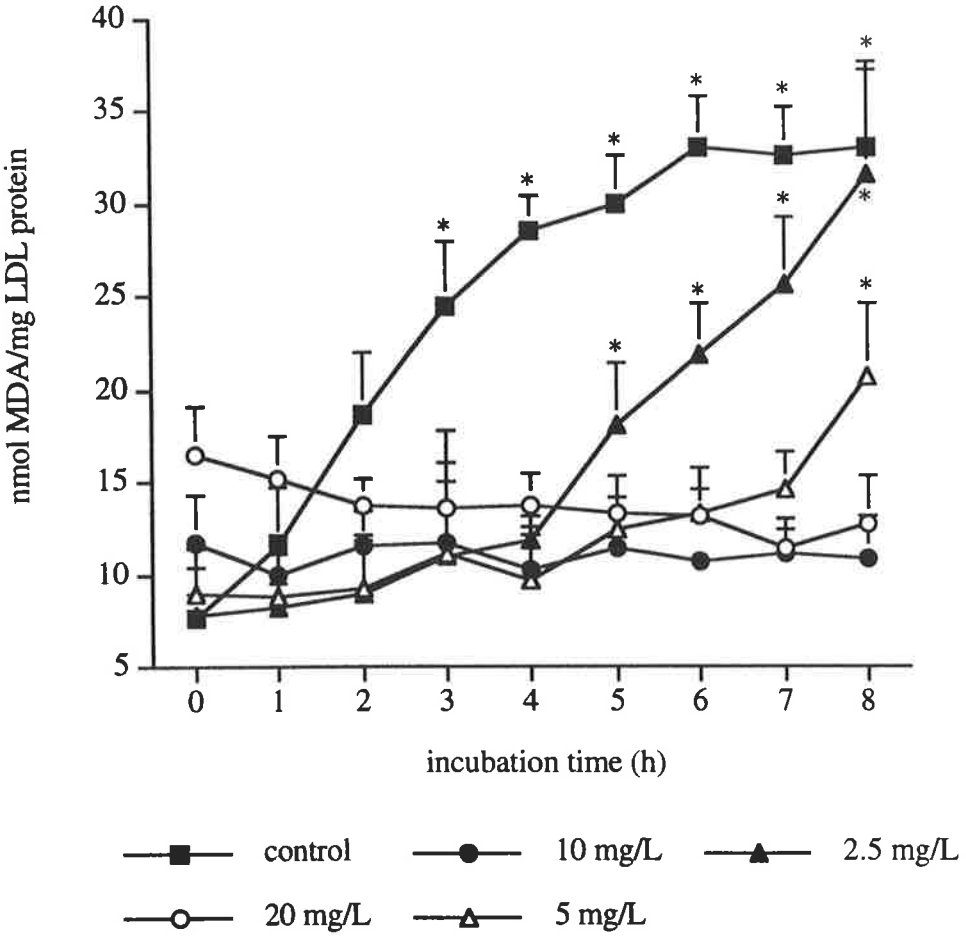


Figure 3.4

The effect of red wine (mg/L GAE) on malondialdehyde concentrations in azo-oxidised LDL.

LDL (100 mg protein/L) was oxidised by peroxy radicals formed from azo-initiator (5 mmol/L) in the presence of red wine (2.5 to 20 mg/L GAE) or control (0.7% ethanol) during incubations in 37°C waterbath. Oxidation of LDL was measured hourly as the concentration of malondialdehyde (MDA) formed as determined by the TBARS assay. Incubations were performed in duplicate and values are mean ± standard deviation (n=3). The (*) denotes significantly different to MDA concentrations prior to azo-oxidation of LDL for each red wine concentration at $P < 0.05$ as determined by parametric ANOVA with Tukey Kramer multiple comparisons.

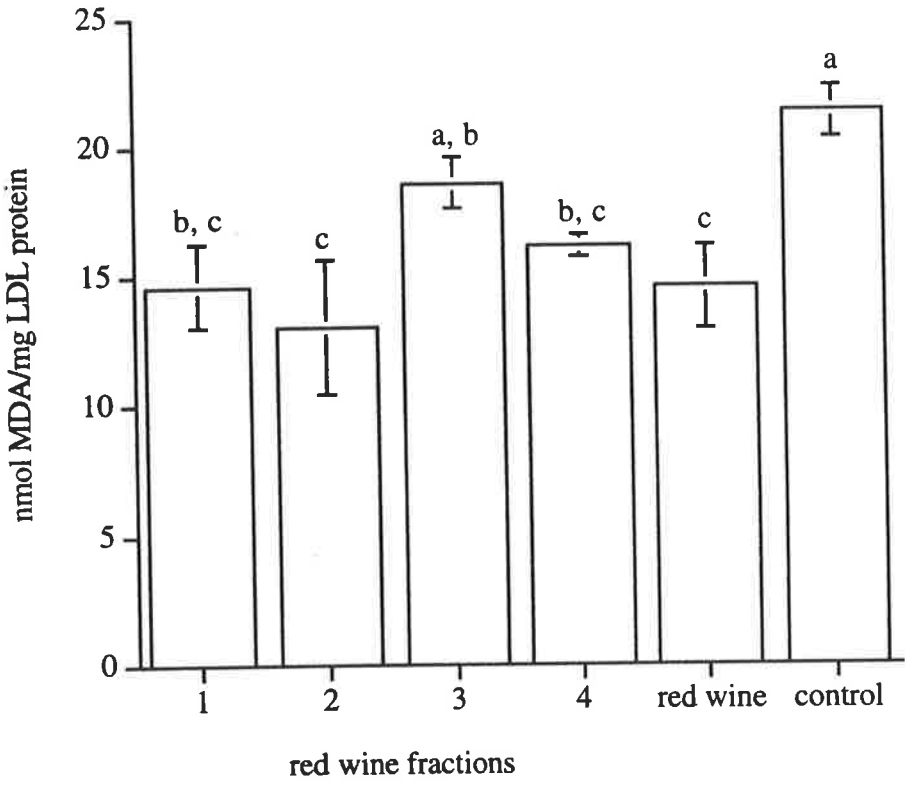


Figure 3.5

The effect of different phenolic fractions (2.5 mg/L GAE) prepared from red wine on malondialdehyde levels in azo-oxidised LDL.

LDL was oxidised by peroxy radicals in the presence of whole red wine (2.5 mg/L GAE), red wine fractions 1 to 4 (2.5 mg/L GAE) or in the absence or red wine (control, 0.7% v/v ethanol). Oxidation of LDL was measured after 6 h incubation with azo-compound as the concentration of malondialdehyde. All oxidations were performed in duplicate and values are mean ± standard deviation (n=3). Columns with differing alphabetic notations are significantly different at $P < 0.05$ as determined by parametric ANOVA with Tukey Kramer multiple comparisons.

3.4 DISCUSSION

This study investigated the effect of differing methods of LDL preparation on α-tocopherol concentrations and subsequent copper-mediated oxidation of LDL. The α-tocopherol concentration in LDL following removal of EDTA, by dialysis or gel filtration chromatography, was the same as LDL which contained EDTA. As expected, LDL containing EDTA was protected against copper-mediated LDL oxidation. The lag time and oxidation rate of copper-mediated LDL were not different between dialysed or gel filtered LDL preparations. Although gel filtration techniques have been proposed as an improved method of LDL preparation, no differences in either LDL-α-tocopherol or oxidation parameters were observed between dialysed and gel filtered LDL.

Scheek *et al.* (1995) reported that LDL which was dialysed to remove EDTA prior to copper-mediated LDL oxidation had a significantly lower α-tocopherol concentration compared to undialysed LDL. The results from the present experiment do not support the findings of Scheek *et al.* (1995). These authors also reported that other endogenous antioxidants in LDL, such as β-carotene and lycopene were also significantly decreased following dialysis, however these antioxidant concentrations were not measured in the present study.

The function of α-tocopherol in *in vitro* models of LDL oxidation is considered to be very important whether it be via an antioxidant or prooxidant role. Some authors support the claim that α-tocopherol is one of the most important lipophilic antioxidants in LDL since it has been reported to be consumed prior to the formation of lipid oxidation products (Esterbauer *et al.* 1989b, Jessup *et al.* 1990) and supplementation of LDL with α-tocopherol protects

against *in vitro* oxidation (Jessup *et al.* 1990, Dieber-Rotheneder *et al.* 1991, Esterbauer *et al.* 1991, Jialal *et al.* 1992, Suzukawa *et al.* 1994). Yet others provide evidence of a prooxidant role of α-tocopherol during *in vitro* LDL oxidation (Bowry *et al.* 1992, Stocker *et al.* 1994, Maiorino *et al.* 1995, Kontush *et al.* 1996a). Although the relevance of *in vitro* oxidation models to the *in vivo* situation are still uncertain, results from the present study provide evidence that the concentration of the endogenous antioxidant, α-tocopherol, is the same in undialysed, dialysed and gel filtered LDL.

The present study has confirmed the *in vitro* antioxidant activity of red wine by inhibiting the oxidation of LDL by copper and azo-initiated systems. The active antioxidant components in red wine appear to be the phenolic compounds since ethanol and wine stripped of phenols did not affect any of the indices of LDL oxidation measured. Furthermore, the LDL isolated from plasma preincubated with red wine displayed a significant 60% increase in lag time (Figure 3.3). When red wine is incubated with isolated LDL and Cu^{2+}, it is not known whether the phenolic compounds are inhibiting oxidation in the surrounding aqueous medium. By incubating red wine with plasma, and then isolating LDL by ultracentrifugation and dialysis, it is assumed that the active antioxidants in red wine are associated with the lipoprotein. The red wine derived antioxidants associated with LDL are capable of inhibiting copper-mediated oxidation of the lipoproteins.

The concentration of red wine required to inhibit copper-mediated LDL oxidation when added directly to LDL *in vitro* or following pre-incubation with plasma differ greatly. In Figure 3.1, red wine containing approximately 0.15 mg/L GAE caused a 60% inhibition in the time for conjugated diene production. In comparison, plasma was incubated with red wine containing 75 mg/L GAE in order to achieve an equivalent inhibitory effect toward LDL oxidation. From this, it is estimated that a 500-fold greater concentration of red wine is required to cause an equivalent inhibition in conjugated diene formation in the two

experiments. Assuming no degradation of red wine polyphenols following incubation with plasma at 37 °C for 3 h, these results suggest that only a very small fraction of the antioxidant compounds in red wine are associated with LDL (approximately 0.2%).

Vinson *et al.* (1995) recently published the results of similar experiments involving the pre-incubation of flavonoid antioxidants with plasma in order to investigate whether these flavonoid compounds could bind to LDL and inhibit copper-mediated LDL oxidation. The authors measured the lipoprotein-bound antioxidant activity of isolated phenols but not in whole red wine. Catechins, phenolic acids and isoflavones inhibited LDL and VLDL oxidation *in vitro* and when bound to lipoproteins with concentrations in the order of 200-600 times greater in the latter experiments. These authors did not measure the concentrations of flavonoids in plasma or LDL.

These experiments involving the preincubation of plasma with antioxidant compounds are relevant to the *in vivo* situation. It is generally hypothesised that lipoprotein oxidation occurs predominantly in the arterial wall following the influx of lipoproteins into the subendothelial space and intima layer (Nordestgaard *et al.* 1994). Antioxidants incorporated within the lipoprotein particle itself, rather than circulating in plasma, may be more effective in protecting against lipid peroxidation and atherogenesis (Steinberg *et al.* 1989). A study by Fuhrman *et al.* (1995) has provided evidence of incorporation of red wine phenols into lipoproteins *in vivo* and its subsequent effectiveness as an antioxidant. Although the concentration of red wine phenols incorporated into LDL particles was not quantified in the present study, Fuhrman *et al.* (1995) have reported polyphenolic levels in LDL equivalent to 1 to 2 μg polyphenolics/50 μg LDL protein following supplementation of healthy subjects with red wine.

In this study it was demonstrated that red wine, by inhibiting LDL oxidation, can also protect against macrophage uptake of [^{3}H]CE-LDL in culture. In this way, antioxidants such as

those in red wine may be incorporated into LDL particles where they protect against lipid peroxidation and subsequent uptake of oxidised LDL by macrophages. The unregulated uptake of oxidised LDL leads to the formation of lipid laden foam cells (Brown and Goldstein 1983). The relevance of the present finding is that red wine protects against changes to the apo B molecule in LDL and prevents the accumulation of lipids by cultured macrophages.

Red wine was effective in inhibiting the oxidation of LDL induced by peroxy radical generation as indicated by the decreased formation of lipid peroxidation products *in vitro* (Figure 3.4). In addition, LDL incubated with red wine did not display an increase in relative electrophoretic mobility which is characteristic of oxidative modification of the apo B molecule in LDL. This study presents evidence that phenolic compounds in red wine can inhibit LDL oxidation in both a radical-dependent and metal-dependent model of oxidation, indicating that phenolic antioxidants are capable of scavenging peroxy radicals *in vitro* and do not elicit their action by merely chelating redox metal ions. Previously there was doubt as to whether the antioxidant activity of red wine was due predominantly to chelation of copper ions or active metal-catalysed oxidation of pre-existing lipid peroxide molecules within LDL (Frankel *et al.* 1993a).

The oxidation of LDL initiated by azo-compound was measured by the formation of malondialdehyde using the TBARS assay. This assay involves the formation of a pink chromophore by the reaction of lipid peroxidation products (carbonyl groups) and thiobarbituric acid. At the highest concentration of red wine (20 mg/L phenols), the anthocyanidins that are responsible for the colour of red wine interfere with the absorbance of the TBAR substances (Figure 3.4). This caused an relatively high MDA value for red wine prior to azo-oxidation of LDL (time 0). The lipid peroxidation products measured by the TBARS assay did not increase following incubation of LDL with the azo-initiator, demonstrating that red wine inhibited LDL oxidation.

The ability of red wine to inhibit plasma and LDL oxidation initiated by an azo compound after human consumption has been documented. A study by Fuhrman *et al.* (1995) recently demonstrated that red wine inhibited azo-initiated MDA formation in whole plasma following a 2 week supplementation of volunteers with red wine. Kondo *et al.* (1994) published similar results which showed that red wine consumption for 14 days significantly decreased azo-initiated oxidation of LDL.

These is currently much interest in the antioxidant activity of red wine and phenolic compounds in general. It is uncertain exactly which of the phenols in red wine exhibit the greatest antioxidant effect. By separating red wine into fractions of differing phenolic composition using a simple rapid separation technique, the contributions of 4 red wine fractions to the antioxidant activity of red wine were investigated. This fractionation technique allows the separation of red wine polyphenols according to their hydrophobicity and degree of polymerisation. The phenolic concentration in each fraction was similar to levels reported elsewhere. (Kerrigan *et al.* 1995).

The red wine fraction with the highest phenol content was fraction 4 (polymeric anthocyanidins), followed by fractions 1 (phenolic acids), 2 (monomeric catechins, procyanidins and monomeric anthocyanidin) and 3 (flavonols). All red wine fractions increased the lag time for copper-mediated LDL oxidation compared to LDL incubated in the absence of red wine. The polymeric anthocyanidins (fraction 4) displayed an antioxidant effect *in vitro* which was similar to that of the phenolic fractions 1 and 3. These highly polymerised phenolic compounds in fraction 4 may be considered to be quantitatively important as antioxidants since they constitute the majority of the phenolic compounds present in red wine, as measured by the fractionation technique. In contrast fraction 3 (flavonols), which contains the lowest level of phenolic compounds in red wine, also possess the lowest antioxidant capacity when compared to the other phenolic compounds in

red wine. In summary, the antioxidant properties of red wine were due primarily to monomeric catechins, procyanidins, monomeric anthocyanidins (fraction 2) and phenolic acids (fraction 1) (see Figure 3.2).

Azo-initiated LDL oxidation was inhibited by red wine fractions 1, 2 and 4. The same antioxidant potency observed for red wine fractions in the copper-mediated LDL oxidation was apparent in the peroxy radical initiated oxidation, that is fraction 2 > fraction 1 fraction 4 > fraction 3 in decreasing order of antioxidant activity. The present experiment provides new evidence that phenolic compounds fractionated from red wine, inhibit LDL oxidation in both metal-dependent and radical-dependent prooxidant systems. One previous study has demonstrated the antioxidant activity of fractionated wine (Petite Syrah wine) (Teissedre *et al.* 1996). Red wine was fractionated by HPLC by using a mobile phase with increasing concentrations of methanol and the antioxidant activity of the phenolic compounds was determined using a copper prooxidant model only. Their findings are in agreement with the present observations which identified phenolic catechins as the most potent inhibitors of copper-mediated LDL oxidation.

In conclusion, red wine is an effective antioxidant *in vitro*. The antioxidant activity is due to phenolic compounds in red wine, since red wine stripped of phenols did not inhibit LDL oxidation. Similarly ethanol alone did not alter the oxidation of LDL in any prooxidant model. It has been demonstrated that phenolic fractions, prepared from red wine, significantly inhibit metal-catalysed and peroxy radical-initiated LDL oxidation. The catechin compounds and phenolic acids present in red wine display similar antioxidant activities, while catechins exhibit a greater antioxidant activity than flavonols and polymeric anthocyanidins. Red wine derived antioxidants can associate with LDL particles following the incubation of red wine with plasma since these LDL samples were less susceptible to copper-mediated LDL oxidation. The amounts of red wine required to achieve this effect though are comparatively high and further studies investigating the bioavailability and the

subsequent antioxidant activity of red wine phenols *in vivo* need to be undertaken.

CHAPTER 4

THE ISOFLAVONE GENISTEIN INHIBITS LDL OXIDATION.

4.1 INTRODUCTION

A naturally occurring polyphenolic compound known to have antioxidant properties is the isoflavone genistein. Genistein is present in soybeans and in soy products, including miso, tofu and soy milk. Much of the research impetus, with regard to genistein, has focused on its potential anticancer properties since it has been shown to possess antiestrogenic effects (Setchell *et al.* 1984), inhibit growth of cancer cells (Akiyama *et al.* 1991), inhibit cellular signal transduction systems (Akiyama *et al.* 1987) and inhibit angiogenesis (Fotsis *et al.* 1993). Several investigators have proposed that soy consumption is one dietary component that may contribute to the lower rates of some cancers in Asian countries compared to Western countries (reviewed by Messina *et al.* 1994).

Genistein exhibits antioxidant effects *in vitro* including the inhibition of ADP and NADPH-dependent lipid peroxidation in rat liver microsomes (Jha *et al.* 1985) and inhibition of the coupled oxidation of β-carotene and linoleic acid (Pratt *et al.* 1979). Record *et al.* (1995) have demonstrated genistein's antioxidant activity in liposomes following a number of prooxidant challenges including UV exposure, peroxy radical and hydroxy radical generating systems. These authors (Record *et al.* 1995) and others (Wei *et al.* 1993) have reported that genistein is an effective scavenger of hydrogen peroxide. These antioxidant effects of genistein *in vitro* have been proposed to contribute to its anticancer properties since there is evidence that reactive oxygen species can cause protein, RNA or DNA damage that could lead to carcinogenesis (Dreosti 1991).

Hertog *et al.* (1993) described an inverse relationship between intakes of dietary flavonoids and the relative risk of coronary heart disease in an elderly Dutch population. A proposed mechanism for the apparent protective effect of flavonoids is the inhibition of lipoprotein oxidation. The oxidation of low density lipoproteins has been implicated in the development of atherosclerosis (reviewed by Steinberg *et al.* 1989).

There have been some reports in the literature concerning genistein's antioxidant properties toward serum and LDL oxidation (Hodgson *et al.* 1996, Rifici *et al.* 1994, Tsai and Chait 1995). Very recently, Hodgson *et al.* (1996) published detailed kinetics describing the effect of genistein and other flavonoids on copper-mediated oxidation. Their results demonstrate that 1 and 10 μmol/L genistein inhibited conjugated diene formation in diluted human serum. Rifici *et al.* (1994), in a paper investigating the effect of tyrosine kinase inhibitors on insulin mediated LDL oxidation, reported that a single concentration of genistein (150 μmol/L) could inhibit copper and cell-mediated oxidation of LDL by 67 and 77%, respectively. Previously Tsai and Chait (1995) reported that genistein was able to inhibit copper-mediated LDL oxidation *in vitro*. These authors performed experiments in which LDL was incubated with genistein and then re-isolated. They found that genistein, in this instance, did not protect LDL against oxidation, suggesting that genistein was not incorporated into LDL, however genistein concentrations in LDL were not measured. The aim of the present study was to investigate the antioxidant effects of genistein against LDL oxidation *in vitro* using two models of prooxidant challenge. In addition to copper-mediated LDL oxidation, the effect of genistein on peroxy radical-mediated oxidation of LDL was investigated. Furthermore the incorporation of genistein into LDL following pre-incubations with plasma was quantitated and the subsequent antioxidant effects were determined.

4.2 METHODS

4.2.1 Copper-mediated oxidation of LDL in the presence of genistein

Genistein was dissolved in 2:1 (v/v) ethanol solution and the concentration was determined using the molar extinction coefficient value ε_{284} (Walter 1941). LDL was oxidised by copper in the presence of 0.2 to 5 μmol/L of genistein (final concentration) or 1.3% ethanol solution (final concentration) as a control sample.

4.2.2 Incorporation of genistein into LDL

To determine whether genistein could be incorporated into LDL, plasma was incubated with 25, 50 or 100 μmol/L genistein (final concentrations) for up to 24 h in a shaking waterbath

set at 37 °C. For control experiments, plasma was incubated with ethanol at the equivalent concentration to that present in the genistein samples. The final ethanol concentration in all plasma samples was approximately 1%. The LDL was isolated from the plasma and dialysed against PBS buffer prior to copper-mediated oxidation in the conditions previously described.

4.2.3 HPLC measurements of LDL and plasma genistein concentrations

The concentrations of genistein in plasma and LDL were determined by HPLC. The method was based on that of King *et al.* (1996). Standard concentrations of genistein in the range 0.05 mg/L to 20 mg/L (0.2 μmol/L to 75 μmol/L) were made up in human plasma obtained from a healthy volunteer. One hundred μl of sample or standard was added to 1.5 ml eppendorf tube containing 100 μl of 0.1 M ammonium acetate buffer (pH 4.6) and 300 μl of water. Genistein was extracted with 500 μl of diethyl ether. The samples were mixed on a rotating wheel for 10 min before being centrifuged at 3000 x *g* for 10 min to separate the organic and aqueous layers. The diethyl ether fraction was removed and the aqueous layer was extracted again with 500 μl of diethyl ether. The two diethyl ether fractions were combined and evaporated to dryness under a stream of nitrogen. The samples were reconstituted in 100 μl of mobile phase for direct injection onto the HPLC.

The column used for the separation of genistein was a C18 (5 μ) radial pak (Waters Associates, Millipore, MA, USA) using a mobile phase of 35 % 0.1 M ammonium acetate buffer (pH 4.6) in 65 % methanol with 1 mM EDTA at a flow rate of 1 ml/min. Dual ultraviolet (UV) detection and electrochemical detection (ECD) were used for the measurement of genistein. The UV detection was set at 260 nm to detect genistein in the concentration range of 0.5 mg/L to 20 mg/L and ECD was set at a voltage of +0.7 to detect genistein at lower concentrations of 0.05 mg/L to 0.5 mg/L. All plasma and LDL samples were assayed in duplicate for the quantification of genistein.

4.2.4 Azo-initiated oxidation of LDL in the presence of genistein

Genistein (final concentration of 50 to 200 μmol/L) was incubated with azo-initiator and LDL. Control LDL samples were also oxidised by azo-initiator with ethanol which was equivalent to that present in the genistein samples. The MDA concentrations and REM of azo-oxidised LDL were determined as described in Chapter 2.

4.2.5 Statistical Analysis

All values are expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) with Tukey Kramer multiple comparisons test was performed on data using Graphpad Instat V2.04 software (Graphpad software, California, USA).

4.3 RESULTS

4.3.1 Copper-mediated oxidation of LDL in the presence of genistein

Copper-mediated LDL oxidation was significantly inhibited by 1, 2.5 and 5 μmol/L genistein ($P < 0.001$) as shown by an increase in lag time (Figure 4.1). There was a decrease in the rate of conjugated diene formation (Figure 4.2) with 2.5 μmol/L ($P < 0.01$) and 5 μmol/L ($P < 0.001$) genistein when compared to control samples.

4.3.2 Incorporation of genistein into LDL

The LDL isolated from plasma after incubation with genistein, displayed no change in copper-mediated LDL oxidation. The lag times before the onset of oxidation in control LDL and LDL isolated from plasma incubated with 25, 50 and 100 μmol/L genistein, were 60.5 ± 9.2 min, 60.9 ± 11.3 min, 63.0 ± 6.3 min and 58.6 ± 8.9 min, respectively.

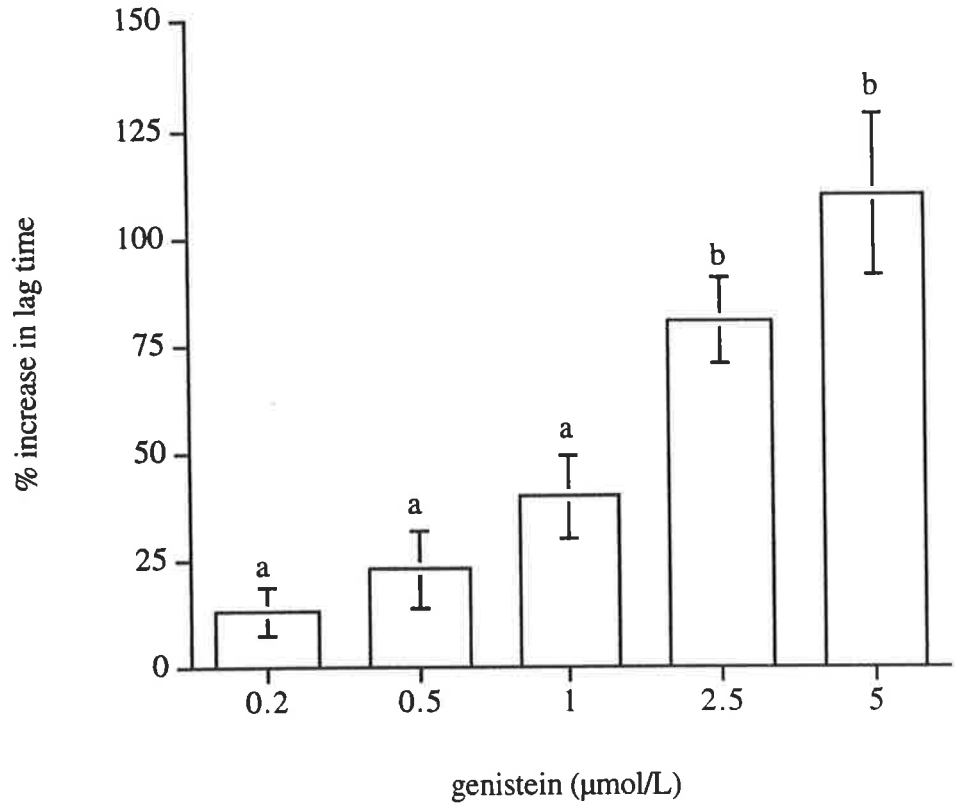


Figure 4.1

The effect of genistein on the lag time of copper-mediated LDL oxidation.

LDL (50 mg protein/L) was oxidised by copper (5 µmol/L) at 37 °C in the presence of 0.2 to 5 µmol/L genistein. Oxidation was determined as the lag time for conjugated diene formation and was expressed as % increase in control LDL lag time. Control LDL was oxidised in the presence of 0.7% ethanol. All oxidations were performed in duplicate and values are mean ± standard deviation (n=3). Columns with differing alphabetic notations are significantly different at $P < 0.05$ as determined by parametric ANOVA with Tukey Kramer multiple comparisons.

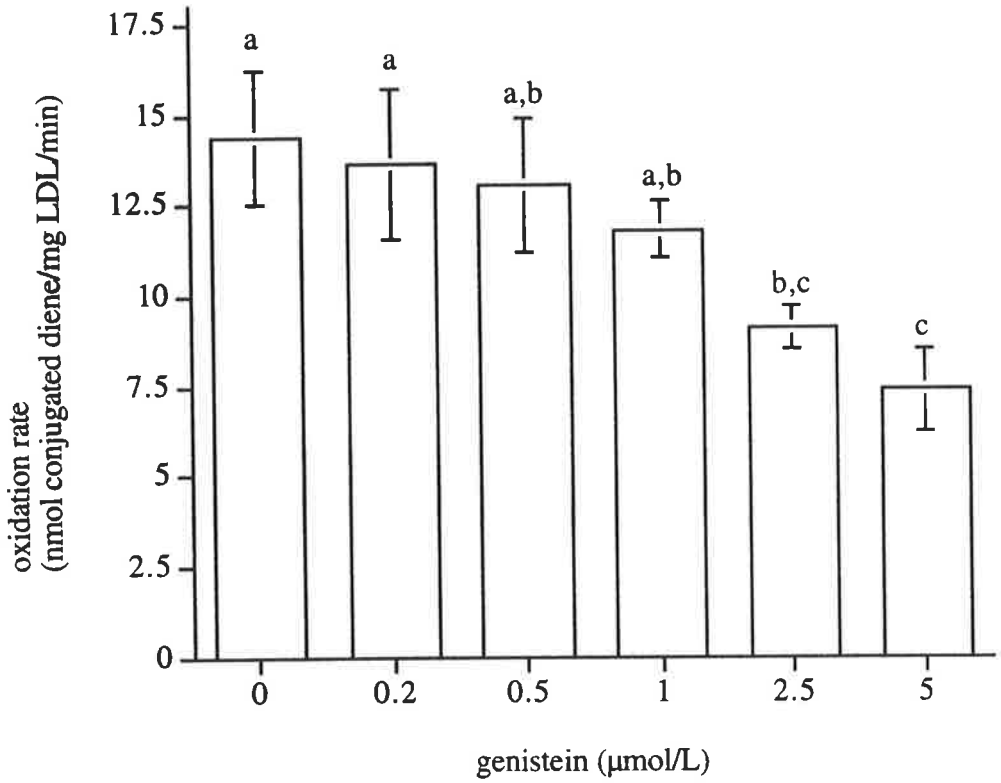


Figure 4.2

The effect of genistein on the oxidation rate of copper-mediated LDL oxidation .

LDL (50 mg protein/L) was oxidised by copper (5 µmol/L) at 37 °C in the presence of 0.2 to 5 µmol/L genistein. Oxidation rate was determined as the slope of the propagation phase of the absorbance profile and was expressed as nmol conjugated diene/mg LDL protein. Control LDL was oxidised in the presence of <% ethanol. All oxidations were performed in duplicate and values are mean ± standard deviation (n=3). Columns with differing alphabetic notations are significantly different at $P < 0.05$ as determined by parametric ANOVA with Tukey Kramer multiple comparisons.

4.3.3 HPLC measurements of LDL and plasma genistein concentrations

Standard curves of genistein concentration versus peak area were linear as indicated by correlation coefficient of r >0.99 for UV detection (0.5 to 20 mg/L or 2 to 75 μmol/L) and ECD (0.05 to 0.5 mg/L or 0.2 to 2 μmol/L). The interassay coefficients of variation for low and high concentrations of genistein measured by UV detection were 7.0% and 1.7% for 0.5 mg/L and 20 mg/L, respectively and 8.8% and 0.9% for 0.05 mg/L and 0.5 mg/L of genistein detected by ECD. The recovery of genistein following extraction with diethyl ether was 84.3% with an interassay coefficient of variation of 10.1%. All the genistein concentrations determined by HPLC have been corrected for this recovery value.

The genistein concentrations in plasma and LDL are presented in Table 4.1. As expected, the plasma genistein concentrations are significantly different to each other (ANOVA, $P<0.0001$). The actual concentrations of genistein in plasma determined by HPLC are approximately equivalent to the theoretical genistein concentration added to plasma indicating a good precision of the HPLC assay.

The incubation of plasma with 25 μmol/L genistein resulted in incorporation of genistein into LDL at a concentration of 0.7 nmol genistein/mg LDL protein. The LDL-genistein concentration was significantly increased (ANOVA $P=0.0006$) to 2.1 and 3.7 nmol genistein/mg LDL protein following plasma incubations with 50 μmol/L and 100 μmol/L genistein, respectively, however the percentage of genistein which was incorporated into LDL was not significantly different with increasing plasma genistein concentrations (Table 4.1).

	Plasma incubation with genistein (µmol/L)		
	25	50	100
plasma genistein (µmol/L)	27.7 ± 3.7 [a]	44.8 ± 1. 3 [b]	90.5 ± 7.0 [c]
LDL genistein (nmol genistein/mg LDL protein)	0.7 ± 0.2 [a]	2.1 ± 0.1 [b]	3.7 ± 0.8 [c]
% incorporation of genistein into LDL	2.8 ± 1.3 [a]	4.0 ± 0.4 [a]	3.6 ± 0.5 [a]

Table 4.1

The concentration of genistein in plasma and LDL samples following incubation experiments.

Plasma was incubated at 37°C for 24 h with genistein (25, 50 or 100 µmol/L). LDL was isolated from plasma by ultracentrifugation and dialysed. Plasma and LDL genistein concentrations were determined by HPLC with dual UV and ECD. Values with differing alphabetic superscripts in the same row are significantly different ($P < 0.05$). All values are means ± standard deviation (n=3).

4.3.4 Azo-initiated oxidation of LDL in the presence of genistein

Two separate comparisons of TBARS data were performed. Firstly comparisons between the TBARS formation over time (0 to 8 h) were performed at each genistein concentration. Significant differences in TBARS formation as determined by malondialdehye equivalents (MDA) are denoted by differing alphabetic superscripts in Table 4.2. In control LDL samples, MDA was significantly increased at 3 h incubation with the azo-initiator compared to MDA levels before oxidation ($P < 0.01$). MDA concentrations increased in control LDL reaching a maximum of 16.0 ± 2.1 nmol MDA/mg LDL protein at 7 h. There were significant delays in azo-initiated LDL oxidation, from 3 h in control LDL, to 4 h ($P < 0.01$) and 7 h ($P < 0.05$) in LDL incubated with 100 and 200 μmol/L genistein, respectively. The lower concentration of genistein, 50 μmol/L, did not significantly alter the time required for LDL oxidation compared to control LDL.

Secondly, comparisons between TBARS formation with varying genistein concentrations at individual time points were performed. Significant differences in MDA are denoted by asterisks in Table 4.2. The formation of MDA in LDL oxidised in the presence of 200 μmol/L of genistein was significantly reduced at 3, 4 and 5 h compared to control incubations and at 4 and 5 h with 100 μmol/L genistein. The lower concentration of genistein, 50 μmol/L, did not significantly decrease MDA levels compared to control samples at any time point.

Following azo-initiated oxidation, the negative charge on the apolipoprotein B-100 moiety of LDL, as measured by relative electrophoretic mobility (REM), was significantly decreased in the presence of 200 μmol/L genistein, 1.13 ± 0.02, when compared to control LDL, 1.31 ± 0.01 ($P < 0.001$, unpaired t-test).

Table 4.2

The effect of genistein on malondialdehyde concentrations in azo-oxidised LDL.

	Incubation time (h)								
	0	1	2	3	4	5	6	7	8
control	4.3±0.9 [a]	6.5±0.7 [a]	7.8±0.3 [a,b]	10.1±0.1 [b,c]	13.8±1.3 [c,d]	15.1±1.2 [d]	15.5±2.3 [d]	16.0±2.1 [d]	14.2±1.8 [d]
50µmol/L genistein	4.6±1.3 [a]	5.6±0.2 [a,b]	7.3±0.5 [a]	8.7±0.6 [b,c]	11.4±0.3 [c,d]	12.5±0.6 [c,d]	12.9±1.8 [d]	14.9±1.4 [d]	14.4±1.8 [d]
100µmol/L genistein	4.8±0.9 [a]	5.0±0.9 [a]	7.0±0.3 [a,b]	8.2±0.1 [a,b,c]	10.0±0.3 [b,c,d,*]	11.1±0.4 [b,c,d,*]	11.7±1.4 [c,d]	13.5±2.1 [d]	12.8±2.3 [d]
200µmol/L genistein	6.2±2.1 [a]	5.9±0.3 [a]	6.1±0.7 [a]	6.9±0.2 [a,b,*]	7.8±0.7 [a,b,*]	8.2±0.1 [a,b,*]	9.0±0.6 [a,b]	10.0±1.2 [b]	9.0±0.6 [a,b]

The effect of genistein on the formation of MDA (nmol MDA/mg LDL protein) following azo-initiated LDL oxidation. LDL (100 mg protein/L) was incubated with azo-compound (5 mmol/L) at 37°C for up to 8 h. MDA levels were measured hourly during incubation. All samples were measured in duplicate. All values are mean ± standard deviation of n=3 experiments. An ANOVA with Tukey Kramer multiple comparison was performed on the data comparing the MDA concentration at individual genistein concentrations (0, 50, 100 and 200 µmol/L) over time (0 to 8 h). Values with differing alphabetic superscripts in the same row are significantly different from other time points at the same genistein concentration. In a separate ANOVA, the effect of differing genistein concentrations on MDA levels were compared at each time point (0 to 8 h). The asterisk (*) denotes significantly different to MDA concentration in control LDL at the same time point.

4.4 DISCUSSION

This present study demonstrated that genistein can inhibit LDL oxidation *in vitro*. The results are in agreement with reports from previous authors (Hodgson *et al.* 1996, Rifici *et al.* 1994, Tsai *et al.* 1995) which have demonstrated an antioxidant effect by genistein during copper or cell-mediated oxidation in LDL and serum. In the present study a concentration dependent antioxidant effect by genistein was observed. An increase in lag time (13 to 109%) of copper mediated oxidation of LDL was observed in the presence of 0.2 to 5 μmol/L of genistein. In addition, genistein inhibited LDL oxidation initiated by an azo compound, albeit at high concentrations (200 μmol/L genistein). This is the first report of an antioxidant effect of genistein in peroxy radical-initiated oxidation of LDL.

Genistein was a more effective antioxidant in the metal-dependent prooxidant system compared to peroxy radical-initiated LDL oxidation. These results may indicate that genistein inhibits LDL oxidation by a predominantly ion chelating mechanism. However, Record *et al.* (1995) have reported that genistein did not exhibit significant iron chelating ability *in vitro* as determined by changes in the absorption spectrum of micelles in the presence of $FeCl_3$/catechol solution. These authors also reported that genistein was an ineffective antioxidant against peroxy radical-mediated oxidation of linoleic acid micelles.

The precise antioxidant mechanism of genistein is unclear. Some authors have described the structure activity relationship for flavonoids and their antioxidant effects (Rice-Evans *et al.* 1996, van Acker *et al.* 1996). Genistein is a trihydroxy compound which does not possess the 3 hydroxy substitute on the C ring which has been identified as an important requisite for both chelating and radical scavenging activity. Genistein does however have a ketone functional group and 2,3 carbon double bond on the C ring and 3 phenolic hydroxy groups attached to the ring structures which also contribute to the antioxidant properties of flavonoid compounds.

An aim of this study was to determine whether genistein could be incorporated into LDL and exhibit antioxidant properties. The amount of incorporation into LDL following pre-

incubations of plasma with genistein was equivalent to 0.7 to 3.6 nmol genistein/mg LDL protein. The LDL-genistein concentration was dependent on the concentration of genistein in plasma and the overall incorporation of genistein into LDL was approximately 3-4% of the plasma concentration. These levels of genistein in LDL are greater than that of endogenous antioxidants such as lycopene (0.3 nmol/mg LDL protein) and ubiquinol-10 (0.2 nmol/mg LDL protein), although lower than α-tocopherol (11.6 nmol/mg LDL protein) (Esterbauer *et al.* 1991). However at these concentrations in LDL, genistein did not inhibit copper-mediated oxidation. Genistein was an effective antioxidant at concentrations of 2.5 μmol/L in copper-mediated LDL oxidation when it was added directly to the aqueous solution containing LDL. In comparison, the concentration of genistein incorporated into LDL following pre-incubation with plasma was approximately 30-fold lower (0.7 to 3.6 nmol genistein/mg LDL protein) than the effective antioxidant concentration in the aforementioned experiments.

In a previous study by Tsai and Chait (1995) genistein was incubated with LDL and the oxidisability of the reisolated LDL was determined. These authors reported no changes in the copper-mediated oxidation of LDL. Although the levels of genistein were not quantified in that study, the authors suggested that genistein was not incorporated into LDL. The lack of antioxidant effect by genistein following preincubation with plasma in the present experiments support the findings of Tsai and Chait (1995). In addition, HPLC analysis performed in the present study revealed that genistein was present in LDL at very low concentrations which were not sufficient to protect against *in vitro* LDL oxidation. The concentration of genistein in plasma, as measured by HPLC, was within 90% of the theoretical concentration of genistein that was added to plasma. These results indicate very little or no oxidation or degradation of genistein during plasma incubations at 37°C for 24 h. The poor binding of genistein to LDL is therefore not due to a decreased concentration of genistein in plasma. Although genistein was poorly incorporated into LDL and did not inhibit LDL oxidation in the present study, the possibility that genistein acts as an antioxidant

in plasma or tissues remains to be determined. Indeed, Hodgson *et al.* (1996) have demonstrated that genistein is an effective antioxidant in diluted human serum.

The poor incorporation or binding of genistein to LDL in the present study is supported by observations from Lehtonen *et al.* (1996) which demonstrated that genistein did not bind to liposomal bilayers *in vitro*. In these experiments by Lehtonen *et al.* (1996), daidzein, another soy derived isoflavone, was incorporated into phospholipid bilayers. These studies demonstrate the ability of some isoflavones, but not others, to bind to phospholipids. Daidzein and its metabolites, equol and O-desmethylangolensin are also effective inhibitors of copper-mediated oxidation of serum *in vitro* (Hodgson *et al.* 1996). It may be that the isoflavone daidzein, with its antioxidant activity and phospholipid binding properties, could afford protection against *in vivo* plasma and LDL oxidation.

A study investigating the oxidisability of LDL following genistein or soy supplementation in humans has been performed. Nestel *et al.* (personal communication) did not find a difference in LDL oxidation in menopausal women consuming 40 or 80 mg of isoflavones for up to 10 weeks. The plasma concentrations of isoflavones, genistein and daidzein, were not measured in the study by Nestel *et al.* (personal communication) so it is possible that the concentrations of genistein in LDL were too low to inhibit oxidation, as was observed in the present *in vitro* study.

Much of the information regarding the bioavailability of genistein in human subjects has been determined following dietary supplementation of isoflavone rich soy products rather than genistein itself. The plasma concentrations of genistein used in the present *in vitro* studies were greater than plasma concentrations in Japanese men consuming a traditional soy diet (116 to 652 nmol/L, Adlercreutz *et al.* 1993) and subjects receiving 80 mg of genistein daily in the form of a soy supplement (900 nmol/L, Gooderham *et al.* 1996). Recently King *et al.* (personal communication) measured plasma genistein concentrations of 4 μmol/L, eight hours after ingestion of a soy meal containing 3.6 μmol genistein/kg body weight. The plasma concentrations of genistein achieved in that study are comparable to the

concentrations of genistein (2.5 μmol/L) required to inhibit copper-mediated LDL oxidation in the present study. However, in the *in vitro* situation it was shown that genistein was poorly incorporated into LDL, so although plasma genistein concentrations of 4 μmol/L are attainable following ingestion of isoflavone rich soy meals, the concentration of genistein in LDL would probably be low.

Taken together, the results of this study and those performed by others (Tsai and Chait 1995, Hodgson *et al.* 1996), indicate that genistein would be a more effective antioxidant in plasma rather than LDL. This is assuming that genistein is present in plasma in the aglycone form. Studies have shown that genistein is present in plasma predominantly as a glucuronide following dietary supplementation of isoflavones (Adlercreutz *et al.* 1993). In the present study, the antioxidant activity of genistein in the aglycone or free form was measured. The antioxidant activity of genistein in the conjugated form is not known, but from what is understood about isoflavones structure-antioxidant activity relationship, it is likely that the presence of glucuronide conjugates would decrease genistein's antioxidant activity (Cook *et al.* 1996). Further studies investigating the *in vivo* distribution of genistein and its antioxidant properties in whole plasma following dietary intervention would need to be performed in order to clarify these *in vitro* observations.

CHAPTER 5

THE EFFECT OF RED WINE ON PLASMA LIPIDS, LIPOPROTEINS AND PHYTOSTEROLS IN CHOLESTEROL-FED RABBITS.

5.1 INTRODUCTION

Cholesterol-fed rabbits are a commonly used model of diet-induced hypercholesterolemia and atherosclerosis. Dietary cholesterol in rabbits leads to elevated plasma cholesterol which is incorporated into lipoproteins, particularly VLDL. The VLDL from cholesterol-fed rabbits displays β-migratory properties on agarose gel electrophoresis and is often referred to as β-VLDL (Shore *et al.* 1974, Thompson *et al.* 1983, Kroon *et al.* 1985). VLDL in cholesterol-fed rabbits could originate from an increased hepatic synthesis and secretion of VLDL or from intestinal chylomicron remnants (Thompson *et al.* 1983, Kroon *et al.* 1985, MacKinnon *et al.* 1985). MacKinnon *et al.* (1985) demonstrated an increase in hepatic secretion of VLDL in cholesterol-fed rabbits as measured by recirculating liver perfusion experiments in anaesthetised rabbits. The mechanism for hypercholesterolemia in cholesterol-fed rabbits was further elucidated in studies by Kovanen *et al.* (1981) which demonstrated a decrease in hepatic lipoprotein receptors normally involved in the removal of VLDL containing apolipoproteins E and B from the plasma. Therefore rabbits fed cholesterol are unable to increase sterol excretion resulting in an increased residency time of lipoproteins in the circulation and accumulation of cholesterol-rich lipoproteins in aortic tissue.

With this established model of diet induced hypercholesterolemia and atherosclerosis in rabbits, the aim of this experiments was to investigate the effects of red wine and ethanol on lipoprotein metabolism. In the present study, lipid and lipoprotein data from rabbits fed a normal chow diet have been included. Although these results are interesting for comparison with cholesterol-fed rabbits, the normocholesterolemic group of rabbits is not a necessary control for comparing the effects of red wine or ethanol on lipoprotein measurements since rabbits in these treatment groups were fed a cholesterol-enriched diet.

Both epidemiological (Kannel *et al.* 1988, Castelli *et al.* 1977, Hulley *et al.* 1981) and intervention studies (Valimaki *et al.* 1988, Clevidence *et al.* 1995, Lecomte *et al.* 1996) have

demonstrated that alcohol elevates plasma triglycerides, HDL-cholesterol and apolipoprotein-AI and II levels. These changes in lipids and lipoproteins may contribute to the cardioprotective effects of moderate doses of alcohol observed in epidemiological studies (St Leger *et al.* 1979, Renaud *et al.* 1992a). Only one study has investigated the effect of alcoholic beverages in animals consuming an atherogenic diet. Klurfeld *et al.* (1981) demonstrated an increase in HDL-cholesterol levels in rabbits consuming a casein-cholesterol diet for 12 weeks in combination with ethanol alone, red and white wine. This effect was not observed in rabbits fed the atherogenic diet and other alcoholic beverages such as beer or whisky. The aim of the present study was to fully characterise the plasma lipid profiles and lipoprotein compositions in rabbits fed 0.25 to 0.5% cholesterol in conjunction with red wine or ethanol consumption.

A second aim of this study was to investigate whether red wine containing polyphenols could alter the absorption of dietary cholesterol in cholesterol-fed rabbits. Previous studies have shown that green tea catechins (Chisaka *et al.* 1988, Ikeda *et al.* 1992) and grape seed tannins (Besancon *et al.* 1994) inhibit intestinal absorption of labelled cholesterol in rats. Alternatively Klurfeld *et al.* (1979) demonstrated that ethanol consumption enhances cholesterol absorption in rats. Based on these observations, it was hypothesised that red wine containing grape-derived polyphenols and ethanol could alter dietary cholesterol absorption in rabbits. Differences in dietary cholesterol absorption by rabbits could also help to explain the large variability in diet-induced hyperlipidemia common in this animal model as previously demonstrated by Beynen *et al.* (1989).

5.2 METHODS

5.2.1 Animal Study: Dietary intervention with red wine and ethanol in cholesterol-fed rabbits

Twenty four adult (5 to 6 months old) male New Zealand White rabbits (IMVS, Gilles Plains, SA, Australia) were housed in individual cages at CSIRO animal facility (O'Halloran Hill, SA, Australia). Ethics approval for the study was obtained from the University of Adelaide and CSIRO Division of Human Nutrition Animal Ethics Committees. The rabbits were housed in surroundings of controlled temperature (20 ± 1°C) and a 12 h light cycle (06:00 to 18:00). All rabbits were maintained in this environment for at least 4 weeks prior to the commencement of experiments. In addition rabbits selected for red wine or ethanol treatment were involved in a 2 to 3 week lead-in phase prior to cholesterol feeding, whereby the beverage was administered at increasing concentrations of ethanol in order to maximise intake.

The rabbits were grouped into 4 different treatment protocols. Control rabbits (n=6) were maintained on a normal commercially available rabbit pellet diet (Ridley Agriproducts, Murray Bridge, SA, Australia) and the remaining 18 rabbits were fed a cholesterol-enriched diet. The cholesterol diet was prepared by simply grinding the rabbit pellets and mixing with powdered cholesterol before repelleting. Initially all cholesterol-fed rabbits were fed a diet containing 0.25% cholesterol (wgt/wgt). Following 5 weeks of treatment the cholesterol content in the diet was increased to 0.5% (wgt/wgt). Of the 18 cholesterol-fed rabbits, 6 rabbits were administered red wine, 6 rabbits ethanol and 6 rabbits water, while the 6 rabbits fed normal diet were also administered water.

The red wine used in the study was made from grapes of the Cabernet Sauvignon variety. The total phenol content of red wine was measured by the Folin-Ciocalteu assay (see Chapter

2). In addition the amounts of catechins, procyanidins, anthocyanidins, hydroxycinnimates and non-phenolic compounds in red wine were measured by the Australian Wine Research Institute, Glen Osmond, SA, Australia (see Table 5.1). The alcohol content of the red wine was diluted from 13.7% to 7.5% (v/v) with water. A similar concentration of aqueous ethanol (7.5%, v/v) was prepared as a control for this experiment. Bottled red wine was diluted with water and purged with nitrogen for 10 mins to minimise any oxidation of the sample. Sterile 50 ml screw top tubes were filled to the top with red wine or ethanol, capped, sealed with parafilm and stored at -20°C. These tubes were stored for no longer than two weeks thereby minimising wine oxidation. Drinking water was withdrawn from rabbits in the morning and replaced with a 40 ml aliquot of red wine, ethanol or water in a 50 ml tube which was capped with a rubber stopper fitted to a drinking spout. Daily consumption of beverages was measured over a 4 hour period.

Rabbits were fasted overnight and blood samples for lipid analysis were taken from the ear artery of rabbits prior to dietary intervention and then at weeks 3, 6 and 9 of treatment. On days of blood sampling, rabbits were not administered any red wine or ethanol. In some rabbits, a non-fasted blood sample was taken following red wine consumption in order to quantify plasma catechin levels. Following 12 weeks of diet treatment rabbits were sacrificed. Rabbits were anaesthetised by inhalation of halothane in oxygen (4%) and exsanguinated by aortic puncture at the ileac bifurcation. Blood was collected into EDTA-tubes. Plasma was isolated by centrifugation at 3000 x *g* for 10 min at 4°C. Plasma samples were stored at -80°C. The entire aorta, from the ascending arch to the ileac bifurcation was carefully removed, divided into 3 segments and placed in ice-cold physiological salt solution (PSS). The upper thoracic segment of the aorta was used in organ bath experiments to investigate vascular function (see Chapter 7). The remaining segment of descending thoracic aorta adjacent to the abdominal aorta was frozen in liquid nitrogen and stored at -80 °C for determination of artery cholesterol content (see Chapter 6). The aortic arch and abdominal aorta were fixed and stained for atheroma assessment (see Chapter 6).

Table 5.1

Composition of red wine

Compound		Red Wine (Cabernet Sauvignon)
Catechin (mg/L)		60
Epicatechin (mg/L)		80
Procyanidin (Seed tannin equivalents, mg/L)		400
Anthocyanidins (Malvidin equivalents, mg/L)		300
Hydroxycinnamates (Caffeic acid equivalents, mg/L)		83
Alcohol	(%)	13.8
Glucose/Fructose	(mg/L)	300
Sulphur dioxide free	(mg/L)	9
Sulphur dioxide total	(mg/L)	40
Acetic acid	(mg/L)	570
Glycerol	(mg/L)	8700
Ascorbic acid	(mg/L)	9
Citric acid	(mg/L)	100
Succinic acid	(mg/L)	1000
Malic acid	(mg/L)	100
Lactic acid	(mg/L)	1500
Tartaric acid	(mg/L)	2300
pH		3.25

All analysis was performed at the Australian Wine Research Institute, Glen Osmond, SA,

5.2.2 Plasma lipid measurements

Plasma cholesterol and triglyceride concentrations were measured (see Chapter 2) at time 0, 3, 6, 9 and 12 weeks of treatment. HDL-cholesterol measurements in whole plasma using a polyethylene glycol precipitation method could not be performed due to the hyperlipidemic nature of some rabbit plasma samples.

5.2.3 Isolation of lipoprotein fractions by sequential ultracentrifugation

Lipoprotein fractions containing VLDL + IDL (density 1.006 to 1.019 g/ml), LDL (density 1.019 to 1.063 g/ml) and HDL (density 1.063 to 1.21 g/ml) were isolated from fasting rabbit plasma at completion (week 12) of the dietary intervention. The cholesterol, triglyceride and protein levels were determined in each of these lipoprotein fractions. The identity of LDL was confirmed by comparing the electrophoretic mobility of rabbit LDL (d 1.019 to 1.063 g/ml) to that of human LDL on a 3 to 13% precast polyacrylamide electrophoresis gel (Gradipore, North Ryde, NSW, Australia).

5.2.4 Dietary cholesterol absorption

Cholesterol absorption was determined by measuring plasma concentrations of the phytosterols, campesterol and sitosterol, following 12 weeks of dietary treatment. Standard solutions of the sterols were prepared in hexane in the concentration ranges of 1 to 40 mg/L campesterol and 0.4 to 12 mg/L sitosterol. The method for sterol detection in plasma was published by Wolthers *et al.* (1991). Two hundred µl of standard solutions were added to glass tubes containing the internal standard, 5β-cholestan-3α-ol, dried under a stream of nitrogen and then reconstituted in 200 µl of water. For preparation of plasma samples, internal standard was added to glass tubes and evaporated to dryness under nitrogen. Two hundred µl of plasma was then added to these tubes. The standards and plasma samples were hydrolysed by the addition of 100 µl of 33% potassium hydroxide and 2 ml ethanol and incubated for 30 min in a waterbath set at a temperature of 60°C. Sterols were extracted

by the addition of 1 ml distilled water and 2 ml hexane. After mixing, the upper hexane layer was collected and evaporated to dryness under nitrogen. Methyl esters were formed by incubating the samples with 100 µl of the derivatising agent, Trisil, for 30 min at 80°C. Liquid extraction was again performed by the addition of 4 ml hexane and 4 ml water. After mixing, the lower aqueous phase was discarded and the remaining hexane phase was washed with a further 4 ml of water. The hexane phase was evaporated to dryness under nitrogen and reconstituted in hexane for injection onto a gas chromatograph (GC).

The GC conditions were as described by Wolther *et al.* (1991). The GC used was a DANI 6500 with a split/splitless injection system (split ratio 1:20) set at a temperature of 250°C and a vitreous silica capillary column (25 cm x 0.25 mm, 1 mm film thickness). The carrier gas was hydrogen. The retention times of the sterols were campesterol 11 min, sitosterol 13 min and internal standard 7 min.

5.3 RESULTS

5.3.1 Phenolic content of red wine

The phenolic content of the red wine was 2906 ± 163 mg/L (n=10) gallic acid equivalents (GAE). The red wine was diluted with water to give a final ethanol concentration of 7.5% (v/v) and in so doing, the final phenolic concentration was also diluted to 1614 mg/L GAE before being administered to rabbits.

5.3.2 Daily intakes of red wine

The consumption of alcoholic beverages by rabbits is expressed as an average daily intake over the 12 week intervention period. The daily average intake of red wine by rabbits (n=6) was 22.5 ± 3.3 ml which was equivalent to 36.3 ± 5.3 mg GAE and 1.7 ± 0.2 g ethanol per day. Although ethanol-treated rabbits (n=6) were provided with the same volume of alcoholic beverage as red wine-treated rabbits (40 ml/d) they consumed a smaller volume of ethanol (12.4 ± 0.4 ml) which was equivalent to an ethanol intake of 0.9 ± 0.1 g/d.

5.3.3 Rabbit body weights

The body weights of the rabbits at the commencement of the study were the same between all treatment groups (control group 3.27 ± 0.12 kg, cholesterol 3.31 ± 0.06 kg, cholesterol + red wine 3.34 ± 0.08 kg and cholesterol + ethanol 3.34 ± 0.07 kg). After 12 weeks of dietary intervention the body weights of the rabbits were similar for control group 3.43 ± 0.18 kg, cholesterol 3.35 ± 0.08 kg, cholesterol + red wine 3.33 ± 0.12 kg and cholesterol + ethanol 3.32 ± 0.08 kg. The body weights of the rabbits did not significantly change over the 3 month intervention with any dietary treatment.

5.3.4 Plasma lipid measurements

Plasma cholesterol levels at the beginning of the dietary intervention study were not significantly different between treatment groups (control 0.67 ± 0.16 mmol/L, cholesterol 0.55 ± 0.12 mmol/L, cholesterol + red wine 0.66 ± 0.06 mmol/L and cholesterol + ethanol 0.63 ± 0.09 mmol/L). The plasma cholesterol concentration versus time profiles are shown in Figure 5.1. Throughout the 12 week experimental period, the plasma cholesterol concentration in control rabbits remained unchanged (week 0, 0.67 ± 0.16 mmol/L and week 12, 0.52 ± 0.04 mmol/L). Cholesterol feeding alone and in combination with red wine or ethanol increased plasma cholesterol levels ($P<0.001$) at weeks 3, 6, 9 and 12 of treatment compared to control rabbits. There were no significant differences in plasma cholesterol levels between cholesterol-fed rabbits and those drinking red wine or ethanol at any time. The cholesterol exposure in rabbits was determined by calculating the area under the curve of the plasma cholesterol versus time graph shown in Figure 5.1. The cholesterol exposure in cholesterol-fed rabbits 129.9 ± 31.4 mmol/L ($P <0.001$), cholesterol + red wine rabbits 139.2 ± 31.8 mmol/L ($P <0.001$) and cholesterol + ethanol 100.0 ± 19.8 mmol/L ($P <0.001$) were significantly greater than that in control rabbits 7.0 ± 0.5 mmol/L. There were no significant differences in the cholesterol exposure between red wine or ethanol treated rabbits and rabbits fed cholesterol alone.

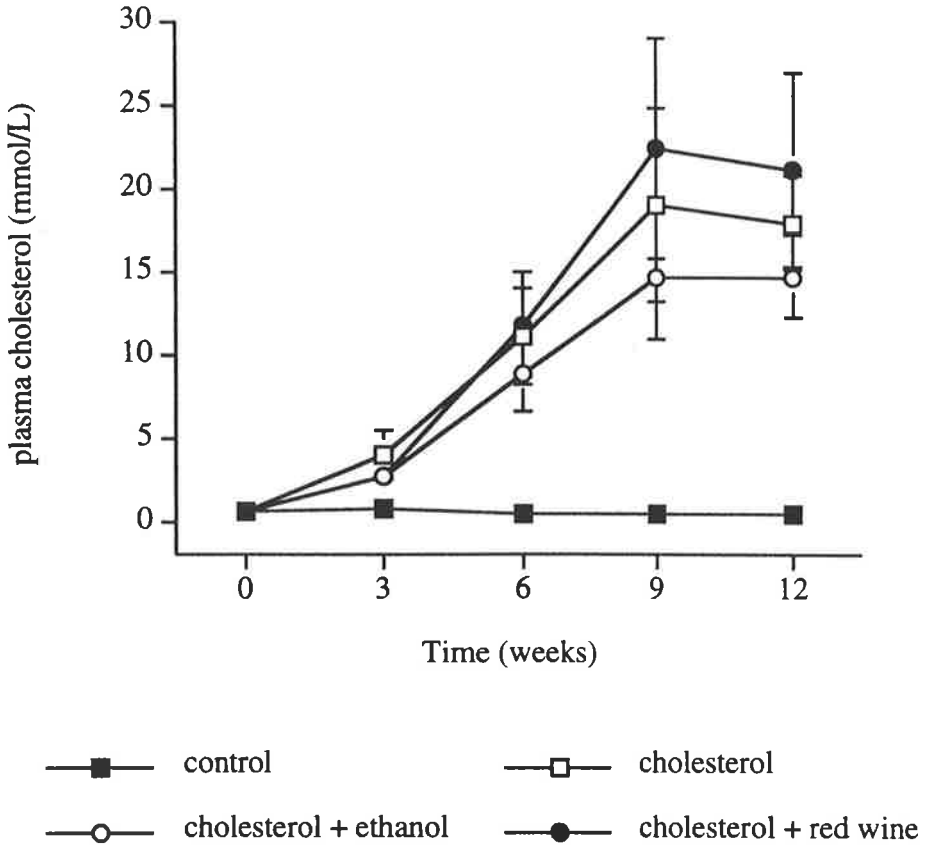


Figure 5.1

Plasma cholesterol levels in rabbit plasma following dietary intervention with cholesterol, red wine or ethanol.

Rabbits were fed a diet containing 0.25 or 0.5% cholesterol (n=6), cholesterol + red wine (n=6), cholesterol + ethanol (n=6) or a normal chow diet (n=6) for 12 weeks. Blood from fasted rabbits was collected from the marginal ear artery at weeks 0, 3, 6, 9 and 12 and the plasma cholesterol concentrations were determined by enzymatic assay on Cobas Bio automated centrifugal analyser. Plasma cholesterol levels in normocholesterolemic controls were significantly lower ($P < 0.001$) compared to all other treatment groups at weeks 3, 6, 9 and 12 as determined by parametric ANOVA with Student Newman Keuls multiple comparison test of the logged data.

Table 5.2 shows the triglyceride concentrations in rabbit plasma. At commencement of the dietary intervention there were no significant differences in plasma triglyceride levels between control rabbits 0.82 ± 0.13 mmol/L, cholesterol 0.55 ± 0.04 mmol/L, cholesterol + red wine 0.85 ± 0.22 mmol/L and cholesterol + ethanol 0.63 ± 0.03 mmol/L. Red wine in combination with cholesterol caused a significant lowering of plasma triglyceride levels after 3 weeks of treatment ($P < 0.05$) compared to rabbits fed a normal chow only. Although mean triglyceride levels appeared to increase at weeks 6, 9 and 12 in cholesterol-fed, red wine and ethanol treated rabbits, there was more variation which meant no statistically significant increases in plasma triglyceride levels were reached.

5.3.5 Plasma lipoprotein composition

Cholesterol concentrations increased in the VLDL + IDL, LDL and HDL fractions in cholesterol-fed rabbits. Overall, red wine or ethanol treatment in conjunction with dietary cholesterol did not markedly alter the composition of the lipoproteins compared to rabbits fed cholesterol alone. Triglyceride and protein concentrations in the VLDL + IDL fraction increased in cholesterol-fed rabbits and this was unchanged following red wine or ethanol-treatment (Table 5.3).

The mobility of rabbit LDL was the same as human LDL isolated in the density range 1.019 to 1.063 g/ml as determined by gradient polyacrylamide gel electrophoresis (data not shown), indicating that the lipoproteins in this density were appropriate to use for LDL oxidation experiments. The composition of the LDL fraction was characterised by elevated cholesterol, triglyceride and protein concentrations in rabbits fed a high cholesterol diet alone or in combination with red wine or ethanol (Table 5.4). Cholesterol-feeding increased HDL-cholesterol, decreased HDL-triglyceride, while HDL-protein levels remained unchanged even in rabbits supplemented with red wine or ethanol (Table 5.5).

	Rabbit treatment				
Triglyceride (mmol/L)	control	cholesterol	cholesterol + red wine	cholesterol + ethanol	ANOVA P value
week 0	0.82 ± 0.13	0.55 ± 0.04	0.85 ± 0.22	0.63 ± 0.03	0.277
week 3	0.61 ± 0.09 [a]	0.45±0.03 [a,b]	0.38 ± 0.02 [b]	0.52±0.03 [a,b]	0.041
week 6	0.51 ± 0.03	0.64 ± 0.11	0.80 ± 0.16	0.71 ± 0.11	0.332
week 9	0.48 ± 0.04	0.83 ± 0.22	0.94 ± 0.22	0.89 ± 0.15	0.216
week 12	0.94 ± 0.14	2.00 ± 0.50	4.13 ± 1.93	1.63 ± 0.10	0.067

Table 5.2

Plasma triglyceride concentrations in rabbits following dietary intervention with cholesterol, red wine or ethanol.

Plasma triglycerides were measured by enzymatic assay on Cobas Bio centrifugal autoanalyser. Values are mean ± sem (n=6). Values in the same row with differing alphabetic superscripts are significantly different as determined by ANOVA with Student Newman Keuls multiple comparison test of the logged data.

Table 5.3

Lipid and protein concentrations in VLDL + IDL isolated from rabbit plasma following dietary intervention with cholesterol, red wine or ethanol.

	Rabbit treatment				
VLDL + IDL fraction (d 1.006 to 1.019 g/ml)	control	cholesterol	cholesterol + red wine	cholesterol + ethanol	ANOVA P value
cholesterol (mmol/L)	0.09 ± 0.03 [a]	13.61 ± 2.53 [b]	17.14 ± 5.63 [b]	10.65 ± 2.39 [b]	<0.0001
triglyceride (mmol/L)	0.38 ± 0.08 [a]	1.55 ± 0.32 [b]	1.54 ± 0.43 [b]	1.22 ± 0.17 [b]	0.0013
protein (g/L)	0.12 ± 0.02 [a]	1.95 ± 0.35 [b]	2.14 ± 0.84 [b]	1.59 ± 0.42 [b]	<0.0001

Lipoprotein fractions were isolated by sequential density ultracentrifugation and cholesterol and triglyceride were determined by enzymatic assays performed on Cobas Bio centrifugal autoanalyser. Protein determinations were performed by Lowry method adapted for Cobas Bio centrifugal autoanalyser using BSA standards. Values with differing alphabetic superscripts in the same row are significantly different as determined by parametric ANOVA with Student Newman Keuls multiple comparison test of the logged data. Values are mean ± sem (n=6).

Table 5.4

Lipid and protein concentrations in LDL isolated from rabbit plasma following dietary intervention with cholesterol, red wine or ethanol.

	Rabbit treatment				
LDL fraction (d 1.019 to 1.063 g/ml)	control	cholesterol	cholesterol + red wine	cholesterol + ethanol	ANOVA P-value
cholesterol (mmol/L)	0.05 ± 0.01 [a]	2.18 ± 0.35 [b]	2.61 ± 0.36 [b]	1.53 ± 0.24 [b]	<0.0001
triglyceride (μmol/L)	21.4 ± 2.9 [a]	63.9 ± 9.8 [b]	80.2 ± 23.8 [b]	82.5 ± 8.8 [b]	0.0012
protein (mg/L)	27.8 ± 8.2 [a]	397 ± 36 [b]	430 ± 84 [b]	313 ± 50 [b]	<0.0001

Values with differing alphabetic superscripts in the same row are significantly different as determined by parametric ANOVA with Student Newman Keuls multiple comparison test of the logged data. Values are mean ± sem (n=6).

Table 5.5

Lipid and protein concentrations in HDL isolated from rabbit plasma following dietary intervention with cholesterol, red wine or ethanol.

	Rabbit treatment				
HDL fraction (d 1.063 to 1.21 g/ml)	control	cholesterol	cholesterol + red wine	cholesterol + ethanol	ANOVA P-value
cholesterol (µmol/L)	233 ± 30[a]	609 ± 84[b]	1006 ± 165[b]	600 ± 61[b]	<0.0001
triglyceride (µmol/L)	146 ± 7[a]	58 ± 8[b]	69 ± 13[b]	69 ± 11[b]	0.0009
protein (mg/L)	646 ± 48	461 ± 64	573 ± 67	531 ± 61	0.184

Values with differing alphabetic superscripts in the same row are significantly different as determined by parametric ANOVA with Student Newman Keuls multiple comparison test of the logged data. Values are mean ± sem (n=6).

The recovery of cholesterol in the lipoprotein fractions isolated from cholesterol-fed rabbits was approximately 90% and the distribution of cholesterol within the 3 main lipoprotein fractions (VLDL + IDL, LDL and HDL) is shown in Figure 5.2. The % distribution of cholesterol in VLDL + IDL fraction was approximately 75 to 80% in cholesterol-fed rabbits, 12 to 15% in LDL fraction and 5% in HDL fractions. In rabbits fed cholesterol, the % distribution of cholesterol in VLDL + IDL and HDL fractions, but not LDL fraction, were significantly greater ($P < 0.001$) than control rabbits. Dietary treatment with red wine or ethanol did not alter the % cholesterol distribution in any lipoprotein fraction when compared to the group fed cholesterol alone.

The ratios of VLDL:HDL-cholesterol, LDL:HDL-cholesterol and VLDL + IDL + LDL: HDL-cholesterol are shown in Table 5.6. The ratios of atherogenic lipoproteins to HDL-cholesterol were increased in all rabbits with dietary supplementation with cholesterol when compared to control rabbits. The ratios of VLDL + IDL and LDL to HDL-cholesterol were lower in red wine and ethanol-treated rabbits compared to cholesterol-fed rabbits, although these results did not reach significance.

5.3.6 Dietary cholesterol absorption

Plasma concentrations of phytosterols were not significantly different in cholesterol-fed rabbits (cholesterol 49.2 ± 8.9 mg/L, cholesterol + red wine 40.3 ± 15.8 mg/L and cholesterol + ethanol 37.4 ± 6.2 mg/L). The plasma ratios of phytosterols (campesterol + sitosterol): cholesterol were determined in rabbits fed a high cholesterol diet. These ratios provide an index of dietary cholesterol absorption efficiency. The dietary cholesterol absorption by rabbits consuming cholesterol alone (7.18 ± 0.97) or cholesterol in combination with red wine (6.14 ± 0.66) or ethanol (6.84 ± 0.59) were not significantly different. Cholesterol absorption in rabbits fed a normal chow diet was not measured since these rabbit's diet was not supplemented with dietary cholesterol.

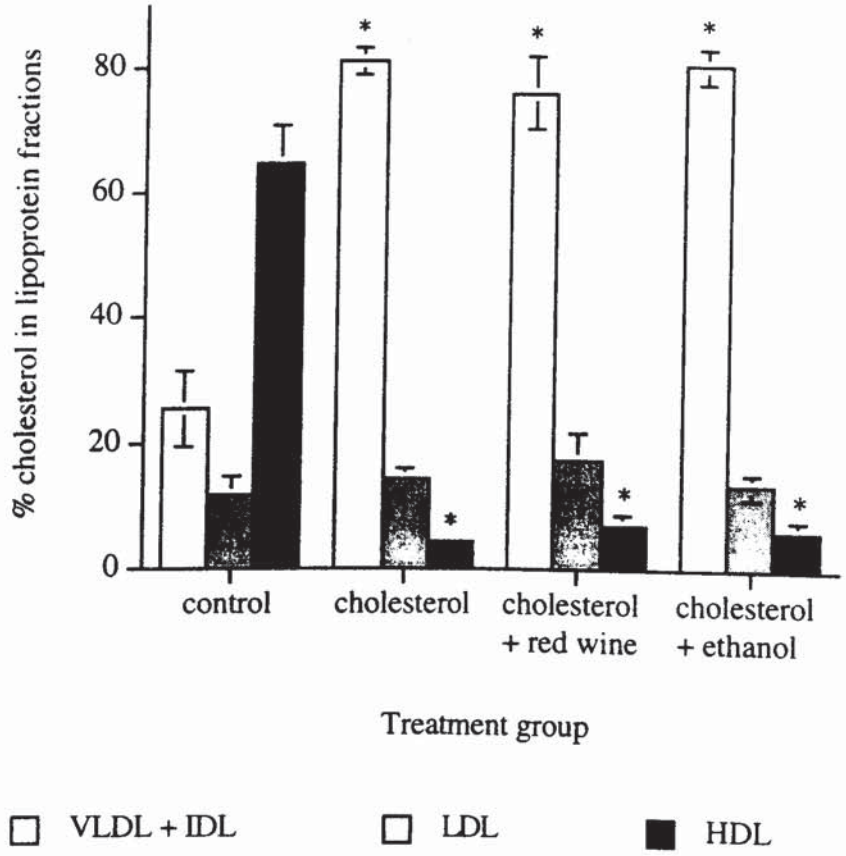


Figure 5.2

Distribution of cholesterol in lipoprotein fractions isolated from rabbit plasma following dietary intervention with cholesterol, red wine or ethanol.

Lipoproteins, VLDL + IDL (1.006 to 1.019 g/ml), LDL (1.019 to 1.063 g/ml) and HDL (d 1.063 to 1.21 g/ml) were isolated from rabbit plasma by sequential ultracentrifugation. Cholesterol levels in lipoproteins were determined by enzymatic kits on Cobas Bio automated centrifugal analyser. The recovery of lipoproteins, in terms of cholesterol concentrations, were approximately 90% in all fractions. The % cholesterol in each lipoprotein fraction was expressed as a percentage of total plasma cholesterol levels. All columns are means ± sem (n=6). The (*) denotes significantly different to control values at P <0.001 as determined by parametric ANOVA and Student Newman Keuls multiple comparisons of the logged data.

Ratio of cholesterol in lipoprotein fractions	Rabbit treatment			
	control	cholesterol	cholesterol + red wine	cholesterol + ethanol
VLDL + IDL: HDL-cholesterol	0.5 ± 0.1 [a]	23.7 ± 4.3 [b]	19.0 ± 6.1 [b]	18.6 ± 5.0 [b]
LDL: HDL-cholesterol	0.2 ± 0.1 [a]	4.1 ± 0.9 [b]	3.0 ± 0.6 [b]	2.8 ± 0.6 [b]
VLDL + IDL + LDL: HDL-cholesterol	0.7 ± 0.2 [a]	27.8 ± 5.1 [b]	22.0 ± 6.4 [b]	21.4 ± 5.5 [b]

Table 5.6

Ratios of cholesterol concentrations in lipoproteins isolated from rabbit plasma following dietary intervention with cholesterol, red wine or ethanol.

Lipoproteins, VLDL + IDL (1.006 to 1.019 g/ml), LDL (1.019 to 1.063 g/ml) and HDL (d 1.063 to 1.21 g/ml) were isolated from rabbit plasma by sequential ultracentrifugation. Cholesterol concentrations in lipoprotein fractions were determined by enzymatic kits on Cobas Bio automated centrifugal analyser and were expressed as a ratios in the different lipoprotein fractions. All values are means ± sem (n=6). Values with different alphabetic superscripts in the same row are significantly different ($P < 0.001$) as determined by parametric ANOVA with Student Newman Keuls multiple comparison test of the logged data.

5.4 DISCUSSION

In this present study lipid and lipoprotein measurements in control rabbits and rabbits fed cholesterol with or without red wine or ethanol were determined. Rabbits fed a cholesterol-rich diet had elevated plasma cholesterol levels which were associated with increases in cholesterol in lipoprotein fractions. In normolipidemic rabbits the major lipid in VLDL + IDL fraction is triglyceride. Following diet-induced hypercholesterolemia, VLDL + IDL becomes the major cholesterol carrying lipoprotein, which in this experiment was characterised by a 100 to 200-fold increase in VLDL-cholesterol levels. Similarly, the cholesterol concentration in the atherogenic LDL fraction also increased approximately 30 to 50-fold in cholesterol fed rabbits. The lipid and protein compositions of VLDL + IDL, LDL and HDL fractions isolated from rabbits consuming cholesterol with red wine or ethanol were not different to rabbits fed cholesterol alone.

The intake of red wine by rabbits in this study is similar to that in humans consuming moderate amounts of red wine (Fuhrman *et al.* 1995, Kondo *et al.* 1994, Maxwell *et al.* 1994, Whitehead *et al.* 1995). Rabbits weighing 3.35 ± 0.19 kg consumed approximately 22.5 ml red wine/d which is equivalent to 6.7 ml/d/kg body weight. This is comparable to a red wine intake of 300 to 600 ml/d in human intervention trials assuming an average body weight of 70 kg in healthy volunteers (4 to 9 ml/d/kg body weight). These levels of red wine consumption over a period of 2 to 4 weeks in normocholesterolemic or hypercholesterolemic subjects were effective in inhibiting both plasma and LDL oxidation (Fuhrman *et al.* 1995, Kondo *et al.* 1994, Maxwell *et al.* 1994, Whitehead *et al.* 1995).

Plasma cholesterol profiles in rabbits supplemented with cholesterol in combination with moderate amounts of red wine or ethanol were not significantly different to rabbits fed cholesterol alone. There have been no studies showing altered total plasma cholesterol levels following dietary intervention with red wine in hypercholesterolemic or normocholesterolemic humans (Struck *et al.* 1994, Lavy *et al.* 1994), rabbits (Klurfeld *et al.* 1981) or rats

(Ruf *et al.* 1995). In comparison ethanol has been shown to increase plasma cholesterol levels in non-human primates (Hojnacki *et al.* 1994) and cholesterol-fed rabbits (Shiash *et al.* 1997).

In the present study the levels of plasma triglyceride were consistently higher in all cholesterol-fed rabbits after 12 weeks of dietary intervention, although not significantly. The concentrations of triglycerides in VLDL + IDL and LDL fractions increased by 3 to 4-fold, however triglyceride levels decreased in HDL fraction in rabbits fed cholesterol. A hypertriglyceridemic effect of either alcoholic beverage, red wine or ethanol, over and above the already raised triglyceride levels associated with cholesterol supplementation in rabbits was not observed in this study.

Ethanol consumption increases plasma triglyceride levels, HDL-cholesterol and HDL protein concentrations in human epidemiological studies (Kannel *et al.* 1988, Castelli *et al.* 1977, Hulley *et al.* 1981) and intervention studies (Valimaki *et al.* 1988, Clevidence *et al.* 1995, Lecomte *et al.* 1996). In healthy volunteers, Lavy *et al.* (1994) investigated the effects of red or white wine supplementation on many biochemical and haematological parameters including plasma lipids and lipoproteins concentrations. In normocholesterolemic subjects (plasma cholesterol 5.2 mmol/L) consuming 400 ml of red wine daily (equivalent to 40 g alcohol/d) for 2 weeks, plasma triglyceride concentrations significantly increased, as did HDL-cholesterol and apo A-I levels. The increases in plasma triglyceride, HDL-cholesterol and HDL protein reported by Lavy *et al.* (1994) were not observed in subjects consuming the same amount of white wine even though the alcohol concentration was the same. These observations indicate that raised plasma triglyceride and increased HDL levels in these subjects are independent of ethanol, or that some other constituent present in red, but not white wine, contributes to the changes in lipid and lipoprotein parameters.

Assuming an average body weight of 70 kg in healthy human subjects, the intake of ethanol in the study by Lavy *et al.* (1994) was equivalent to 0.6 g/d/kg body weight. In the present study no alterations in lipid or lipoprotein profile were apparent in rabbits consuming ethanol at a dose of 0.3 g/d/kg body wgt or red wine consisting of 0.5 g ethanol /d/kg body wgt. The most likely explanation for the lack of effect of red wine or ethanol on plasma triglycerides and HDL levels could be that the rabbits in our study were also consuming moderately high levels of cholesterol, which may have outweighed any ethanol or red wine-mediated alterations in lipid and lipoprotein measurements. Alternatively, ethanol may undergo different hepatic metabolism in rabbits which alter its interaction with lipoproteins although no evidence to support this conclusion were provided by these experiments.

In animals, the effect of ethanol on the levels and composition of circulating lipoproteins is variable. Interpretation of the available data is difficult since many studies differ on a number of critical details including the animal model used, the dose of ethanol administered and the duration of ethanol administration. In non-human primates, administration of incremental doses of ethanol (1.2 to 7.1 g ethanol/d), in the form of vodka, produced differences in plasma lipids and lipoproteins (Hojnacki *et al.* 1994). Linear increases in plasma cholesterol, HDL-cholesterol and apolipoprotein-A1 were apparent in the ethanol dose range of 3.5 to 7.1 g ethanol/d. This is many times greater than the dose of ethanol administered to rabbits in our study, either as red wine or ethanol. Lower doses of ethanol (< 3.5 g ethanol/d) had modest or no effect on lipid or lipoprotein profiles in non-human primates as reported by Hojnacki *et al.* (1994). These results indicate that the effect of ethanol on lipids and lipoproteins is dose related but that at lower doses of ethanol these effects are absent.

Furthermore, lipoprotein metabolism differs between animal models. HDL is the major circulating lipoprotein in non-human primate models, such as that used by Hojnacki *et al.* (1994) in their studies investigating the effects of ethanol on lipoprotein metabolism. At least in humans it is proposed that alcohol can increase plasma HDL levels by increasing the

hepatic synthesis and secretion of apolipoproteins, as well as by altering the activity of enzymes such as lipases and cholesteryl ester transfer protein which may also contribute to increases in plasma HDL (reviewed by Savolainen and Kesaniemi 1995). The major lipoprotein in cholesterol-fed rabbits is VLDL, and little is known about the effect of ethanol on lipoproteins of this density.

Rabbits seem to be a little used model for investigating the effects of ethanol on lipoprotein metabolism. One study by Klurfeld *et al.* (1981) administered red wine and other alcoholic beverages to rabbits fed an atherogenic diet for 12 weeks. The major findings of that study, in terms of lipoprotein measurements, was that red wine and ethanol increased HDL-cholesterol concentrations. A more comprehensive study by Shaish *et al.* (1997) investigated ethanol consumption in rabbits fed an atherogenic diet, and its effect on lipoprotein levels and atherosclerosis. Their results indicate an atherogenic effect of alcohol as measured by increases in lesion area and artery cholesterol content. This apparent atherogenic effect was associated with alcohol induced increases in total, VLDL and LDL-cholesterol concentrations, while HDL-cholesterol and hepatic mRNA levels of apo A-I, a major apolipoprotein in HDL, remained unchanged.

In the present study rabbits consumed more ethanol in the red wine treatment group (1.7 g/d) compared to rabbits supplemented with 7.5% (v/v) ethanol (0.9 g/d). Despite diluting alcoholic beverages to the same ethanol concentration, red wine consumption was greater than ethanol consumption in rabbits. Prior to the commencement of the dietary experiment, rabbits were exposed to the alcoholic beverages at very dilute concentrations of ethanol which were gradually increased over a period of days and weeks until consumption of at least 10 ml (0.75 g ethanol/d) was achieved. Throughout the study period the volume of alcoholic beverage available to the rabbits was the same (40 ml/d), however the palatability of ethanol was inferior to that of red wine. This difference in ethanol consumption between the treatments groups did not however affect lipid and lipoprotein profiles in cholesterol-fed

rabbits.

The major cholesterol carrying lipoprotein fraction in our study of cholesterol-fed rabbits was VLDL+IDL (density <1.019 g/ml). This observation in cholesterol-fed rabbits has been well documented in the literature (Shore *et al.* 1974, Brattsand 1976, Thompson *et al.* 1983, Kroon *et al.* 1985, Daley *et al.* 1994a). In this present study, cholesterol feeding increased the circulating levels of VLDL + IDL and LDL (1.019 to 1.063 g/ml) as shown by increases in both lipid and protein concentrations in lipoproteins. The % distribution of cholesterol in VLDL + IDL fraction increased from 25% in control rabbits to 80% in cholesterol-fed rabbits but remained unchanged at 12% in the LDL fraction. Daley *et al.* (1994a) reported that in rabbits fed 0.125 to 0.5% cholesterol for 24 weeks, 30% of plasma cholesterol was present in the LDL fraction (density 1.019 to 1.063 g/ml) with another 60% cholesterol in VLDL + IDL (density < 1.019 g/ml). Although the LDL-cholesterol levels in rabbits fed cholesterol by Daley *et al.* (1994a) reached approximately 3.5 mmol/L, the % of total plasma cholesterol present in LDL was not different to control rabbits. This is comparable to our results in which total plasma and LDL-cholesterol were significantly greater in cholesterol-fed compared to control rabbits, but the % distribution of cholesterol in LDL between these treatment groups was not different. These results indicate that cholesterol feeding increases the amount of LDL particles without altering their composition at least with respect to cholesterol. The findings of the present study are supported by results published by Shiash *et al.* (1997). Dietary treatment with 10, 20 and 30% ethanol (% of calories as alcohol) in rabbits raised VLDL+IDL and LDL levels, while the relative proportions of cholesterol, triglyceride and phospholipid remained the same in these lipoprotein fractions.

The balance between VLDL, LDL and HDL-cholesterol concentrations provide an index of atherogenic versus anti-atherogenic lipoprotein levels. It has been demonstrated in data from the Framingham study that elevated LDL-cholesterol is associated with an increased risk of

CAD and inversely, elevated HDL-cholesterol (Kannel *et al.* 1977) and lower LDL:HDL-cholesterol ratio (Castelli 1984) are linked to a reduced risk. Since VLDL is the major cholesterol carrying lipoprotein in cholesterol-fed rabbits, the ratios of lipoprotein cholesterol concentrations in VLDL + IDL (density 1.006 to 1.019 g/ml) and LDL (density 1.019 to 1.063 g/ml) to the cholesterol levels in HDL (density 1.063 to 1.21 g/ml) were determined. In this present study, rabbits drinking red wine displayed slight but not significant increases in HDL-cholesterol concentrations which resulted in favourable VLDL:HDL-cholesterol and LDL:HDL-cholesterol balances. Ethanol consumption by rabbits appeared to lower VLDL and LDL cholesterol concentrations compared to rabbits fed cholesterol alone, also leading to lower VLDL:HDL-cholesterol and LDL:HDL-cholesterol ratios. These results from the present study should be considered with caution since they did not reach significance at the 0.05 level, but do in part follow the trends of previous studies which have demonstrated beneficial LDL:HDL cholesterol ratios (Klurfeld *et al.* 1981).

Dietary cholesterol absorption efficiency was not different in rabbits fed cholesterol alone or in combination with red wine or ethanol. Dietary cholesterol and plant sterols form water-soluble micelles which are absorbed from the gastrointestinal tract. In the present study, the ratio of plasma phytosterol:cholesterol was used as a marker for dietary cholesterol absorption. Studies by Tilvis *et al.* (1986) and Miettinen *et al.* (1990) demonstrated that concentrations of plasma phytosterols correlates with fractional absorption of labelled dietary cholesterol and that this relationship was further strengthened when plasma phytosterol levels were expressed as a ratio of plasma cholesterol. In this way the ratio of phytosterol:cholesterol in plasma can be used as an index of cholesterol absorption efficiency.

Previous studies have shown that green tea catechins (Chisaka *et al.* 1988, Ikeda *et al.* 1992) and grape seed tannins (Besancon *et al.* 1994) inhibit intestinal absorption of labelled cholesterol in rats, whereas ethanol consumption enhances cholesterol absorption (Klurfeld

et al. 1979). Based on these findings, it was hypothesised that red wine containing catechins, procyanidins (condensed tannins) and ethanol, or ethanol alone, could alter dietary cholesterol absorption in rabbits. Furthermore, Beynen *et al.* (1989) have shown that differences in dietary cholesterol absorption efficiency contributes to the variations in plasma cholesterol concentrations common in rabbit models of diet-induced hypercholesterolemia. In their study, rabbits with elevated plasma cholesterol concentrations in response to dietary cholesterol displayed greater cholesterol absorption efficiency than non-responding rabbits as measured by the fractional absorption of ^{3}H-cholesterol and ^{14}C-sitosterol. It was hypothesised that differences in dietary cholesterol absorption capacity between rabbits could contribute to the variability observed in plasma cholesterol concentrations in our study. However using the phytosterol technique, no differences in dietary cholesterol absorption efficiency in rabbits fed a high cholesterol diet were apparent.

Red wine may contain phytosterols which originate from grapes. Phytosterols in red wine could have increased the dietary intake of phytosterols in rabbits drinking red wine and in turn increased plasma phytosterol concentrations. Although the phytosterol content of red wine was not measured, the plasma campesterol and sitosterol concentrations were not different between rabbit groups. If phytosterols were present in red wine, they failed to affect the dietary cholesterol absorption. In summary, the ability to absorb dietary cholesterol was the same in cholesterol-fed rabbits and rabbits fed cholesterol in combination with red wine or ethanol.

In summary, the extensive hyperlipidemia induced by cholesterol feeding in rabbits may conceal any red wine or ethanol mediated changes to plasma lipids and lipoproteins. Overall, red wine or ethanol drinking did not markedly alter plasma lipid profiles or lipoprotein compositions in cholesterol-fed rabbits. Modest changes in LDL and HDL-cholesterol concentrations and favourable LDL:HDL cholesterol ratios were observed following red wine and ethanol intervention, although the changes were not significant.

CHAPTER 6

THE EFFECT OF RED WINE ON LDL OXIDATION, FATTY ACIDS, α-TOCOPHEROL AND AORTIC FATTY STREAK DEVELOPMENT IN CHOLESTEROL-FED RABBITS.

6.1 INTRODUCTION

It is hypothesised that antioxidants can decrease the development of atherosclerosis by inhibiting oxidation of LDL. Many studies have demonstrated that dietary antioxidants such as vitamin E (Williams *et al.* 1992), probucol (Kita *et al.* 1987, Carew *et al.* 1987, Daugherty *et al.* 1989, Mao *et al.* 1991, Nagano *et al.* 1992) and butylated hydroxytoluene (BHT) (Bjorkhem *et al.* 1991) can decrease the extent of aortic lesion development in hypercholesterolemic animal models. In most instances, this inhibition of atherosclerosis is associated with a decrease in oxidisability of LDL and occasionally a cholesterol-lowering effect is also observed.

Klurfeld *et al.* (1981) has published a study investigating the effect of alcohol consumption on atherosclerosis development in cholesterol/casein fed rabbits. The extent of atherosclerotic lesions in the aortic arch, thoracic aorta and small myocardial arteries was decreased in rabbits supplemented with red wine. The authors did not examine the effect of red wine on lipid peroxidation in these rabbits nor did they quantitate the absorption of any red wine polyphenols. They could not attribute the antiatherogenic effect of red wine to ethanol since the other alcoholic beverages had no effect on atherosclerosis progression. It was suggested that components in red wine other than ethanol may be responsible for the observed antiatherogenic effect. These findings have not been confirmed by other researchers. In light of the emerging evidence that red wine possesses antioxidant properties (Chapter 3), it would be meaningful to conduct a study investigating the effect of red wine consumption on LDL oxidation and fatty streak development in rabbits fed a high cholesterol diet.

6.2 METHODS

6.2.1 Animal Study: Dietary intervention with red wine and ethanol in cholesterol-fed rabbits

The dietary intervention with red wine or ethanol in cholesterol-fed rabbits is described in Chapter 5 (5.2.1). All experiments were performed in n=6 rabbits for each treatment group.

6.2.2 LDL isolation and oxidation

Following 12 weeks of treatment the LDL fraction (density 1.019 to 1.063 g/ml) was isolated from rabbit plasma. The LDL from cholesterol-fed rabbits could not be isolated by the single-step, rapid ultracentrifugation technique (Chung *et al.* 1987) described in Chapter 2, due to the hyperlipidemic nature of the rabbit plasma. Furthermore rabbit LDL appears to be low in β-carotene which is responsible for the distinctive orange colour of the LDL fraction isolated from human plasma, therefore the LDL band was not visible. Instead, the LDL fraction was isolated from rabbit plasma by sequential ultracentrifugation as described in Chapter 2. Following isolation, the LDL fraction was immediately dialysed in phosphate-buffered saline for LDL oxidation experiments.

Copper-mediated oxidation of LDL was performed as described in Chapter 2 with the following modifications, 2.5 μmol/L of copper was used instead of 5 μmol/L and the oxidation was conducted for 6 hours so as to complete the 3 phases of the LDL oxidation absorbance profile. The lower concentration of copper was used in the present experiments to lengthen the time prior to LDL oxidation so that small changes in lag time may be detected. The concentration of LDL used, 50 mg/L protein, was the same as previous experiments. The indices of LDL oxidation determined were (i) lag time (mins), (ii) oxidation rate (nmol conjugated diene/mg LDL protein/min), (iii) maximum diene concentration (nmol diene/mg LDL protein) and (iv) TBARS (nmol malondialdehyde/mg LDL protein). The calculation of

these parameters using the extinction coefficients of conjugated diene and malondialdehyde are outlined in Chapter 2.

6.2.3 LDL fatty acid and α-tocopherol measurements

The methyl esters of fatty acids in LDL were measured by gas chromatography as described in Chapter 2. The α-tocopherol content in LDL fractions was determined by HPLC with ultraviolet detection as described in Chapter 2 and expressed as μmol α-tocopherol/mmol LDL-cholesterol.

6.2.4 Assessment of lipophilic staining of aortic arch

Segments of aortic arch and abdominal aorta were fixed and stained to quantitate fatty streak development. The vessels were gently dissected of gross adherent fatty and connective tissue on the adventitia surface and then cut open longitudinally. The arterial segments were pinned flat onto a piece of cork so that the luminal surface was exposed. The cork was then placed in a solution of fixative containing 10% buffered formalin for approximately 24 h after which time the tissue was placed briefly in a solution of 70% alcohol. The tissue was then stained for lipid deposits by immersing in a solution of 0.1% oil red O (Chroma-Gesellschraft, Schnid and Co., Stuttgart, Germany) in 70:30 (v/v) alcohol/acetone for 15 min. The tissue was then immersed in 80% alcohol for 15 min and gently washed in running water for a further 30 min. The stained tissue segments were stored in 10% buffered formalin. The development of aortic fatty streaks was quantified using TM/TC Image Analysis Systems (Digithurst, Herts, England) using MicroScale software and was expressed as a % of total aortic surface area exhibiting lipophilic staining. The preparation of aortic tissue and measurement of lipophilic staining by image analysis was performed with the technical assistance of Ms Thelma Bridle.

6.2.5 Artery cholesterol measurements

The total cholesterol in the descending segment of the thoracic aorta was measured. Approximately 15 to 20 mm segments of aorta, weighing 40 to 80 mg, were homogenised with a glass pestle in 2 ml of buffer containing 10 mmol/L Tris-HCl, 154 mmol/L NaCl, 2 mmol/L $CaCl_2$ and 1 mmol/L phenylmethyl-sulfonyl fluoride. The homogenate was then sonicated on ice for 30 sec. The cholesterol was extracted by the Folch method (Folch *et al.* 1957). Standards were prepared which contained cholesterol in the concentration range of 2.5 to 25 µg/ml and 5α-cholestane was used as an internal standard. Briefly 1 ml of homogenate or standard containing 30 µl of internal standard were mixed with 4 ml of 2:1 (v/v) chloroform: methanol. The chloroform layer was collected and evaporated to dryness under a stream of nitrogen. The cholesterol was hydrolysed by incubating with 100 µl of 33% (v/v) aqueous KOH and 2 ml ethanol in a shaking waterbath set at 60 °C for 30 min. When cool, the cholesterol was extracted from the aqueous phase with 2 ml of hexane. The samples were evaporated to dryness under a stream of nitrogen and redissolved in 50 µl of hexane for GC injection.

The GC conditions were as described by Wolther *et al.* (1991). The GC instrument was a DANI 6500 with a split/splitless injection system (split ratio 1:20) set at a temperature of 250°C and a vitreous silica capillary column (25 cm x 0.25 mm, 1 mm film thickness) was used with hydrogen as the carrier gas. The retention times of cholesterol and internal standard were 8.3 and 8.9 min respectively. Cholesterol concentrations were calculated from a standard curve plotted as cholesterol concentration (µg/ml) versus the peak area ratio of cholesterol to internal standard. The aortic cholesterol concentration was expressed as µg cholesterol/mg wet weight of aortic tissue.

6.3 RESULTS

6.3.1 LDL oxidation

The results of copper-mediated LDL oxidation in the various rabbit treatment groups are shown in Table 6.1. The LDL isolated from rabbits treated with cholesterol + red wine displayed a significantly shorter lag time ($P < 0.05$) prior to conjugated diene formation compared to rabbits fed cholesterol only. The lag time of LDL oxidation in ethanol-treated rabbits was not significantly different to LDL isolated from rabbits fed cholesterol alone or in combination with red wine. There were no significant differences in oxidation rate, maximum diene and malondialdehyde concentrations between rabbit treatment groups. The LDL isolated from control rabbits did not oxidise when incubated with copper for up to 6 h.

6.3.2 LDL fatty acid and α-tocopherol measurements

The fatty acid composition of the LDL fraction isolated from rabbits is shown in Table 6.2. There were no differences in fatty acids following dietary treatment with either red wine or ethanol when compared to hypercholesterolemic rabbits. Fatty acids in LDL isolated from normocholesterolemic rabbits were barely detectable due to the low concentration of LDL isolated from control rabbit plasma so the data is not included.

The α-tocopherol concentration in LDL fractions isolated from rabbit plasma were similar for all treatment groups (cholesterol group 1.06 ± 0.13 μmol α-tocopherol/mmol cholesterol, cholesterol + red wine 1.24 ± 0.26, cholesterol + ethanol 1.02 ± 0.03). The α-tocopherol concentrations in the LDL fractions from control rabbits could not be detected again due to the low concentration of LDL isolated from control rabbit plasma.

6.3.3 Assessment of lipophilic staining of aortic arch

In control rabbits there was virtually no lipophilic staining on the luminal surface of the aortic arch (<1 %). In comparison there were significant increases ($P < 0.05$, nonparametric ANOVA) in the % surface area of the aortic arch that were stained with lipophilic oil red O in rabbits fed cholesterol, cholesterol + red wine and cholesterol + ethanol (Table 6.3). There were no differences in the extent of fatty streaks between cholesterol-fed rabbits following treatment with either red wine or ethanol although it can be observed from Figure 6.1 that there were large variations in % lipophilic stained area in all treatment groups. The presence of fatty streaks on the abdominal aortas in cholesterol-fed rabbits were negligible with values of 1 to 5% or no lipophilic staining (data not shown).

6.3.4 Artery cholesterol content

The concentration of cholesterol in the descending thoracic segment of the aorta from rabbits is shown in Table 6.3. In rabbits fed a cholesterol diet, the aortic cholesterol concentration increased, but was not significantly different to control animals. Rabbits consuming red wine or ethanol in combination with cholesterol displayed significant increases in aortic cholesterol concentrations compared to control rabbits ($P < 0.01$) and cholesterol-fed rabbits ($P < 0.05$).

6.3.5 Correlations

The lag time of copper-mediated LDL oxidation was not correlated with LDL α-tocopherol ($r=0.004$, $n=18$) or % PUFA in LDL ($r= -0.430$, $n=18$, $P=0.075$). The rate of LDL oxidation was significantly correlated with linoleic acid, oleic acid and the ratio of linoleic:oleic acid in LDL (Figure 6.2). The relationship between % lipophilic staining of aortic surfaces and aortic cholesterol concentrations in cholesterol-fed rabbits was not significant ($r=0.39$, $n=18$, $P=0.11$). Plasma cholesterol exposure, determined as the area under the concentration versus time curve, was not significantly correlated with thoracic aorta cholesterol or with % lipophilic staining of the aortic arch.

Table 6.1

Copper-mediated oxidation of LDL isolated from rabbit plasma following dietary intervention with cholesterol, red wine or ethanol.

	cholesterol	cholesterol + red wine	cholesterol + ethanol	ANOVA P value
lag time (min)	160.8 ± 16.8 [a]	112.1 ± 11.1 [b]	124.2 ± 9.6 [a,b]	0.044
oxidation rate (nmol diene/mg LDL protein/min)	6.88 ± 0.50	7.08 ± 0.57	6.90 ± 0.47	0.956
maximum diene concentration (nmol diene/mg LDL protein)	385.3 ± 10.0	395.0 ± 18.4	402.2 ± 10.4	0.681
malondialdehyde (nmol MDA/mg LDL protein)	38.7 ± 1.4	39.5 ± 1.0	36.3 ± 1.6	0.279

LDL (d 1.019 to 1.063 g/ml) was isolated from rabbit plasma by sequential density ultracentrifugation. Dialysed LDL (50 mg/L protein) was oxidised by incubation with 2.5 µmol/L cupric sulphate and the formation of conjugated dienes was measured spectrophotometrically at 234 nm. Malondialdehyde (MDA) formation was measured in oxidised LDL by TBARS method. Control LDL did not oxidise during 6 h incubation with copper. Statistical analysis was performed using one-way parametric ANOVA with Student Newman Keuls multiple comparison tests. Values with differing superscripts in the same row are significantly different ($P < 0.05$). Values are mean ± sem (n=6).

Table 6.2

Fatty acid composition of LDL fraction isolated from rabbit plasma following dietary intervention with cholesterol, red wine or ethanol.

Treatment	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4	Total PUFA
cholesterol	0.5±0.1	20.4±0.8	7.5±0.8	7.9±0.2	37.8±0.6	21.6±1.0	1.9±0.2	2.4±0.1	25.8±1.1
cholesterol + red wine	0.9±0.4	21.5±1.1	8.1±0.7	7.4±0.2	36.4±1.4	21.7±0.6	1.8±0.1	2.2±0.2	25.7±0.6
cholesterol + ethanol	0.5±0.1	21.1±0.8	7.0±0.8	8.4±0.7	34.9±1.9	23.7±0.8	2.1±0.2	2.4±0.1	28.2±0.8

LDL (d 1.019 to 1.063 g/ml) was isolated from rabbit plasma by sequential density gradient ultracentrifugation. Methyl esters of fatty acids were determined by GC. The peak area ratios of fatty acids to internal standard were determined and are expressed as % of total fatty acids measured. The fatty acids in LDL measured were 14:0 (myristic acid), 16:0 (palmitic acid), 16:1 (palmitoleic acid), 18:0 (stearic acid), 18:1 (oleic acid), 18:2 (linoleic acid), 18:3 (linolenic acid) and 20:4 (arachidonic acid). No statistical differences were observed between values in the same columns as determined by one-way parametric ANOVA with Student Newman Keuls multiple comparison test. Values are mean ± sem (n=6).

Table 6.3

Fatty streak assessment and cholesterol content in aorta dissected from rabbits following dietary intervention with cholesterol, red wine or ethanol.

	control	cholesterol	cholesterol + red wine	cholesterol + ethanol	ANOVA P value
aortic arch fatty streak (% total surface area with lipophilic stain)	0.06 ± 0.04 [a]	19.3 ± 7.0 [b]	21.1 ± 4.1 [b]	15.9 ± 6.1 [b]	0.0034
thoracic aortic cholesterol (µg cholesterol/mg wet wgt)	0.54 ± 0.03 [a]	0.65 ± 0.03 [a]	1.13 ± 0.16 [b]	1.11 ± 0.20 [b]	0.0011

Fatty streak formation was measured in aortic arch by lipophilic (0.1% oil red O) staining and image analysis and is expressed as a % of total aortic surface area with lipophilic stain. Statistical analysis was performed using non-parametric ANOVA (Kruskal-Wallis) with Dunn's multiple comparison. Cholesterol extracted from thoracic aorta using Folch method was quantified by GC analysis and expressed as µg total cholesterol relative to the wet weight of the aortic segment. Statistical analysis was performed using one-way parametric ANOVA with Student Newman Keuls multiple comparison test of the logged data. Values with different alphabetic superscripts in the same row are significantly different ($P < 0.05$). Values are mean ± sem (n=6).

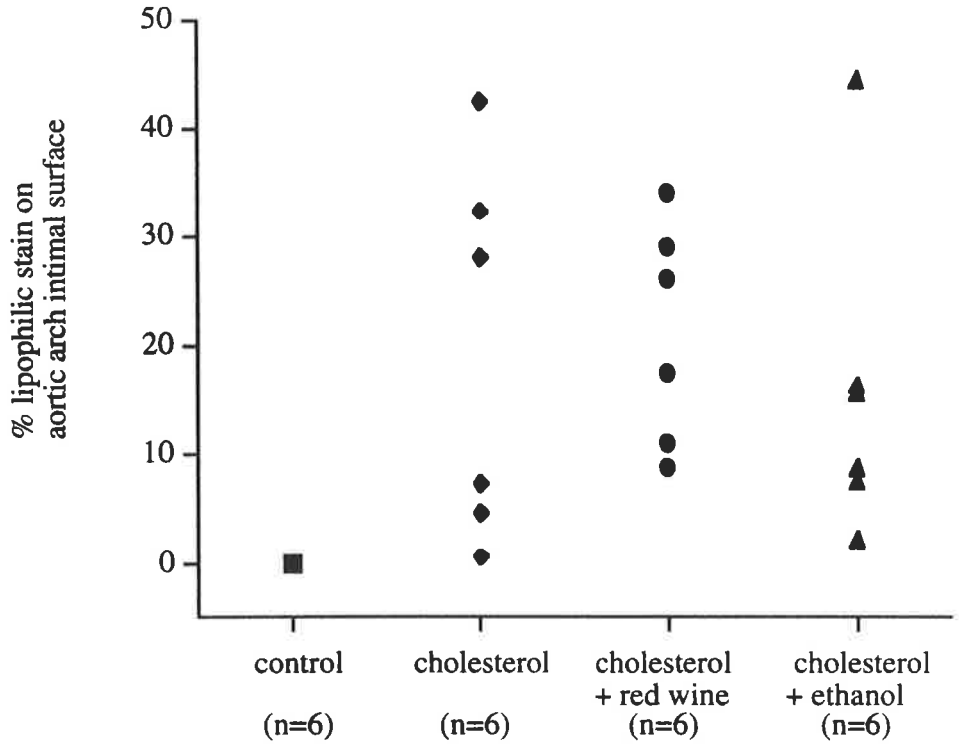


Figure 6.1

Fatty streak development as measured by % lipophilic stain in aortic arch segments from rabbits following dietary intervention with cholesterol, red wine or ethanol.

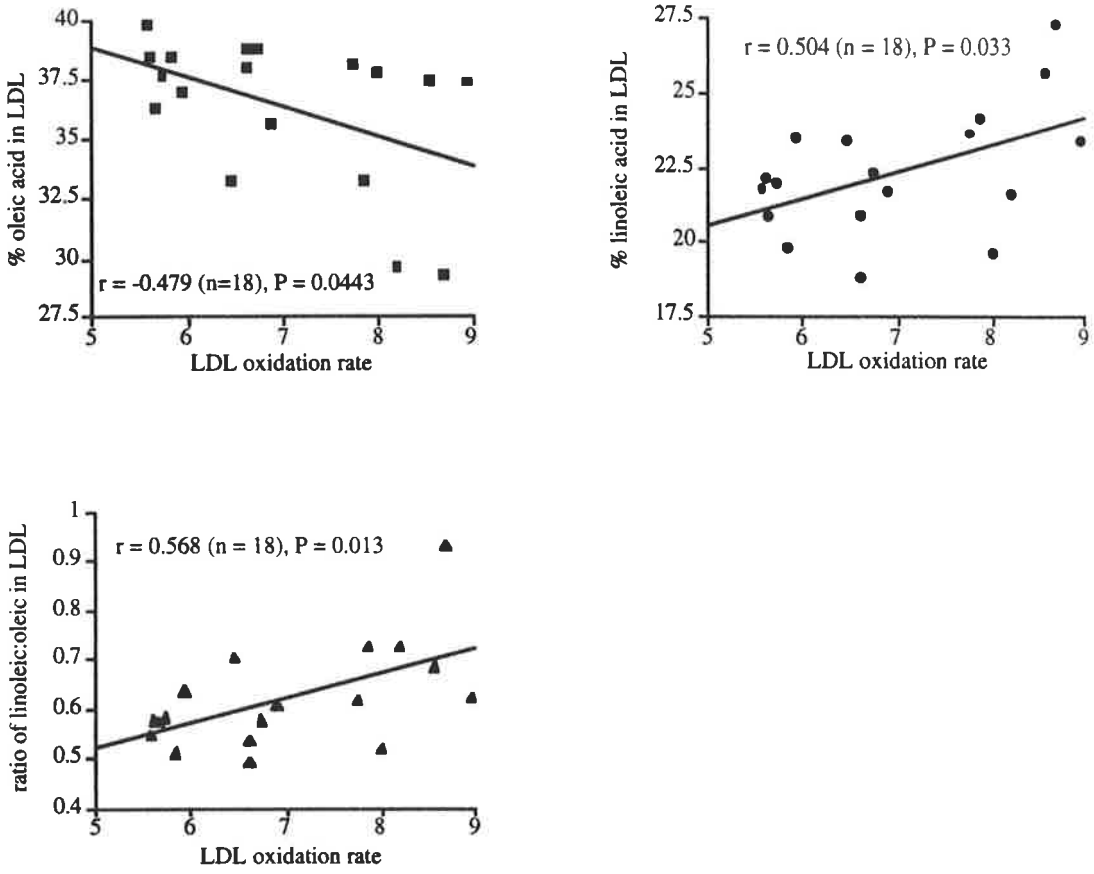


Figure 6.2

The relationship between LDL oxidation rate and LDL fatty acid composition in cholesterol-fed rabbits

LDL was isolated from rabbit plasma and oxidised by copper. Oxidation rate (nmol conjugated diene/mg LDL protein/min) was calculated from the slope of the linear propagation phase of the absorbance profile. Fatty acids in LDL were measured by gas chromatography. Correlations between LDL oxidation rate and LDL fatty acid compositions were determined using Pearson correlation statistical test.

6.4 DISCUSSION

The major findings of these series of experiments is that red wine and ethanol did not prevent the development of fatty streaks in cholesterol-fed rabbits as measured by lipophilic staining of the luminal surface of the aortic arch. Throughout this thesis it has been hypothesised that the antioxidant properties of red wine could protect against fatty streak development in rabbit aorta by decreasing the oxidisability of LDL. In direct contrast to this, cholesterol-fed rabbits consuming red wine displayed a significant decrease in lag time prior to LDL oxidation compared to rabbits fed cholesterol alone. LDL isolated from ethanol-treated rabbits also displayed a decreased lag time which was not different to red wine-treated rabbits. The differences in LDL oxidisability following dietary intervention were not associated with any other changes in lipid peroxidation parameters (oxidation rate, maximum diene formation or TBARS formation) nor any differences in LDL α-tocopherol content or LDL fatty acid composition. Despite the increased susceptibility for oxidation of LDL isolated from red wine-treated rabbits, the presence of fatty streaks in the aortic arches of these rabbits were not different to rabbits fed cholesterol alone. There was however evidence of elevated cholesterol content in thoracic segments of aorta dissected from red wine and ethanol-treated rabbits which may be indicative of progressive fatty streak development in the lower part of this artery.

The LDL isolated from rabbits consuming a cholesterol diet with daily red wine and ethanol intake displayed 30% and 22% reductions, respectively, in the time required for conjugated diene formation following copper-mediated oxidation compared to LDL from cholesterol-fed rabbits. LDL isolated from rabbits fed a normal chow diet did not oxidise following incubation with copper. The reason for this is most likely the low levels of cholesterol in LDL (see Chapter 5, table 5.4), since esterified cholesterol is a substrate for oxidation. In

addition the ratio of cholesterol to protein in this lipoprotein fraction is at least three-fold lower in control rabbits compared to rabbits fed a cholesterol rich diet.

There are a number of factors that can affect the oxidation of LDL, namely the antioxidant content and the fatty acid composition of LDL. In the present study, the α-tocopherol content of LDL was similar for all treatment groups. The levels of α-tocopherol in LDL isolated from hypercholesterolemic rabbit plasma were approximately 1 to 1.2 μmol/mmol LDL-cholesterol (2.7 to 3 μg/mg LDL protein) which is in agreement with levels reported by Morel *et al.* (1994) in cholesterol-fed rabbits (approximately 0.8 μmol/mmol LDL-cholesterol) and Kleinveld *et al.* (1995) in Watanabe heritable hyperlipidemic (WHHL) rabbits (3.9 μg/mg LDL protein). α-Tocopherol levels were undetectable in LDL isolated from control rabbits due to the low levels of LDL in these rabbits. Furthermore, other authors have reported that the levels of antioxidant are very low in plasma and LDL from normolipidemic rabbits (Yap *et al.* 1995). Hypercholesterolemia is commonly associated with an increase in the levels of α-tocopherol in plasma and LDL probably due to the increase in the formation of lipoproteins which incorporate and transport lipophilic antioxidants in the plasma.

α-Tocopherol is one of the main lipophilic antioxidants present in LDL. Many authors have highlighted this compound as the most important antioxidant in protecting LDL against *in vitro* catalysed oxidation. Pioneering experiments by Esterbauer *et al.* (1989b) and then by many subsequent authors have suggested that the metal-catalysed oxidation of LDL proceeds only after the α-tocopherol in LDL has been completely consumed. Many studies have been able to demonstrate a correlation between plasma and LDL α-tocopherol levels with LDL oxidation parameters in subjects that have supplemented their diet with antioxidant vitamins

(Abbey *et al.* 1993, Jialel *et al.* 1992). Despite this, many studies (Jessup *et al.* 1990, Kleinveld *et al.* 1992) have failed to show a strong correlation between α-tocopherol content and lag time for oxidation in LDL isolated from healthy subjects not participating in vitamin supplementation. This has led authors to conclude that the α-tocopherol content of LDL is not necessarily predictive of overall antioxidant status and that other antioxidants and indeed other factors contribute to the oxidisability of LDL.

Esterified polyunsaturated fatty acids in the LDL particle are the substrates for oxidation. In cholesterol-fed rabbits, linoleic acid comprised approximately 85% of the total PUFA in LDL as measured by gas chromatography of methyl esters of fatty acids. This linoleic acid content was similar to that measured in human LDL (Abbey *et al.* 1993). In the present study no differences in LDL fatty acid composition following consumption of red wine or ethanol in cholesterol-fed rabbits were apparent. Croft *et al.* (1996) measured polyunsaturated fatty acid composition and *in vitro* oxidation of LDL isolated from human subjects consuming 4.9% (v/v) alcohol, for 4 weeks, in the form of beer. The study was a crossover design in which a second treatment phase involved consumption of a low alcohol beer (0.9% v/v) containing a similar phenolic content to the high alcohol beer. Although polyunsaturated fatty acids were reduced in subjects consuming high alcohol beer, LDL was more susceptible to *in vitro* oxidation. This was an anomolous result since reduced polyunsaturated fatty acids would be expected to decrease LDL oxidation. These authors concluded that the effect of alcoholic beverages on LDL oxidation may be determined by a balance between the prooxidant effects of alcohol and the antioxidant properties of the phenolic components of beer.

Treatment of cholesterol-fed rabbits with red wine or ethanol did not change the overall fatty acid composition, the total PUFAs or the ratio of linoleic: oleic acid in LDL. Dietary fatty acid intakes were the same in all treatment groups since all rabbits were fed the same commercial chow diet. Significant correlations between the oxidation rate of LDL and

linoleic acid, oleic acid and the ratio of linoleic: oleic acid were observed in the present study, indicating the LDL fatty acids influence oxidation. Many dietary intervention studies in humans have shown that altering the LDL fatty acid profiles can affect LDL oxidation rate resulting in significant correlations between fatty acids and LDL oxidation parameters (Parthasarathy *et al.* 1990, Reaven *et al.* 1993, Abbey *et al.* 1993, Kleinveld *et al.* 1995). A diet rich in linoleic acid can increase the linoleic acid content of LDL which results in an increased oxidation of these lipoproteins and conversely an increase in LDL-oleic acid can decrease the rate of LDL oxidation (Abbey *et al.* 1993).

Another factor which could contribute to the overall oxidisability of LDL, which was not measured in these experiments, could be the amount of pre-existing lipid hydroperoxides (LOOH) in LDL. In copper-mediated LDL oxidations, copper (II) ions are reduced to their more reactive redox state, Cu (I). The Cu (I) ions readily abstract H• from bis-allylic carbon bonds in PUFA. It was demonstrated by Kontush *et al.* (1996b) that lipoproteins could reduce Cu (II) to Cu (I) and enriching these lipoproteins with α-tocopherol increased the rate of Cu (II) reduction, whereas Cu (II) reduction was slower in vitamin E deficient subjects. Furthermore, Cu (II) reduction was related to a decrease or consumption of α-tocopherol. Alternatively Patel *et al.* (1997) proposed that preexisting lipid hydroperoxides within lipoproteins are the reductant responsible for donating electrons to Cu (II). Presumably the concentrations of LOOH within LDL could affect the lag phase and oxidation of LDL *in vitro*. Frei *et al.* (1988) developed a method for detecting lipid hydroperoxides and demonstrated that concentrations of these peroxides was related to LDL oxidation parameters. Since the concentrations of LOOH were not determined in the present study, no conclusions can be made regarding the levels of LOOH in LDL from cholesterol-fed rabbits.

The atherosclerosis induced by cholesterol feeding in rabbits has been well characterised. Most recently Kolodgie *et al.* (1996) used immunocytochemical techniques to elucidate the

composition of aortic lesions following cholesterol feeding in rabbits. These experiments identified the cellular constituents of aortic lesions using specific monoclonal antibodies to muscle actin and rabbit macrophages. These studies and those of Daley *et al.* (1994b) reveal that fatty streaks are composed almost exclusively of macrophages with limited amounts of smooth muscle cells. In rabbits, cholesterol feeding (0.125 to 0.5%) for 6 to 8 months led to the development of more advanced lesions composed of larger amounts of smooth muscle cells and extracellular matrix (collagen, proteoglycan and necrotic material) which are similar to the lesion morphology and composition seen in humans (Kolodgie *et al.* 1996).

In the present study, rabbits were fed a diet containing 0.25 or 0.5% cholesterol. The objective of this feeding protocol was to achieve plasma cholesterol levels of approximately 20 mmol/L to induce the formation of aortic foam cells and fatty streaks which are characteristic of the early stages of atherosclerosis in rabbits. Diet-induced hyper-cholesterolemia in rabbits leads to the formation of macrophage-derived foam cells and fatty streaks in the intimal layer of the aorta, depending on the amount of cholesterol in the diet (Kolodgie *et al.* 1996). A review of the literature shows a very wide range of atherosclerosis development in response to dietary cholesterol. High levels of dietary cholesterol (2%, w/w) fed to adult New Zealand White rabbits for nearly 12 weeks resulted in atherosclerotic lesions covering 55% of the thoracic aorta (Daugherty *et al.* 1989), however other studies have shown only 10 to 20% aortic lesions on the surface of the aortic arch by feeding rabbits a 1% cholesterol diet for 12 to 16 weeks (Del Rio *et al.* 1995, Bjorkhem *et al.* 1991). Feeding rabbits lower levels of cholesterol, 0.05, 0.15, 0.2 and 0.25% (w/w), for 32 weeks results in about 2, 14, 50 and 65% lesion area in the thoracic aorta, respectively as measured by % sudanophilia aortic surface area (Kolodgie *et al.* 1996).

The development of atherosclerosis in rabbits was measured as the percent of lipophilic stain in the aorta of rabbits. Fatty streaks were quantified by lipophilic staining of the luminal surface of the aortic arch using oil red O. The percent of fatty streaks on the intima surface of

the aortic arch were not different in all cholesterol-fed rabbits. Lipophilic staining was present in the aortic arch of all cholesterol-fed rabbits with the exception of a single rabbit which had < 1% staining, despite having a plasma cholesterol concentration of 26 mmol/L. In all cholesterol-fed rabbits only very small areas of the abdominal aorta was covered in fatty deposits as measured by lipophilic staining and were entirely absent in some cases. The section of the aorta used to assess lipid deposits was important since relatively little fatty deposits in abdominal segments of the aorta was observed following staining with lipophilic oil red O even though lipophilic staining was present in the arch region. These observations indicate that fatty streak development decreases markedly as one descends the length of the aorta. For this reason, no significant correlation between fatty streak formation in the aortic arch and cholesterol deposition in the descending thoracic aorta were observed.

The total cholesterol concentration in segments of the thoracic aorta were quantified to give a measure of intracellular and extracellular deposits of cholesterol in the artery wall. Aortic cholesterol content was measured in the descending thoracic segment of the aorta adjacent to the abdominal aorta. This techniques involved homogenisation of the artery segment and therefore measured cholesterol in the entire tissue including the intima, media and adventitia layers. The cholesterol content of aortae dissected from rabbits administered red wine or ethanol were greater than control rabbits (fed normal chow diet) and those fed cholesterol alone. The influx of cholesterol-carrying lipoproteins into the artery is involved in atherogenesis (Nordestgaard *et al.* 1992, Herrmann *et al.* 1994). The increase in aortic cholesterol content in rabbits consuming red wine or ethanol indicates an atherogenic effect of these treatments since aortic cholesterol deposition represents an early stage of fatty streak development (Kuhn *et al.* 1994). There are reports in the literature of artery cholesterol measurements in rabbits. The total cholesterol content of aortae ranges from 14 to 23 μg/mg wet weight in rabbits fed 0.25 to 0.5 % cholesterol (Sparrow *et al.* 1992, Del Rio *et al.* 1995, Kolodgie *et al.* 1996) as measured by either commercial enzyme kit or gas chromatography compared to a level of 0.6 μg/mg wet weight in normocholesterolemic

rabbits (Del Rio *et al.* 1995). Interestingly the aortic cholesterol content in normo-cholesterolemic control rabbits in this present study are comparable to that of other studies (Del Rio *et al.* 1995), however the aortic cholesterol content in cholesterol-fed rabbits was relatively low. Although the present experiment was similar in design to those of Sparrow *et al.* (1992), Del Rio *et al.* (1995) and Kolodgie *et al.* (1996) the severity of fatty staining and aortic cholesterol deposition in the cholesterol-fed rabbits from the present study were markedly lower.

Neither aortic cholesterol content or % lipophilic staining of arch segments were significantly correlated with cholesterol exposure as measured by the area under the curve of plasma cholesterol concentration versus time, in cholesterol-fed rabbits. These results indicate that plasma cholesterol levels are not predictive of fatty streak development in this study. A recent study by Kolodgie *et al.* (1996) comprehensively studied the relationship between increasing dietary cholesterol intakes, aortic cholesterol levels and sudanophilic lesion development in New Zealand White rabbits. These authors found a weaker correlation between aortic fatty streaks (measured by sudanophilic staining) and plasma cholesterol when cholesterol exposure was below a threshold value of approximately 5000 mg/dl (130 mmol/L). In the present study, rabbits fed a diet containing 0.25 or 0.5% cholesterol displayed cholesterol exposures of 100 to 140 mmol/L. These cholesterol levels fall near or below the threshold value reported by Kolodgie *et al.* (1996) and may explain the lack of correlation between lesion development and cholesterol exposure in rabbits in the present study.

It is hypothesised that LDL oxidation promotes atherosclerosis. In rabbits, this theory is supported by results from dietary intervention studies with antioxidants. The anti-atherogenic effects of antioxidants such as N, N'-diphenyl-phenylenediamine (Sparrow *et al.* 1992). probucol (Daugherty *et al.* 1989) and BHT (Bjorkhem *et al.* 1991) have been successfully demonstrated in cholesterol-fed rabbits. In these studies the decrease in atherosclerosis is associated with a protective effect against LDL and VLDL oxidation without any cholesterol-

lowering activity (Sparrow *et al.* 1992, Bjorkhem *et al.* 1991). More evidence indicating a role of lipoprotein oxidation in atherosclerosis development emerges from a study by Rosenfeld *et al.* (1991) in which oxidation specific adducts were identified in atherosclerotic lesions from cholesterol-fed rabbits. These observations lend some support to the involvement of oxidative mechanisms in the development of atherosclerosis in cholesterol-fed rabbits.

In contrast, Stein *et al.* (1989) and Morel *et al.* (1994) have shown that antioxidants do not slow the progression of atherosclerosis in rabbits despite decreasing lipoprotein oxidation. In the latter study Morel *et al.* (1994) investigated the effect of probucol or vitamin E and C supplementation to cholesterol-fed New Zealand White rabbits. These authors hypothesised that the lack of anti-atherogenic effect of vitamin E could be due to a species difference in rabbits, since previous authors have demonstrated an anti-atherogenic effect of vitamin E in WHHL rabbits (Williams *et al.* 1992). In the study by Stein *et al.* (1989), rabbits were fed a diet supplemented with cholesterol and probucol. Throughout the course of the study dietary intakes of cholesterol were adjusted to achieve comparable plasma cholesterol levels between probucol-treated and control rabbits. In these rabbits probucol did not attenuate aortic cholesterol content, indicating a cholesterol-lowering mechanism was at least in part responsible for probucol's antiatherogenic properties. These results were not supported by Mao *et al.* (1991) in which probucol analogues possessing antioxidant, but not cholesterol-lowering properties, were effective in inhibiting atherosclerosis in cholesterol-fed rabbits.

WHHL rabbits are the more commonly used rabbit model to demonstrate antioxidant and antiatherogenic effects of vitamin E, BHT and probucol (Williams *et al.* 1992, Kita *et al.* 1987, Carew *et al.* 1987 and Mao *et al.* 1991). One notable exception is the study by Kleinveld *et al.* (1994) which did not demonstrate an anti-atherogenic effect of vitamin E or probucol in WHHL rabbits, although vitamin E was an effective antioxidant as measured by increases in lag time and apo B fluorescence of copper oxidised LDL. In that study, lower doses of vitamin E and probucol were administered to 6 month old WHHL rabbits with

already established plaques. Similarly, probucol administered to 9 month old WHHL for a period of 6 months did not reduce atherosclerotic lesions (Daugherty *et al.* 1991). These differences in study design by Kleinveld *et al.* (1994) and Daugherty *et al.* (1991), using older rabbits with established plaques rather than young rabbits, may explain the lack of anti-atherogenic effect in their respective studies since most other studies are performed in younger WHHL rabbits (2 to 3 months old) without lesion progression (Carew *et al.* 1987, Kita *et al.* 1987 and Williams *et al.* 1992).

The WHHL strain of rabbit does not have functional LDL receptor and therefore exhibit elevated LDL-cholesterol which lead to non-diet induced hypercholesterolemia and atherosclerosis. The WHHL rabbit may be a more useful model to investigate antioxidant therapies since hyperlipidemia is associated with elevations in LDL-cholesterol and oxidation of this lipoprotein is implicated in atherogenesis. As previously mentioned the major cholesterol carrying lipoprotein in cholesterol-fed rabbits is VLDL, often referred to as β-VLDL due to its migratory properties on agarose gel. This VLDL has been shown to be atherogenic in both its native (Mahley *et al.* 1980) and oxidised form (Parthasarathy *et al.* 1989b) since it is taken up by macrophage scavenger receptors. From these observations it has been proposed that oxidation of LDL in cholesterol-fed rabbits is not a necessary requisite for fatty streak lesion development, since VLDL is the major atherogenic lipoprotein in this model. Furthermore, the atherogenicity of β-VLDL in cholesterol-fed rabbits may not be dependent on oxidative changes.

Another short-coming in using rabbits as an animal model of hypercholesterolemia and atherosclerosis is their large variability in responses. This can be overcome by using larger numbers of animals or by screening rabbits to identify those that respond well to diet-induced changes in plasma cholesterol (reviewed by Kolodgie *et al.* 1996). These factors should be considered in any future studies.

The antioxidant and antiatherogenic hypothesis of red wine must be questioned since dietary intervention with red wine, and to a lesser extent ethanol, increased artery cholesterol content and the susceptibility of LDL to oxidation in cholesterol-fed rabbits. Since rabbits consumed more ethanol during the red wine intervention compared to the ethanol intervention, the effects of red wine on LDL oxidation and artery cholesterol may be due largely to ethanol. Recently Shiash *et al.* (1997) have demonstrated the atherogenicity of ethanol administration in rabbits. Feeding rabbits ethanol at 10, 20 and 30 % of calories in combination with cholesterol leads to dose-dependent increases in aortic fatty lesion development compared to rabbits fed an atherogenic diet alone. It seems in rabbits that alcohol consumption, at least in combination with an atherogenic diet, promotes atherosclerosis.

Wilson *et al.* (1996) recently investigated the effect of resveratrol on hypercholesterol-emia and atherosclerosis in rabbits. Resveratrol is a polyphenolic compound found both in grape skins and red wine. Like whole red wine and other isolated polyphenolic compounds, resveratrol has been shown to inhibit LDL oxidation *in vitro*. (Frankel *et al.* 1993b). However resveratrol, when fed to rabbits consuming a 0.5% cholesterol diet did not alter the peroxy radical initiated oxidation of LDL. Assessment of the atherosclerotic lesions from these rabbits revealed that resveratrol treatment significantly increased the percent of sudanophilic lesions on the aorta. This observation led the authors to conclude that the grape-derived polyphenol, resveratrol, promotes atherosclerosis in this animal model. The results published by Wilson *et al.* (1996) are the first report of a proatherogenic effect of red wine polyphenols.

The only other study investigating the effect of red wine on atheroma development was performed by Klurfeld *et al.* (1981). Red wine inhibited aortic and coronary atherosclerosis as measured by sudanophilic staining in rabbits fed a 9.5% casein and 0.5% cholesterol-suuplemented diet. Like WHHL rabbits, hypercholesterolemia induced by a casein-rich diet

is characterised by elevations in LDL-cholesterol. However the study by Klurfeld *et al.* (1981) used a combination of cholesterol and casein which was associated with a cholesterol distribution similar to that observed in the present study, that is 65% in VLDL + IDL, 10% cholesterol in LDL, and 5% in HDL in control rabbits fed the atherogenic diet (Chapter 5). This study did not include any measurements of lipid peroxidation and could not attribute the anti-atherogenic effect of red wine to ethanol since rabbits consuming ethanol alone did not display reduced aortic or coronary atherosclerosis.

In summary, LDL isolated from cholesterol-fed rabbits consuming red wine, displayed a decrease in the lag time during copper-mediated oxidation. The increase in LDL oxidisability, could not be attributed to changes in LDL α-tocopherol or fatty acid composition. Red wine did not affect the progression of atherosclerosis in the aortic arches from cholesterol-fed rabbits but increased cholesterol deposits in the thoracic aorta. Importantly there were no differences in LDL oxidation or aortic lipid deposits between red wine and ethanol-treated rabbits indicating that any atherogenic effects of red wine may be due to ethanol rather than other components of red wine. These findings do not support the hypothesis that red wine is a dietary antioxidant that inhibits the development of atherosclerotic fatty deposits in aortic tissue in cholesterol-fed rabbits.

CHAPTER 7

THE EFFECT OF RED WINE ON ENDOTHELIUM-DEPENDENT RELAXATION IN AORTIC RINGS ISOLATED FROM CHOLESTEROL-FED RABBITS.

7.1 INTRODUCTION

It has been well documented in the literature that hypercholesterolemia and atherosclerosis are associated with an impairment in endothelium-dependent relaxation (Jayakody *et al.* 1987, Bossaller *et al.* 1987, Verbeuren *et al.* 1986). Equally well founded is the observation that certain strategies, such as antioxidant supplementation, can restore acetylcholine-mediated relaxation in hypercholesterolemic rabbits (Keaney *et al.* 1993, Stewart-Lee *et al.* 1994, Matz *et al.* 1994, Klemsdale *et al.* 1994, Andersson *et al.* 1994, Simon *et al.* 1993, Keaney *et al.* 1995).

Red wine may also be capable of restoring endothelium-derived relaxation based on the *in vitro* findings of Fitzpatrick *et al.* (1993), in which a relaxation response in precontracted rat aorta, following the addition of red wine and grape seed extract was observed. The aim of this study was to measure the endothelium-dependent relaxation of aorta isolated from rabbits consuming red wine in combination with a cholesterol diet.

7.2 METHODS

7.2.1 Animal Study: Dietary intervention with red wine and ethanol in cholesterol-fed rabbits

See chapter 2. Aortic ring studies were performed in rabbits fed a normal chow diet (control, n=6), rabbits fed a cholesterol diet alone (n=5) or a cholesterol diet in combination with red wine (n=5) or ethanol (n=6).

7.2.2 Aortic ring preparations

The procedures for organ bath aortic ring preparations was adapted from Head *et al.* (1987). Following the dietary intervention rabbits were killed by exsanguination from the abdominal aorta and the aorta was resected and placed in cold oxygenated physiological salt solution (PSS, containing 113 mmol/L NaCl, 4.8 mmol/L KCl, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4.7H_2O$, 0.6 mmol/L ascorbic acid, 11.2 mmol/L glucose and 2.5 mmol/L $CaCl_2$). Adherent fatty and connective tissue was gently removed from the adventitial surface of the thoracic aorta. Care was taken with aorta so as to ensure an intact endothelium during tissue

preparation. Aortic ring segments (2 to 3 mm long, n=8 for each animal) were mounted on stainless steel stirrups and placed in organ bath chambers containing 15 ml PSS which was continuously aerated with 95% O_2 and 5% CO_2 and maintained at 37°C. The aortic rings were equilibrated for at least 1 h under isometric conditions at a resting basal tension of 7 g. In preliminary experiments the optimal resting tension for smooth muscle contraction in normal rabbit thoracic aorta was determined by constructing cumulative dose response curves to KCl under increasing vessel tensions. The contractions were recorded on a Graphtec Linearcorder FW33701 (Graphtec, Yokohama, Japan) as changes in isometric tension measured by a FT03 force displacement transducer (Grass Medical Instruments, Quincy, MA, USA), connected to a JRAK WR3701 amplifier (JRAK Biosignals, Windsor, Vic, Australia). The apparatus was calibrated daily with standardised weights.

The preparations were contracted by the addition of potassium-containing PSS (KPSS) in which NaCl (113 mmol/L) was replaced with KCl (117.8 mmol/L) on an equimolar basis in order to test the viability of the tissue preparations. Following maximal contraction, the tissues were repeatedly washed with fresh PSS until vessel tone reached baseline levels. A cumulative dose-response curve (0.01 to 100 μmol/L) to the α_1-contractile agonist, phenylephrine was performed. Relaxation to acetylcholine (1 to 1000 nmol/L) was studies in rings precontracted with a half-maximal concentration (EC_{50}) of phenylephrine. Dose responses to other known vasorelaxant agents, calcium ionophore (A23187, 0.3 to 1000 nmol/L) and sodium nitroprusside (0.1 to 1000 nmol/L), were determined in a similar manner using phenylephrine precontracted rings.

The line fitting for individual cumulative dose-response curves was performed using DResponse program (courtesy of Mr GA Crabb, Department of Clinical and Experimental Pharmacology, University of Adelaide) and the concentrations of vasoactive compounds required to elicit 30, 50 and 70% of the maximal response (EC_{30}, EC_{50} and EC_{70}) were determined from these curves.

7.3 RESULTS

7.3.1 Vascular contractility to potassium and phenylephrine

The maximal contractile responses of rabbit aortic rings to KPSS and phenylephrine were similar in all treatment groups (Table 7.1). Only at lower phenylephrine concentrations of 0.1 to 0.3μmol/L there were significantly greater contractile responses in cholesterol-fed rabbits compared to the cholesterol + ethanol group ($P < 0.01$, Figure 7.1). The aorta from cholesterol + ethanol-treated rabbits also displayed a reduced phenylephrine-mediated contraction compared to all other groups at a single concentration of 0.3 μmol/L ($P < 0.05$). The EC_{30}, EC_{50} and EC_{70} values were not different between any of the rabbit groups (ANOVA P-values of 0.15, 0.11 and 0.13, respectively) nor were the tension developments different in rabbit treatment groups as indicated by the maximum contraction values in Table 7.1. The EC_{50} values for phenylephrine contraction were approximately -6.4 to -6.5 log M irrespective of dietary treatment (Table 7.1). Although this EC_{50} value is roughly equivalent to the concentration of phenylephrine that produced significantly lower contractions in aorta from ethanol-treated rabbits (0.3 μM phenylephrine), the decrease in maximal response in these rabbits meant that the half maximal response to the contractile agent were not different.

7.3.2 Vascular relaxation to acetylcholine, calcium ionophore (A23187) and sodium nitroprusside

Aortic rings from normocholesterolemic control rabbits showed a concentration-dependent relaxation in response to acetylcholine with a maximal relaxation of 94.3 ± 1.4%. The vasodilator response to acetylcholine was significantly reduced in the aortic rings from cholesterol-fed, cholesterol + red wine and cholesterol + ethanol rabbits as shown in Figure 7.2. The concentration-response curves to acetylcholine shifted to the right in all cholesterol-fed treatment groups, however the maximum relaxation did not differ significantly between control (94.3 ± 1.4%) and cholesterol-fed rabbits with values of 74.1 ± 8.8% in cholesterol-fed, 74.7 ± 8.7% in cholesterol + red wine and 81.4 ± 4.8% in cholesterol + ethanol. The impaired relaxations to acetylcholine in all cholesterol-fed rabbits are also summarised in Table 7.2 which lists the estimates of EC_{30}, EC_{50} and EC_{70}. There were significant increases in the concentrations of acetylcholine required to mediate 50 and 70% relaxation in

preparations from rabbits fed cholesterol alone or in combination with red wine or ethanol. There were no differences in smooth muscle relaxation mediated by acetylcholine between cholesterol-fed rabbits and those drinking red wine or ethanol (Figure 7.2).

The responses of rabbit aorta to the calcium ionophore, A23187, were qualitatively similar to that observed with acetylcholine (Figure 7.3). The relaxation response to A23187 was greatest in control rabbits (maximum relaxation 87.6 ± 4.8%) and was attenuated in all rabbits fed a cholesterol supplemented diet (cholesterol group 62.1 ± 18.1%, cholesterol + red wine 61.5 ± 9.6% and cholesterol + ethanol 76.2 ± 3.9%). However there were no statistically significant reductions in A23187 mediated relaxation between treatment groups. A correlation between the concentrations of acetylcholine and A23187 required to achieve 50% relaxation (EC_{50}) in rabbit aorta was observed (Pearson r=0.44, P=0.0529, n=24). The vasorelaxation responses to the endothelium-independent agent, sodium nitroprusside were the same in all rabbit groups (Figure 7.4). Sodium nitroprusside induced relaxations of aortic rings were concentration-dependent and reached approximately 100% relaxation in all rabbits irrespective of dietary treatment.

	Phenylephrine		KPSS
Rabbit treatment	max contraction (g)	EC_{50} (-log M)	max contraction (g)
control	5.43 ± 0.51	6.56 ± 0.05	4.63 ± 0.55
cholesterol	6.09 ± 0.23	6.52 ± 0.05	5.18 ± 0.19
cholesterol + red wine	5.63 ± 0.43	6.51 ± 0.03	4.67 ± 0.36
cholesterol + ethanol	5.13 ± 0.25	6.41 ± 0.04	4.44 ± 0.18

Table 7.1

Phenylephrine and potassium induced contraction of aortic rings.

Contractile responses to phenylephrine and potassium were determined in thoracic aortic rings isolated from rabbits following dietary intervention with cholesterol alone (n=5), cholesterol + red wine (n=5), cholesterol + ethanol (n=6) or normal diet (control, n=4). EC_{50} values are the concentration of agonist required to produce 50% contraction and were determined by curve fitting of the individual concentration versus response graphs. No statistical differences were observed between values using one-way parametric ANOVA with Student Newman Keuls multiple comparison test. Values are mean ± sem.

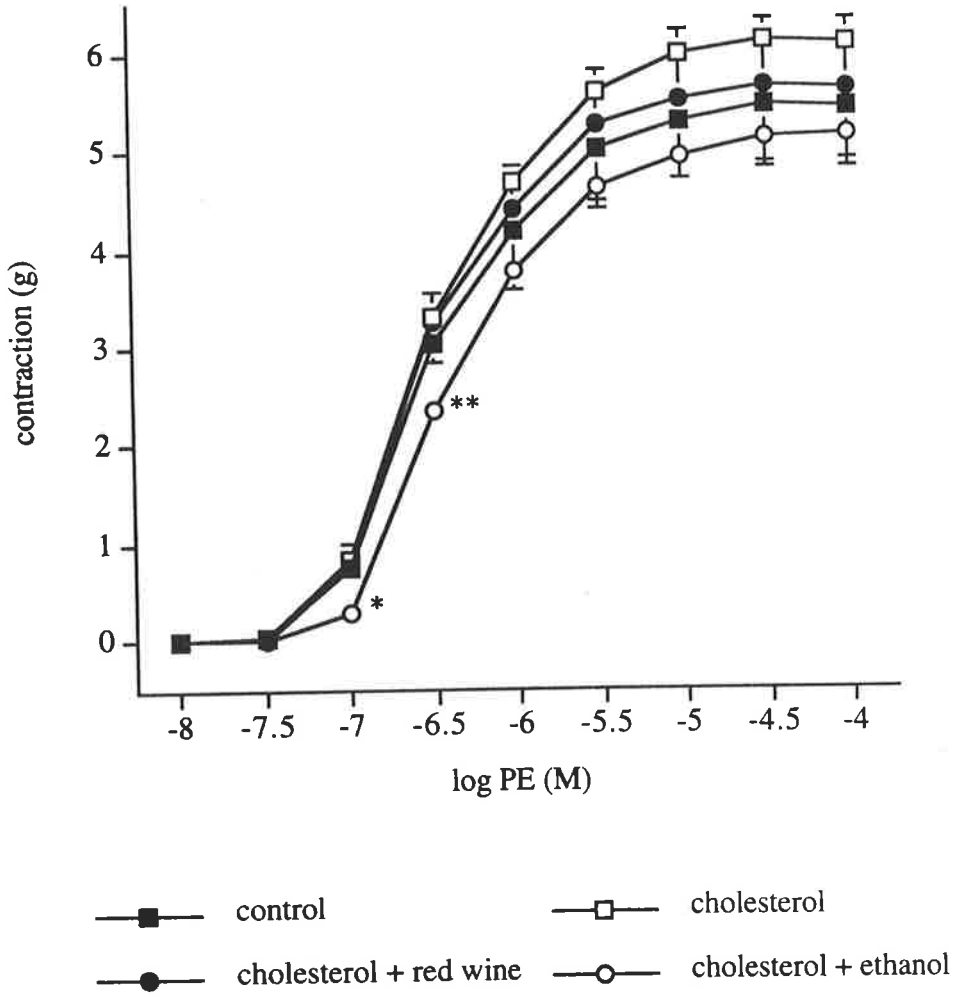


Figure 7.1

Phenylephrine induced contraction in aortic rings.

Contractile responses to phenylephrine were measured in thoracic aortic rings isolated from rabbits following dietary intervention with cholesterol alone (n=5), cholesterol + red wine (n=5), cholesterol + ethanol (n=6) or normal diet (control, n=4). Statistical differences were determined by parametric ANOVA with Student Newman Keuls multiple comparison. The (**) denotes that the cholesterol + ethanol rabbits have significantly lower values compared to all other groups ($P < 0.05$) and (*) denotes that the cholesterol + ethanol rabbits have significantly lower values compared to cholesterol group only ($P < 0.01$). Values are mean ± sem.

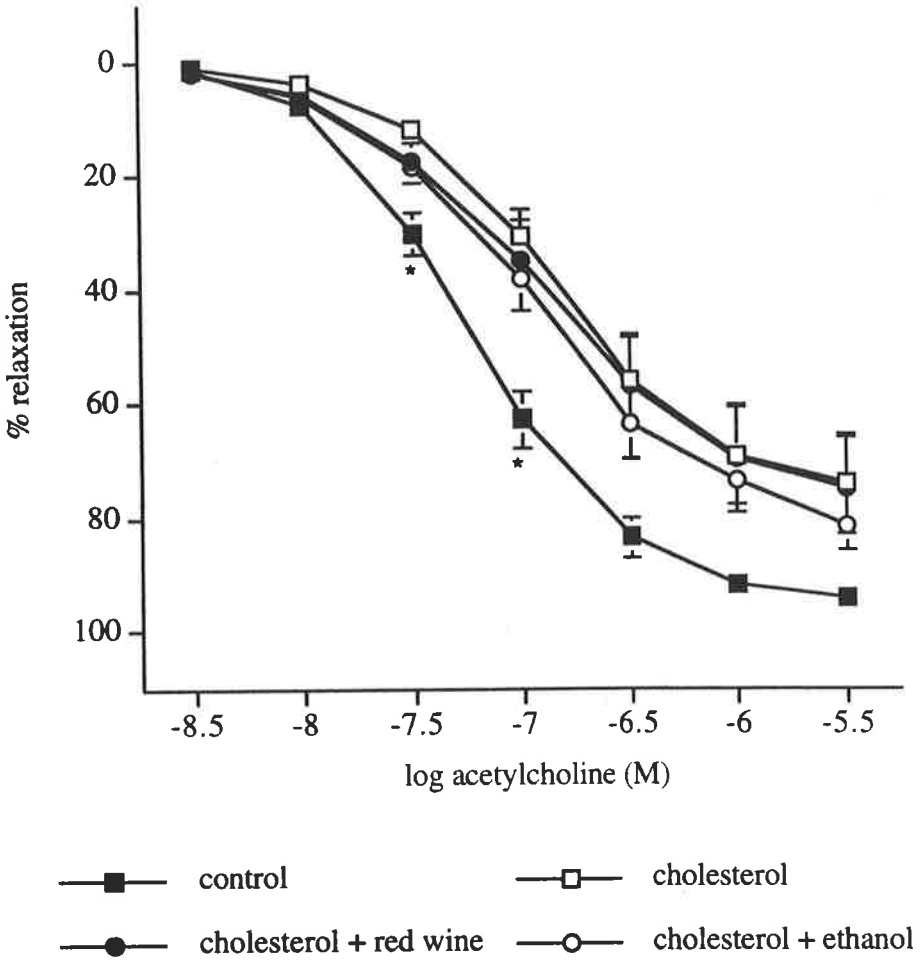


Figure 7.2

Acetylcholine induced relaxation in aortic rings precontracted with phenylephrine

Relaxation responses to acetylcholine were measured in precontracted aortic rings isolated from rabbits following dietary intervention with cholesterol alone (n=5), cholesterol + red wine (n=5), cholesterol + ethanol (n=6) or normal diet (control, n=4). Values are mean ± sem. The (*) denotes significantly different at $P < 0.05$ to all other treatment groups as determined by parametric ANOVA with Student Newman Keuls multiple comparison test.

	Acetylcholine (-log M)		
Treatment group	EC_{30}	EC_{50}	EC_{70}
control	7.51 ± 0.07 [a]	7.24 ± 0.07 [a]	6.92 ± 0.08 [a]
cholesterol	7.19 ± 0.06 [b]	6.87 ± 0.07 [b]	6.56 ± 0.08 [b]
cholesterol + red wine	7.31 ± 0.06 [a,b]	6.92 ± 0.08 [b]	6.57 ± 0.09 [b]
cholesterol + ethanol	7.30 ± 0.08 [a,b]	6.94 ± 0.08 [b]	6.61 ± 0.07 [b]

Table 7.2

Acetylcholine induced relaxation of aortic rings precontracted with phenylephrine.

Relaxation responses to acetylcholine were determined following dietary intervention with cholesterol alone (n=5), cholesterol + red wine (n=5), cholesterol + ethanol (n=6) or normal diet (control, n=4). EC_{30}, EC_{50} and EC_{70} values are the concentrations of agonist required to produce 30, 50 and 70% relaxation and were determined by curve fitting of the individual concentration versus response graphs. Values with differing alphabetic superscripts in the same column are significantly different ($P < 0.05$) as determined by one-way parametric ANOVA with Student Newman Keuls multiple comparison test. Values are mean ± sem.

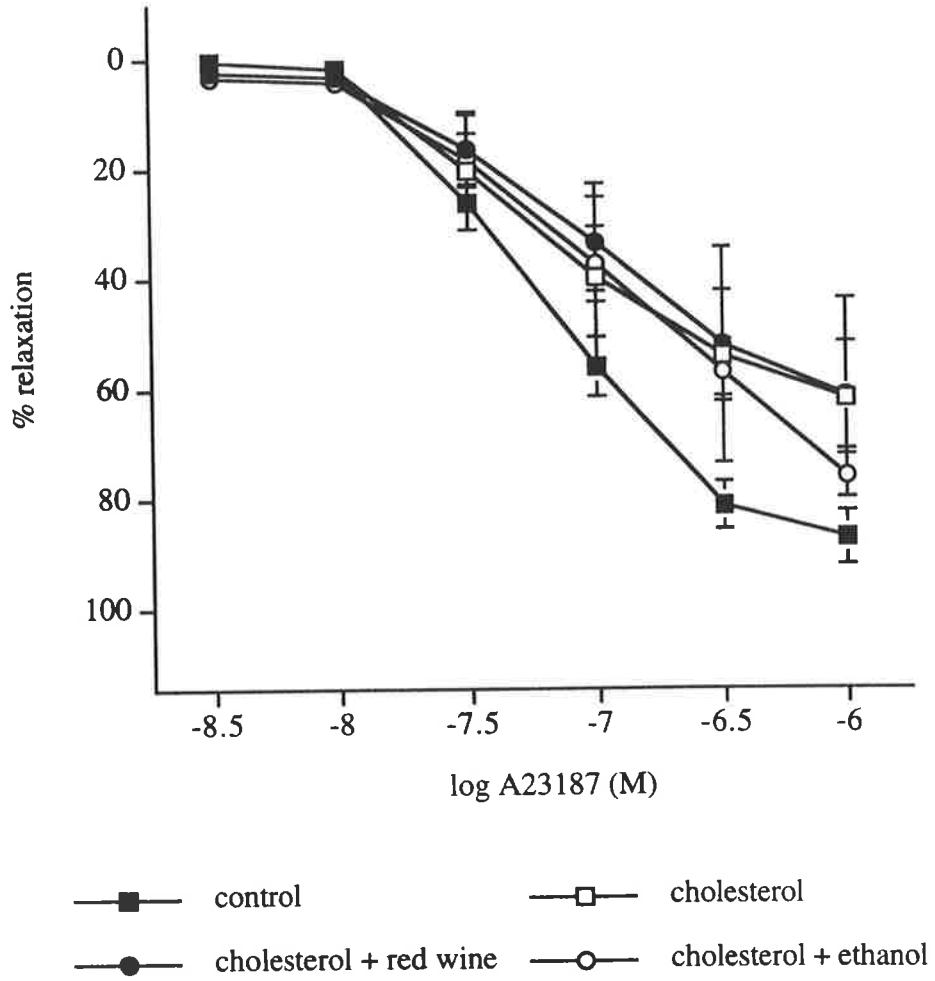


Figure 7.3

Calcium ionophore (A23187) induced relaxation in aortic rings precontracted with phenylephrine.

Relaxation responses to A23187 were measured in precontracted aortic rings isolated from rabbits following dietary intervention with cholesterol alone (n=5), cholesterol + red wine (n=5), cholesterol + ethanol (n=6) or normal diet (control, n=4). Values are mean ± sem. No statistically significant differences were observed following a parametric ANOVA with Student Newman Keuls multiple comparison test.

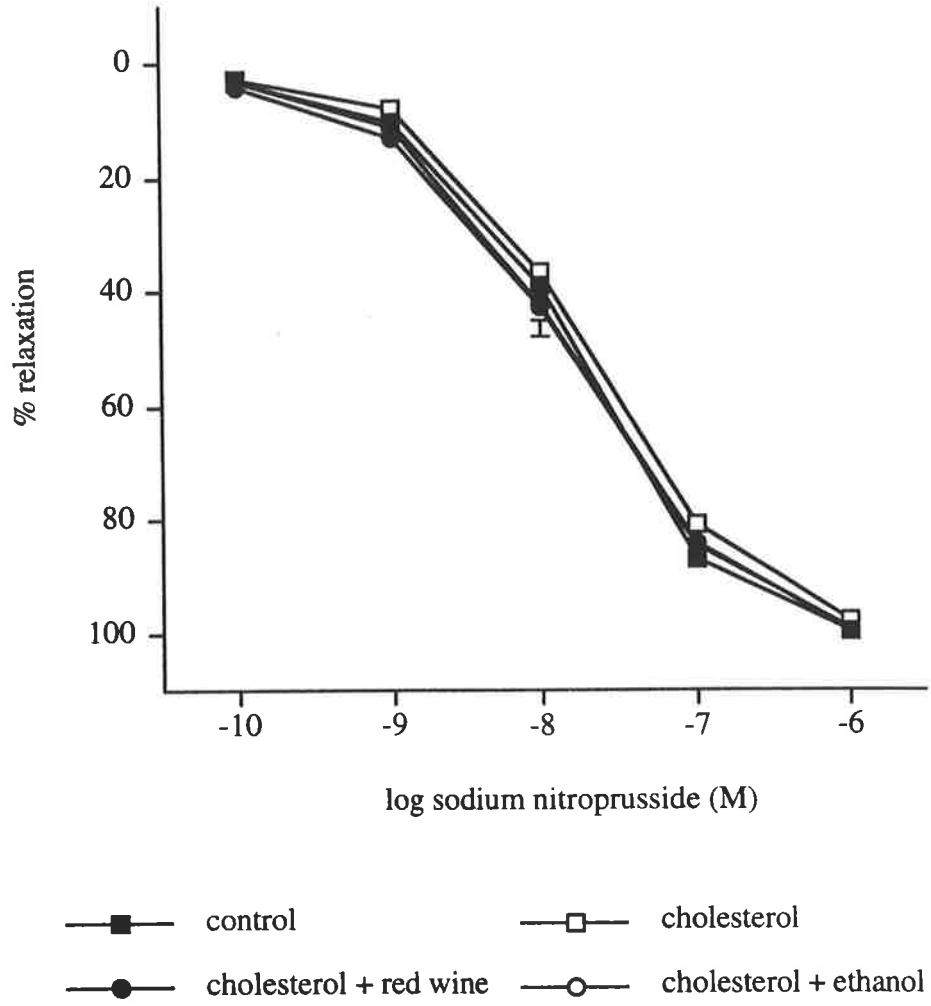


Figure 7.4

Sodium nitroprusside induced relaxation in aortic rings precontracted with phenylephrine.

Relaxation responses to sodium nitroprusside were determined in precontracted aortic rings isolated from rabbits following dietary intervention with cholesterol alone (n=5), cholesterol + red wine (n=5), cholesterol + ethanol (n=6) or normal diet (control, n=4). Values are mean ± sem. No statistically significant differences were observed following a parametric ANOVA with Student Newman Keuls multiple comparison test.

7.4 DISCUSSION

7.4.1 Vascular relaxation

The principal finding of these experiments was that dietary supplementation with red wine or ethanol did not overcome the impairment in acetylcholine-mediated relaxation observed in cholesterol-fed rabbits. The hypothesis at the commencement of this study was that red wine containing polyphenols could restore, at least in part, endothelium-dependent vasodilatation. This was based on the findings of Fitzpatrick *et al.* (1993) in which red wine and grape seed extracts mediated an endothelium-dependent relaxation of rat aortic rings *in vitro*. There were no differences in aortic relaxation to acetylcholine, A23187 and sodium nitroprusside in cholesterol-fed rabbits treated with red wine or ethanol compared to the rabbits fed cholesterol alone.

The mechanisms by which acetylcholine, A23187 and sodium nitroprusside achieve vascular relaxation differs. Acetylcholine is an endothelium-dependent relaxing agent (Furchgott and Zawadzki 1980). It acts via muscarinic receptors on endothelial cells (Jayakody *et al.* 1987). This agonist/receptor interaction catalyses a G-protein transduction system that stimulates nitric oxide synthesis and release. The nitric oxide then mediates smooth muscle relaxation by activation of soluble guanylate cyclase and cytoplasmic guanosine 3' 5' monophosphate (cGMP) (reviewed by Di Rosa *et al.* 1996). In contrast, calcium ionophores such as A23187, although acting on endothelial cells, do not bind to any cell surface receptors. Nitric oxide release is stimulated by direct calcium influxes into the endothelial cells (Dusting *et al.* 1995). Sodium nitroprusside mediates relaxation by donating nitric oxide directly to smooth muscle cells so that it's action is completely endothelium-independent (Dusting *et al.* 1995).

Cholesterol feeding affected the endothelial cell function as evident by the decreased relaxation in response to acetylcholine. The shift to the right in the cumulative dose-response curve to acetylcholine in cholesterol-fed rabbits compared to control rabbits, implies a change in affinity or receptor-agonist interactions in these preparations. Such results are consistent with other studies investigating endothelium-dependent relaxation in

hypercholesterolemic rabbits (Verbeuren *et al.* 1986, Bossaller *et al.* 1987). Aortic relaxation to the calcium ionophore, A23187 was only partially impaired in cholesterol-fed rabbits indicating that calcium entry and nitric oxide function is not impaired. The relaxations to the nitric oxide donor, sodium nitroprusside, were the same in all rabbit treatment groups, suggesting that aortic smooth muscle responses to nitric oxide are unchanged.

Impaired relaxations of aortic preparations in the present experiments are not due to alterations in soluble guanylate cyclase, cGMP or smooth muscle function since sodium nitroprusside produced the same relaxation responses in all rabbits irrespective of dietary treatment. Secondly, A23187 caused only partial impairment of endothelium-dependent relaxation which was not significant indicating that there is no notable effect of cholesterol feeding on calcium dependent nitric oxide synthase. Since the only differences in aortic relaxation were observed in response to acetylcholine and not other vasorelaxing agents, an alteration in endothelial surface receptors and/or the G-protein transduction pathway in endothelial cells is the likely mechanism of impaired endothelium-dependent relaxation in cholesterol-fed rabbits.

There have been some suggestions that the presence of atherosclerotic lesions in arteries can actually impede the diffusion of nitric oxide from endothelial cells to smooth muscle cells in the intima layer (reviewed by Dusting *et al.* 1995). Rabbits fed cholesterol in this study had both elevated plasma cholesterol levels (see Chapter 5) and moderate fatty streak development in the aortic arch (see Chapter 6). The present findings do not allow any conclusions that would support a decreased diffusion of nitric oxide to smooth muscle cells as a result of atherosclerotic lesions. If lipophilic streaks in the artery were to impede nitric oxide diffusion to the site of action, then one would also expect to observe a similar decrease in aortic relaxation in response to A23187, however this was not apparent.

It is not uncommon to find that A23187 maintains its relaxing response despite an impairment of acetylcholine-evoked vasodilatation. A23187-mediated relaxation was unchanged in studies by Bossaller *et al.* (1987), Matz *et al.* (1994) and Stewart-Lee *et al.*

(1994) when rabbits were fed 1% cholesterol diets for 4 to 10 weeks. The rabbits in the present study were maintained on a 0.25 to 0.5% cholesterol diet for 12 weeks. Although significant elevations in plasma cholesterol concentrations and fatty streak development were achieved in cholesterol-fed rabbits, there was no appreciable loss of vasorelaxation to the calcium ionophore. These studies do however show impaired relaxation to acetylcholine, as in the present study, which support the hypothesis for impairment at the agonist-endothelial receptor level.

Red wine, grape seed extract and isolated polyphenolic compounds (quercetin and tannic acid) but not ethanol produce vasorelaxation in precontracted rat aorta *in vitro* (Fitzpatrick *et al.* 1993). This relaxation is characteristic of an acetylcholine-mediated response in that it was endothelium-dependent, produced a concentration dependent increase in aortic cGMP levels, was inhibited by nitric oxide synthase inhibitors and restored by *L*-arginine, a precursor for nitric oxide production. These results published by Fitzpatrick *et al.* (1993) provide the first evidence that red wine containing polyphenols can mediate agonist-like endothelium-dependent relaxation. As well as mediating relaxation, grape seed extract also decreased the contractile response of aorta to phenylephrine as shown by an increase in agonist concentration required to produce 50% contraction (EC_{50}) and a decrease in maximum contraction. These observations provide evidence of an *in vitro* protective effect of red wine and polyphenols on endothelial relaxation which have recently been confirmed by Andriambeloson *et al.* (1997). Interestingly a crude extract of red wine polyphenols was effective in mediating endothelium-dependent relaxation, but catechin alone was not.

Work by van Acker *et al.* (1995) and Verhagen *et al.* (1996) have demonstrated that flavonoids and red wine are effective scavengers of nitric oxide *in vitro*. These authors raise an interesting hypothesis that nitric oxide scavenging could contribute to the beneficial effects of flavonoids, since nitric oxide is a precursor for peroxynitrite, another reactive radical species. Interestingly nitric oxide by itself inhibits *in vitro* LDL oxidation (Yates *et al.* 1992, Jessup *et al.* 1992), yet in the presence of superoxide forms peroxynitrite and hydroxy radicals which have prooxidant effects towards LDL oxidation (Darley-Usmar *et al.*

1992, Hogg *et al.* 1992, 1993). However in terms of vascular function, nitric oxide is an important mediator of vasorelaxation and scavenging of nitric oxide would lead to vasoconstriction. The relevance of these *in vitro* findings of van Acker *et al.* (1995) and Verhagen *et al.* (1996) are unclear, particularly since many studies have focused on optimising nitric oxide function and thus endothelium-dependent relaxation. The role of nitric oxide in atherosclerosis formation is so far unknown but recently peroxynitrite has been identified as a likely catalyst of free radical damage in the subendothelial space contributing to LDL oxidation and atherosclerosis (Darley-Usmar and Halliwell 1996, Leeuwenburgh *et al.* 1997).

To date no published studies have investigated the endothelium-dependent relaxation in aorta from cholesterol-fed animals supplemented with red wine or flavonoids. In the present study, consumption of red wine or ethanol for 12 weeks did not alter the impaired acetylcholine-mediated relaxation of aortic preparations *in vitro* induced by cholesterol feeding. This result suggests that red wine or ethanol does not offer any protective effect toward vascular relaxation in animals with diet-induced hyperlipidemia and fatty streak development. However, other studies have successfully demonstrated restoration of endothelium-dependent relaxation in cholesterol-fed rabbits, with dietary supplementation of antioxidants such as vitamin E (Stewart-Lee *et al.* 1994, Keaney *et al.* 1993, Matz *et al.* 1994, Klemsdale *et al.* 1994, Andersson *et al.* 1994), probucol (Simon *et al.* 1993, Keaney *et al.* 1995) and β-carotene (Keaney *et al.* 1993). A possible reason for the lack of effect of red wine supplementation on vasorelaxation could be due to low plasma and tissue concentrations of red wine derived polyphenols.

The effect of ethanol treatment on endothelium-dependent relaxations is not well understood. It appears from the data available in the scientific literature that ethanol inhibits acetylcholine relaxation and decreases cGMP levels in isolated rat aortic segments (Hatake *et al.* 1993). In rabbits fed a diet supplemented with cholesterol and ethanol, vasorelaxation was comparable to that in rabbits fed cholesterol alone.

7.4.2 Vascular contractility

The α_1-adrenergic-induced contractions by phenylephrine in aortic segments isolated from cholesterol-fed, cholesterol + red wine or normocholesterolemic rabbits were not different. This is consistent with published results in which α-adrenergic contraction of aortic segments isolated from hypercholesterolemic and atherosclerotic rabbits is either decreased or unchanged (Verbeuren *et al.* 1986, Kolodgie *et al.* 1990, Simon *et al.* 1993, Matz *et al.* 1994, Fujiwara *et al.* 1993). Interestingly, the vascular contractility mediated by low concentrations of phenylephrine was attenuated in thoracic aorta isolated from cholesterol-fed rabbits consuming ethanol.

In terms of vascular contractility, chronic ethanol treatment in rats fed a normal diet has also been shown to enhance contractile responses to phenylephrine (Pinardi *et al.* 1992). To my knowledge there are no studies that have examined the effect of ethanol on aortic function in hypercholesterolemic or atherosclerotic models. Results from the present study demonstrate a decreased contractility to phenylephrine following long term ethanol consumption in cholesterol-fed rabbits. This result was not observed in rabbits drinking red wine, despite the fact that these rabbits consumed approximately twice as much ethanol as rabbits in the ethanol-treated group. The attenuated contractile response in red wine-treated rabbits was not different to that observed in rabbits fed cholesterol alone. The differences in phenylephrine-mediated contraction between ethanol and red wine treatments is not due to components of red wine other than ethanol, such as grape-derived polyphenolics, since the responses in red wine-treated rabbits were not different to rabbits fed cholesterol alone. The reason for the different phenylephrine-mediated contraction in ethanol-treated rabbits is unclear particularly since ethanol has been reported to increase vascular contractility in response to phenylephrine (Pinardi *et al.* 1992). It is likely that cholesterol feeding and atherosclerosis in rabbits, which has been reported to attenuate phenylephrine contraction (Verbeuren *et al.* 1986, Kolodgie *et al.* 1990), outweighs any increase in vascular contractility which is associated with chronic ethanol treatment.

To summarise, aorta from cholesterol-fed rabbits displayed an impaired vasorelaxation in response to acetylcholine, an effect that was not restored by supplementation with red wine or ethanol in combination with dietary cholesterol. Red wine does not offer any benefit in the regulation of endothelial function in cholesterol-fed rabbits, with regard to relaxation, mediated by acetylcholine and calcium ionophore, or contraction, mediated by potassium and phenylephrine.

CHAPTER 8

DEVELOPMENT OF A SENSITIVE ASSAY TO MEASURE RED WINE-DERIVED CATECHINS IN RABBIT PLASMA.

8.1 INTRODUCTION

In a previous chapter the antioxidant effect of red wine *in vitro* has been described (Chapter 3). Subsequent experiments have investigated the effects of red wine *in vivo* on lipid profiles, LDL oxidation and atherosclerosis. To make any conclusions regarding the antioxidant and antiatherogenic properties of red wine *in vivo* it is important to ascertain whether these compounds can be absorbed from the gastrointestinal tract following consumption. The aim of the present experiment was to quantitate the amount of polyphenols in rabbit plasma following dietary intervention with red wine. An obvious problem in investigating the effect of red wine on LDL oxidation and atherosclerosis is the lack of quantitative information regarding the bioavailability of polyphenols due mainly to the enormous chemical heterogeneity of the polyphenols in red wine. Red wine contains many different types of polyphenols with various types, patterns and numbers of substituent groups and differing degrees of polymerisation. Since catechin is a potent antioxidant and present in relatively large amounts in red wine, attempts were made to develop an assay to measure catechin levels in rabbit plasma.

A few studies have quantified the plasma concentrations of catechin following the consumption of green tea (Lee *et al.* 1995, Kivits *et al.* 1997). Green tea contains four main catechins namely, epicatechin, epicatechin-3-gallate, epigallocatechin and epigallocatechin-3-gallate (Graham 1992). The current techniques used to measure catechins in plasma include high performance liquid chromatography methods with electrochemical (Lee *et al.* 1995) or diode array detection (Pan *et al.* 1991). The types of techniques used to extract catechins from plasma include liquid solvent extractions (Lee *et al.* 1995) with ether or ethyl acetate or solid phase SEP-PAK extractions (Pan *et al.* 1991). Other techniques for quantifying catechins are based on colorimetric assays in which catechins react with reagents such as 4-dimethylamino-cinnamaldehyde in acidic conditions to produce chromophores with characteristic wavelength absorbances which can be quantified spectrophotometrically. Examples of these assays have been published by Treutter (1989) and Kivits *et al.* (1997). The advantage of these assays is that they allow the quantification of monomeric as well as oligomer catechins referred to as procyanidins (condensed tannins). Since procyanidins are

present in red wine in relatively large concentrations (400 mg/L), compared to catechin (60 mg/L) and epicatechin (80 mg/L), the use of a colorimetric assay involving the reaction of monomeric and polymeric catechins with 4-dimethylamino-cinnamaldehyde would enable us to measure the total concentration of catechins in plasma following red wine consumption.

The aim of these experiments was to develop a sensitive assay for measuring plasma concentrations of catechins. This catechin assay would then by applied in order to quantitate plasma catechin concentrations in plasma following red wine consumption in rabbits.

8.2 METHODS

8.2.1 SEP-PAK C18 extraction and HPLC measurement of monomeric catechins

The extraction of catechin from plasma using solid phase SEP-PAK C18 cartridge was adopted from the published method of Pan *et al.* (1991). SEP-PAK cartridges (Waters Associates, Millipore, MA, USA) were conditioned by successive washes with 2 ml methanol, 5 ml 0.1M sodium acetate (pH 6.4) and 3 ml water. One ml of plasma was loaded onto the column and washed by the addition of 4 ml sodium acetate buffer and 4 ml hexane. The catechin was eluted from the column with 4 ml methanol and 4 ml acetone. These fractions were combined and evaporated to dryness under nitrogen. The samples were reconstituted in 200 µl of mobile phase for HPLC injection. The catechin was separated by HPLC using a C18 column (25 cm x 4 mm) and mobile phase of 16:84 (v/v) methanol: citrate buffer (0.03 M at pH 6.4) with 0.04% tetraethylmethylenediamine (TEMED) and 1 mmol/L EDTA at a flow rate of 1 ml/min. Catechin was detected by electrochemical detection (ECD) set at a voltage of +0.8 to +9 V.

8.2.2 Liquid solvent extraction and HPLC measurement of monomeric catechins

A method using a simple solvent extraction of catechin from plasma was employed as published by Lee *et al.* (1995). These authors described a method involving the extraction of tea catechins from plasma with ethyl acetate followed by quantification with HPLC and

ECD. This method was used, with minor modifications, to quantify catechin and epicatechin in plasma. One ml of plasma was added to a tube containing 100 µl of 0.4 M NaH_2PO_4 with 0.1% EDTA and 20% ascorbic acid and 20 µl of catechol as an internal standard. Lipids were initially extracted from plasma by mixing with 1 ml dichloromethane for 10 min and centrifuged at 3500 x *g* for 10 min to separate the organic and aqueous phases. The aqueous layer was collected and the remaining dichloromethane layer was extracted with 1 ml of water. The aqueous phases were pooled and extracted with an equal volume of ethyl acetate. The samples were mixed for 10 min then centrifuged at 3500 x *g* for 10 min. The ethyl acetate extraction of plasma was repeated. Twenty µl of buffer containing EDTA and ascorbic acid was added to the ethyl acetate phase and evaporated to dryness under a stream of nitrogen. The samples were reconstituted in 200 µl of mobile phase for HPLC injection. The mobile phase was 17.5:82.5 (v/v) methanol: citrate buffer (0.03M) at a flow rate of 1 ml/min. Catechin and epicatechin were separated using a spherisorb C18 column (25 cm x 4 mm) and were quantified by ECD set at a voltage of +0.9 V. The retention times of catechin, epicatechin and internal standard were 10.4, 22.8 and 8.9 mins respectively.

In other experiments measuring total catechin and epicatechin concentrations, plasma (1 ml) was incubated with β-glucuronidase (900 units) for 1 h in a water bath set at temperature of 37°C. The sample was then extracted with dichloromethane and ethyl acetate as described.

8.2.3 Solid phase extraction with aluminium oxide and colorimetric measurement of monomeric and polymeric catechins

A colorimetric method for measuring plasma catechins was adapted from that of Treutter (1989) and Kivits *et al.* (1997). Catechins were extracted from rabbit plasma using the method described by Kivits *et al.* (1997). One ml of plasma was vortexed for 2 min with 3 ml of methanol containing 1% BHT. The samples were centrifuged at 2000 x *g* for 10 min and the supernatant was collected and added to a 10 ml tapered tube containing 100 mg aluminium oxide. These samples were mixed on a rotating wheel at 4°C to allow catechins to adsorb to the aluminium oxide. After 15 min, the samples was centrifuged at 1000 x *g* for 3

min and the supernatant remove. The aluminium oxide was resuspended in diethyl ether and transferred to a glass pipette which had the tapered end blocked with glass wool. The aluminium oxide was washed with a further 3 ml of diethyl ether. The aluminium oxide was then dried by blowing with nitrogen. A 0.5 ml volume of 98:1:1 (v/v/v) methanol: perchloric acid: water containing 6 mmol/L of 4-dimethylamino-cinnamaldehyde (DMACA) was then passed through the column under a stream of nitrogen. The eluant was collected and centrifuged for 1 min at 1000 x *g* and incubated at room temperature for 9 min after which time the absorbance was measured at a visible wavelength of 637 nm. Preliminary experiments demonstrated that maximal absorbance was reached following 9 min at a wavelength of 637 nm.

8.3 RESULTS

8.3.1 Validation of SEP-PAK C18 extraction and HPLC measurement of monomeric catechins

The solid phase extraction method described by Pan *et al.* (1991) performed in the present experiments provided only a 30% recovery of catechin from plasma. With modifications to this method the recovery of catechin from plasma was increased to 75%. The modifications involved the omission of the acetone washing step since no catechin was detected in this solvent and the use of 2 ml of methanol rather than 4 ml since all of the catechin was eluted in this volume. The evaporation of 2 ml of methanol under a stream of nitrogen was more rapid and probably prevented catechin degradation. Despite the improved extraction efficiency of catechin from plasma, the limit of sensitivity measured by HPLC with ECD was only 0.5 mg/L with an accuracy of 20 to 30%. The reason for the low sensitivity and poor accuracy of the assay, despite the good recovery, was due to the poor chromatography of this HPLC method which had many interfering peaks at the catechin retention time of 7.5 min and an unsteady baseline. Modifications of the extraction procedure, mobile phase composition and voltage of ECD failed to improve the chromatography and quantification of catechin.

8.3.2 Validation of ethyl acetate extraction and HPLC measurement of monomeric catechins

Examples of a HPLC chromatograph and standard curves for catechin and epicatechin extracted from plasma with dichloromethane and ethyl acetate are shown in Figures 8.1 and 8.2. The standard curves were linear (r=0.999) between the concentration ranges of 0.1 to 10 mg/L. The limits of quantification of the lowest standard were 0.1 mg/L for both catechin and epicatechin with coefficients of variation of 9.1 and 6.9%, respectively. The extraction efficiencies of catechin and epicatechin were between 75 and 82%. The extractions of catechin and epicatechin were the same regardless of the initial dichloromethane extraction although this step greatly improved sample cleanliness as shown by the good chromatography. The use of ethyl acetate instead of diethyl ether resulted in 3 to 4-fold greater extraction of catechins from plasma samples. Increasing the volume of buffer containing NaH_2PO_4, EDTA and ascorbic acid or increasing the concentration of ascorbic acid did not improve the % recovery of catechins or the sensitivity of the assay.

8.3.3 Validation of solid phase extraction with aluminium oxide and colorimetric measurement of monomeric and polymeric catechins

Standard curves for catechin determined using a solid phase extraction of plasma catechins with aluminium oxide and colorimetric reaction with DMACA were linear between 0.1 to 1 mg/L of catechin (Figure 8.3). The coefficients of variation were acceptable below 10% over this concentration range. The recovery of catechin from plasma using this aluminium oxide adsorption technique was 85% with a coefficient of variation of 9.9%. This value is a small improvement on the published extraction efficiency of 78% (Kivits *et al.* 1997). The slight modification of this method involved transferring the aluminium oxide to a glass pipette and washing through with ether, drying with nitrogen and then washing the column with DMACA. These modification improved the recovery of catechin which was probably due to the increased contact of aluminium oxide with DMACA reagent compared to vortexing aluminium oxide with the reagent in a tube. In addition, the use of the glass pipette column increased the cleanliness of the sample which was reflected by the lower absorbance reading

of blank plasma samples. Increasing the concentration of DMACA did not increase the sensitivity of the assay.

8.3.4 Catechin measurements in red wine and rabbit plasma

The total monomeric and polymeric catechins in red wine were determined. The absorbance profile of red wine following reaction with DMACA reagent was the same as that determined for catechin with a peak absorbance at 637 nm. The catechin concentration in red wine, as measured by the DMACA colorimetric assay, without any extraction procedure was 245.3 ± 12.6 mg/L, compared to a value of 143.7 ± 2.6 mg/L of catechin equivalents following extraction of red wine catechins with aluminium oxide. Compared to the 85% extraction of monomeric catechin from plasma, monomeric and polymeric catechins were extracted with an efficiency of approximately 60% from red wine, indicating that the solid phase extraction with aluminium oxide was more effective in extracting monomeric catechins than it was for polymeric catechins.

Using the method published by Lee *et al.* (1991) with the modifications described, monomeric catechin or epicatechin could not be detected in plasma from rabbits consuming red wine daily for 12 weeks even after hydrolysis of plasma samples with β-glucuronidase. Plasma levels of monomeric catechins and polymeric catechins were also undetectable in plasma from fasted rabbits and in non-fasted plasma samples taken approximately 1 h following red wine consumption using the aluminium oxide extraction method and DMACA reaction.

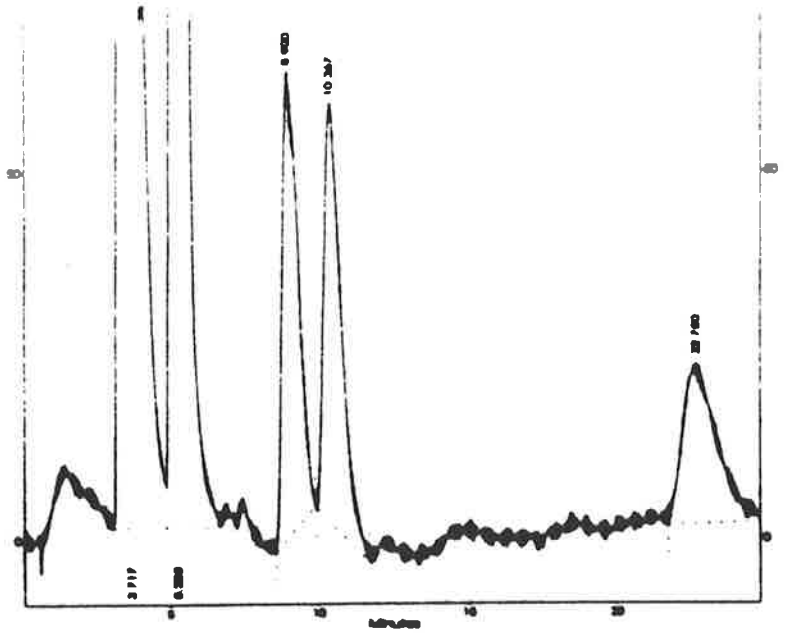


Figure 8.1
HPLC chromatograph of catechin and epicatechin following plasma extraction with ethyl acetate.

HPLC with ECD was performed on plasma samples spiked with known amounts of catechin and epicatechin. The catechins were extracted from plasma with sequential dichloromethane and ethyl acetate steps as described in method 8.2.2. Peaks at retention times of 8.9, 10.4 and 22.8 min correspond to internal standard, catechin and epicatechin respectively.

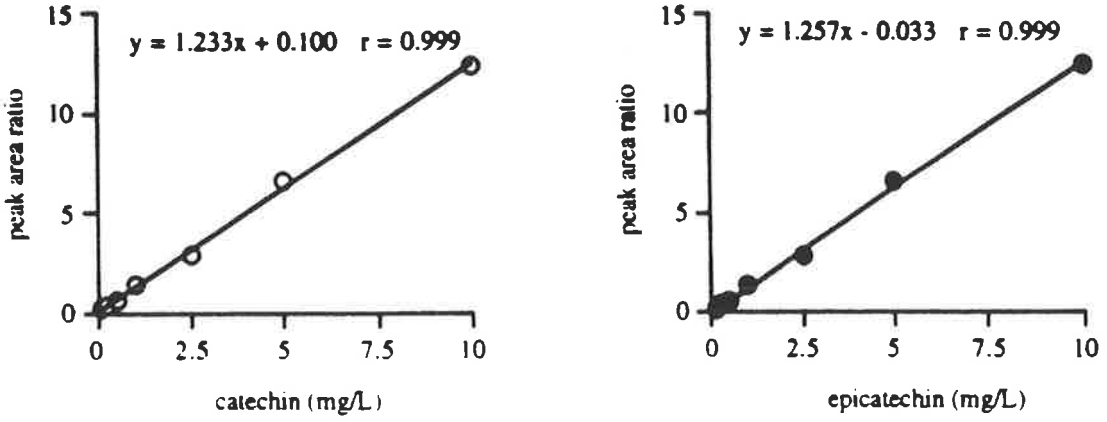


Figure 8.2
Standard curves of catechin and epicatechin measured by HPLC with ECD following plasma extraction with ethyl acetate

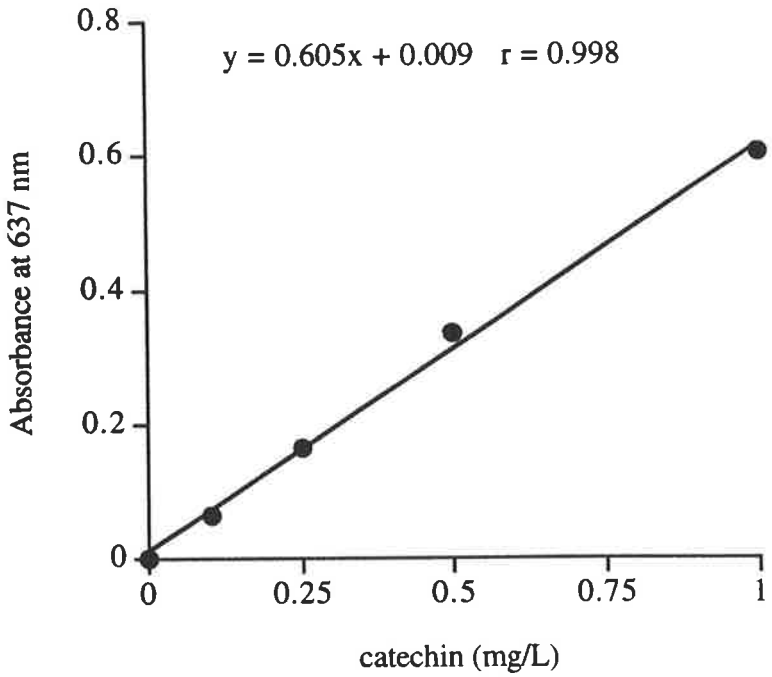


Figure 8.3

Standard curves of catechins measured by colorimetric reaction with DMACA following plasma extraction with aluminium oxide.

8.4 DISCUSSION

In the present experiments plasma concentrations of either monomeric catechin or procyanidins following red wine consumption in rabbits were undetectable. The red wine used in this dietary intervention study contained a total of 140 mg/L of monomeric catechins (60 mg/L catechin and 80 mg/L epicatechin) and 400 mg/L of procyanidins (Table 5.1). Prior to administration to rabbits, the red wine was diluted in water to reduce the ethanol content from 13.5% (v/v) to 7.5% and in so doing the catechin concentrations were diluted to 33, 44 and 220 mg/L of catechin, epicatechin and procyanidins, respectively. The total catechin concentration in diluted red wine was 300 mg/L. Rabbits consumed 22.5 ml of red wine or 36 mg of total polyphenols/d which is roughly equivalent to 7 mg of total catechins/d.

To date only one group have measured the levels of polyphenols in LDL following red wine consumption. Fuhrman *et al.* (1995) measured 25 mg polyphenols/g LDL protein in healthy subjects, as measured by the Folin-Ciocalteu method, following the consumption of 400 ml red wine for two weeks (Singleton 1988). This method is routinely used to measure polyphenols in wine and involves a colorimetric reaction between phenolic hydroxy groups and phosphomolybdic phosphotungistic acid reagent. The application of this method in biological fluids is questionable since it is very non-specific and produces a chromophore that absorbs light at a wavelength of 765 nm, a common absorbance range for proteins and other plasma components. These findings by Fuhrman *et al.* (1995) should be treated with caution since the increase in absorbance may not be necessarily due to red wine derived polyphenols.

The plasma concentrations of tea catechins following green tea consumption in humans have been previously determined (Lee *et al.* 1995, Unno *et al.* 1996, Kivits *et al.* 1997). Tea catechins are rapidly absorbed with peak plasma concentrations 1 to 2 h following tea ingestion as measured by HPLC with coulochem ECD. The main tea catechins quantified in plasma are epigallocatechin-3-gallate, epigallocatechin and epicatechin with concentrations of approximately 0.05 to 0.27 mg/L, 0.08 to 0.20 mg/L and 0.05 to 0.08 mg/L, respectively.

These plasma levels of tea catechins were achieved following the consumption of 1.2 g of green tea in 200 ml of water which contained approximately 210 mg of green tea catechins. (Lee *et al.* 1995).

Kivits *et al.* (1997) have managed to quantify quite high levels of catechins in human plasma following tea drinking by applying a simple colorimetric assay that relies on a chemical reaction between 4-dimethylamino-cinnamaldehyde reagent and compounds with meta-orientated hydroxy groups on the A ring and a single C—C bond at positions 2 and 3 of the C ring such as catechin (see Figure 1.1). Anthocyanidins share these structural similarities, but following reaction with DMACA, exhibit a different maximal absorbance (Treutter 1989). This colorimetric assay is therefore more specific than the method described by Singleton (1988), since the latter assay is based on the reaction of any phenolic hydroxy groups with reagent. Whereas most studies (Lee *et al.* 1995, Unno *et al.* 1996) measure individual tea catechins in the 0.1 to 0.2 mg/L concentration range, Kivits *et al.* (1997) has reported a total catechin concentration of 1.4 mg/L in human plasma 1 h after ingesting 3 g green tea (equivalent to 6 cups).

The method of Kivits *et al.* (1997) did not improve the sensitivity of catechin quantification in the present study. This method did however offer advantages over the HPLC methods because it was much simpler and quicker to perform. Furthermore the use of this colorimetric assay meant that polymeric as well as monomeric catechins could be measured in rabbit plasma. Red wine contains quantitatively more procyanidins than monomeric catechins. The absorbance profile measured in the present study for the reaction between red wine with DMACA, was the same as the absorbance profile for catechin which has been reported by Kivits *et al.* (1997). Experiments revealed that polymeric catechins were not extracted as efficiently as monomeric catechins from red wine using the aluminium absorption technique. In this method polar hydroxy groups bind to aluminium oxide. Procyanidins are very hydrophobic compounds and would be expected to bind well to the aluminium oxide. The reason why procyanidins were not extracted as well as monomeric catechins may be due to the large size of these polymeric compounds.

In summary, plasma catechins were not detected in rabbit plasma 1 h post red wine consumption nor any time thereafter. Rabbits consumed 7 mg of catechins/d in the form of red wine. This dose of catechin is considerable lower than the 210 mg of catechin in green tea consumed in the dietary intervention studies mentioned above (Lee *et al.* 1995). Although approximately 0.2 mg/L of catechins are measured in plasma following consumption of green tea (Lee *et al.* 1995), this is equivalent to a small fraction of the dose, indicating that the bioavailability of oral catechin is poor. With the current knowledge of tea catechin doses and bioavailability, it seems that the lack of catechin detection in rabbit plasma is a result of the comparatively low intake of red wine-derived catechins in this study.

CHAPTER 9

GENERAL DISCUSSION

Red wine and genistein decreased the susceptibility of LDL to copper- and peroxy radical-mediated oxidation *in vitro*. The oxidation of LDL is considered to be an underlying event in the development of atherosclerosis. Oxidised LDL is a ligand for scavenger receptors on macrophages and the accumulation of oxidised LDL intracellularly leads to foam cell formation. By inhibiting free radical catalysed oxidation of LDL, the atherogenic process may also be prevented. However, despite it's *in vitro* antioxidant properties, red wine supplementation in cholesterol-fed rabbits did not inhibit LDL oxidation or prevent the development of aortic fatty lesions.

The isoflavone genistein was poorly incorporated into LDL following incubations of the aglycone form of genistein with plasma (Chapter 4). This observation is supported by experiments performed by Lehtonen *et al.* (1996) who demonstrated that genistein was not incorporated into liposomal bilayers *in vitro*. Genistein may not be a good antioxidant *in vivo* due to its inability to be incorporated into lipoproteins. Indeed Nestel *et al.* (personal communication) did not find a change in LDL oxidisability following dietary intervention with isoflavones (genistein and daidzein) in menopausal women. Further studies investigating the effect of isoflavones on LDL oxidation and atherosclerosis were not performed since previous experiments have shown that dietary intervention with genistein does not inhibit LDL oxidation *in vitro* (Nestel *et al.* personal communication) and that plasma genistein concentrations are relatively low following dietary supplementation in humans (Adlercreutz *et al.* 1993, Gooderman *et al.* 1996). Furthermore, genistein is present in plasma mainly in the conjugated form (Adlercreutz *et al.* 1993) and the antioxidant activity of the glucuronidated genistein is not known.

The impetus for studying red wine, with respect to antioxidant and antiatherogenic effects, stemmed firstly from epidemiological studies. Many cross-cultural epidemiological studies identified wine as a dietary variable inversely associated with CAD mortality (St Leger *et al.* 1979 Renaud *et al.* 1992a). A biochemical basis for these epidemiological observations could

lie with red wines ability to inhibit LDL oxidation (Frankel *et al.* 1993a), increase HDL-cholesterol (Valimaki *et al.* 1988, Clevidence *et al.* 1995, Lecomte *et al.* 1996) and decrease platelet aggregation (Pikkar *et al.* 1987, Renaud *et al.* 1992b). While red wine is considered by many to be protective against heart disease, little evidence by way of controlled intervention studies has been accumulated which confirms that dietary intake of red wine is associated with reduced atherosclerosis and CAD.

One previous study has investigated the effect of red wine and other alcoholic beverages on atherosclerosis (Klurfeld *et al.* 1981). In that study, a reduction in atherosclerosis lesions in the aortic arch, thoracic aorta and small myocardial arteries following red wine consumption in rabbits was observed. The results from the present study do not confirm the antiatherogenic properties of red wine reported by Klurfeld *et al.* (1981). The mechanism for the reduced atherosclerosis development in the study performed by Klurfeld *et al.* (1981) was not elucidated. Apart from determinations of lipoprotein profiles, no other factors which could influence atheroma development, such as lipid peroxidation, LDL fatty acid or antioxidant levels were measured nor did they did make any measurements concerning the absorption or bioavailability of red wine polyphenols in that study.

For an antioxidant compound to be effective *in vivo* it must be absorbed following administration and be present at the site of action at the concentrations required to exert its antioxidant effect. The compounds in red wine responsible for it's antioxidant properties are the grape-derived polyphenols. The main polyphenols in red wine are phenolic acids, flavonols, flavan-3-ols (catechins), procyanidins and anthocyanidins (Singleton 1988). The great heterogeneity of these compounds makes it difficult to identify a single compound or class of compounds that exerts the greatest antioxidant activity. This also leads to difficulties when attempting to quantitate plasma concentrations of red wine polyphenols.

In the present study attempts were made to quantify plasma concentrations of polyphenols following red wine consumption in rabbits. Catechin was chosen to be a relevant marker of red wine bioavailability since levels of these compounds in red wine were known (see Table 5.1) and the fraction of red wine containing catechins displayed antioxidant properties *in vitro* (see Chapter 3). Neither monomeric catechins nor procyanidins were detected in rabbit plasma following red wine consumption, which was due in part to the relatively low dose of catechin and the poor bioavailability of catechins which has been previously reported following green tea ingestion (Lee *et al.* 1995, Unno *et al.* 1996, Kivits *et al.* 1997).

There is a lack of quantitative data in the literature describing the relationship between plasma concentrations of flavonoids and their effects on biochemical parameters such as LDL oxidation. Although the plasma levels of red wine-derived catechins were less than 0.1 mg/L, the concentrations of total polyphenols required to inhibit copper-mediated oxidation of LDL *in vitro* were equivalent to this concentration. Catechins comprised approximately 20% of the total polyphenols in red wine according to the red wine analysis (see Table 5.1) and the composition data following red wine fractionation (Chapter 3). Further studies are needed in order to elucidate the dose-dependent or plasma concentration-dependent effects of red wine polyphenols on LDL oxidation and atherosclerosis, possibly without the confounding influence of ethanol.

Dietary supplementation of red wine in humans has been reported to inhibit oxidation of LDL (Fuhrman *et al.* 1995, Kondo *et al.* 1994, Maxwell *et al.* 1994, Whitehead *et al.* 1995). Only one study conducted by Fuhrman *et al.* (1995) has determined the concentrations of polyphenols in LDL following red wine consumption in humans. Fuhrman *et al.* (1995) observed a decreased susceptibility of LDL to oxidation which was associated with a 5-fold increase in LDL polyphenol concentration. This study was conducted in subjects consuming a fixed amount of red wine. It would be useful to investigate whether increasing dietary intakes of red wine influence the degree of protection against LDL oxidation in these types of

human dietary interventions. A correlation between plasma concentrations of red wine polyphenols and the susceptibility of LDL to *in vitro* catalysed oxidation would strengthen the claim that red wine is an effective *in vivo* antioxidant.

Although the plasma concentrations of red wine polyphenols in rabbits were not detectable, some discussion regarding the dietary intake of red wine by rabbits is appropriate. On a kilogram body weight basis, red wine consumption by rabbits (6.7 ml/d/kg body weight) was comparable to an intake of 2 to 3 glasses (300 to 450 ml) of red wine in human subjects (4 to 9 ml/d/kg body weight). These levels of red wine consumption over a period of 2 to 4 weeks in normocholesterolemic or hypercholesterolemic subjects are effective in inhibiting both plasma and LDL oxidation (Fuhrman *et al.* 1995, Kondo *et al.* 1994, Maxwell *et al.* 1994, Whitehead *et al.* 1995). Furthermore, a consumption of alcohol equivalent to 2 to 3 glasses daily is often categorised as "moderate" alcohol intake which is associated with a reduced risk of myocardial infarction (Maclure *et al.* 1993). The dietary intake of red wine by rabbits in the present study are relevant to the levels of intake in humans which have been shown to slow LDL oxidation and associated with reductions in CAD mortality in cross-cultural epidemiological studies (St Leger *et al.* 1979, Renaud *et al.* 1992a).

An anomaly exists between the *in vitro* antioxidant and the apparent prooxidant properties of red wine following dietary intervention in cholesterol-fed rabbits. Since plasma catechin levels were not detected in cholesterol-fed rabbits consuming red wine, it is difficult to conclude that polyphenolic components of red wine attributed to the proatherogenic effects of red wine observed in this present study. Due to the unpalatability of ethanol, the intake by ethanol-treated rabbits was approximately half the intake of ethanol by red wine-treated rabbits. This is an important consideration when interpreting the results from this dietary intervention study, particularly with respect to the *in vivo* prooxidant effects of ethanol. Croft *et al.* (1996) observed an increase in LDL oxidisability in subjects consuming a high alcohol beverage compared to subjects consuming a low alcohol beverage. In addition,

Shiash *et al.* (1987) reported an ethanol-dependent increase in artery cholesterol and lipophilic staining of the aorta in cholesterol-fed rabbits consuming ethanol. These published studies provide evidence of *in vitro* prooxidant and proatherogenic effects of ethanol in certain models. In the present study, the percent of aortic fatty lesions (Table 6.3) and the increases in LDL oxidisability (Table 6.1) following the intake of red wine, and to a lesser extent ethanol, may have been dependent on the dose of ethanol. Although elevated, there did not appear to be an ethanol dose-dependent effect on cholesterol concentration in descending thoracic aortic segments from red wine- and ethanol-treated rabbits (Table 6.3).

The reason why ethanol increases susceptibility of LDL oxidation *in vitro* (Croft *et al.* 1996) and promotes atherosclerosis in certain animal models (Shiash *et al.* 1997) but is reported to lower the incidences of CAD in epidemiological studies is unclear (St Leger *et al.* 1979). Perhaps ethanol intake and CAD do not have a causal relationship. Alternatively, models of LDL oxidation *in vitro*, like that catalysed by copper, may not be predictive or representative of oxidation *in vivo*. The discrepancy between *in vitro* oxidisability of LDL in the presence of red wine and following dietary intervention with red wine and cholesterol in rabbits may also be due to the prooxidant model used. Future studies investigating the effect of red wine or ethanol on LDL oxidation may benefit by using other catalysts of *in vitro* oxidation that are more relevant to the *in vivo* situation, such as reactive oxygen species, macrophages and lipoxygenases.

Rabbits fed a cholesterol diet exhibited an impaired response to acetylcholine-induced relaxation of aortic smooth muscle. The initial hypothesis was that red wine polyphenols could ameliorate the impairment of vascular relaxation in cholesterol-fed rabbits, since other antioxidants have been shown to restore endothelium-dependent vascular relaxation (Stewart-Lee *et al.* 1994, Keaney *et al.* 1993, Matz *et al.* 1994, Klemsdale *et al.* 1994, Andersson *et al.* 1994). *In vitro* studies by Fitzpatrick *et al.* (1993) and Andriambeloson *et al.* (1997) have demonstrated that red wine and polyphenolic compounds mediate

endothelium-dependent relaxation of rat aorta. In the present study dietary intervention with red wine or ethanol did not alter aortic relaxation to acetylcholine. Since red wine treatment increased LDL oxidisability and thoracic aortic cholesterol in cholesterol-fed rabbits, it is unlikely that red wine would restore endothelium-dependent relaxation by scavenging free radicals in the artery wall. The concentrations of red wine derived polyphenols in aortic segments were not measured in this study. Since plasma levels of catechin were undetectable, aortic concentrations of these compounds may also be low and this may explain the apparent inability of red wine to restore endothelium-dependent relaxation *in vivo*.

In conclusion, the *in vitro* properties of red wine such as inhibiton of LDL oxidation and mediating endothelium-dependent relaxation are not apparent *in vivo*. A controlled dietary intervention study with red wine in cholesterol-fed rabbits did not lend support to the hypothesis that red wine polyphenols can protect against LDL oxidation and atherosclerosis *in vivo*. The low plasma levels of red wine-derived polyphenols and the prooxidant effects of ethanol in cholesterol-fed rabbits may explain the increases in oxidisability of LDL and aortic cholesterol concentrations following dietary intervention with red wine.

BIBLIOGRAPHY

Abbey M, Nestel PJ, Baghurst PA. Antioxidant vitamins and low-density lipoprotein oxidation. *Am J Clin Nutr* 1993; 58: 525-532.

Abu-Amsha R, Croft KD, Puddey IB, Proudfoot JM, Beilin LJ. Phenolic content of various beverages determines the extent of inhibition of human serum and low density lipoprotein oxidation in vitro: identification and mechanism of some cinnamic derivatives from red wine. *Clinical Sci* 1996; 91: 449-458.

Adlercreutz H, Honjo H, Higashi A, Fotsis T, Hamalainen E, Hasegawa T, Okada H. Urinary excretion of lignans and isoflavanoid phytoesterogens in Japanese men and women consuming a traditional Japanese diet. *Am J Clin Nutr* 1991; 54: 1093-1100.

Adlercreutz H, Markkanen H, Watanabe S. Plasma concentrations of phtyo-oesterogens in Japanese men. *Lancet* 1993; 342: 1209-1210.

Afanas'ev IB, Dorozhko AI, Brodskii AV, Kostyuk VA, Potapovitch AI. Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochem Pharmacol* 1989; 38: 1763-1769.

Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M, Fukami Y. Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J Biol Chem* 1987; 262: 5592-5595.

Akiyama T, Ogawara H. Use and specificity of genistein as inhibitor of protein tyrosine kinase. *Methods Enzmol* 1991; 201: 362-370.

Andersson TL, Matz J, Ferns GAA, Anggard EE. Vitamin E reverses cholesterol-induced endothelial dysfunction in the rabbit coronary circulation. *Atherosclerosis* 1994; 111: 39-45.

Andrews HE, Bruckdorfer KR, Dunn RC, Jacobs M. Low-density lipoproteins inhibit endothelium-dependent relaxation in rabbit aorta. *Nature* 1987; 327: 237-239.

Andriambeloson E, Kleschyov AL, Muller B, Beretz A, Stoclet JC, Andriantsitohaina R. Nitric oxide production and endothelium-dependent vasorelaxation induced by wine polyphenols in rat aorta. *Br J Pharmacol* 1997; 120: 1053-1058.

Arai E, Kita T, Yokode M, Narumiya S, Kawai C. Multiple receptors for modified low density lipoproteins in mouse peritoneal macrophage: Different uptake mechanisms for acetylated and oxidised low density lipoproteins. *Biochem Biophys Res Comm* 1989; 159: 1375-1382.

Aust SD, Morehouse LA, Thomas CE. Role of metals in oxygen radical reactions. *J Free Rad Biol Med* 1985; 1: 3-25.

Azen SP, Qian D, Mack WJ, Sevanian A, Selzer RH, Liu CR, Liu CH, Hodis HN. Effect of supplementary antioxidant vitamin intake on carotid arterial wall intima-media thickness in a controlled clinical trial of cholesterol lowering. *Circ* 1996; 94: 2369-2372.

Barter PJ, Jones ME. Rate of exchange of esterified cholesterol between human plasma and high density lipoprotein. *Atherosclerosis* 1979; 34: 67-74.

Berliner JA, Territo MC, Sevanian A, Ramin S, Kim JA, Bamshad B, Esterson M, Fogelman AM. Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. *J Clin Invest* 1990; 85: 1260-1266.

Besancon TK, Rouanet JM. Dietary grape tannins affect lipoproteins, lipoprotein lipases and tissue lipids in rats fed hypercholesterolemic diets. *J Nutr* 1994; 124: 2451-2457.

Beynen AC, Meijer GW, Lemmens AG, Glatz JFC, Versluis A, Katan MB, Van Zutphen LFM. Sterol balance and cholesterol absorption in inbred strains of rabbits hypo- and hyperresponsive to dietary cholesterol. *Atherosclerosis* 1989; 77: 151-157.

Bickle PE, Freeman MW. Rabbit aortic smooth muscle cells express inducible macrophage receptor messenger RNA that is absent from endothelial cells. *J Clin Invest* 1992; 90: 1450-1457.

Bjorkhem I, Henriksson-Freyschuss A, Breuer O, Diczfalusy U, Berglund L, Henriksson P. The antioxidant butylated hydroxytoluene protects against atherosclerosis. *Arterioscl Thromb* 1991; 11: 15-22.

Bonithon-Kopp C, Coudray C, Berr C, Touboul PJ, Feve JM, Favier A, Ducimetiere P. Combined effects of lipid peroxidation and antioxidant status on carotid atherosclerosis in a population aged 59-71 y: The EVA Study. Etude sur le Viellisement Arteriel. *Am J Clin Nutr* 1997; 65: 121-127.

Bossaller C, Habib GB, Yamamoto H, Williams C, Wells S, Henry PD. Impaired muscarinic endothelium-dependent relaxation and cyclic guanosine 5'-monophosphate formation in atherosclerotic human coronary artery and rabbit aorta. *J Clin Invest* 1987; 79: 170-174.

Bowry VW, Ingold KU, Stocker R. Vitamin E in human low-density lipoprotein. When and how this antioxidant becomes a pro-oxidant. *Biochem J* 1992; 288: 341-344.

Bowry VW, Stocker R. Tocopherol-mediated peroxidation. The prooxidant effect of vitamin E on the radical initiated oxidation of human low-density lipoprotein. *J Am Chem Soc* 1993; 115: 6029-6044.

Bowry VW, Mohr D, Cleary J, Stocker R. Prevention of tocopherol-mediated peroxidation in ubiquinol-10-free human low density lipoprotein. *J Biol Chem* 1995; 270: 5756-5763.

Brattsand R. Distribution of cholesterol and triglycerides among lipoprotein fractions in fat-fed rabbits at different levels of serum cholesterol. *Atherosclerosis* 1976; 23: 97-110.

Brown MS, Goldstein JL. Lipoprotein metabolism in the macrophage: Implications for cholesterol deposition in atherosclerosis. *Ann Review Biochem* 1983; 52: 223-261.

Buege JA, Aust SD. Microsomal lipid peroxidation. *Methods in Enzymology* 1978; 52: 302-310.

Bui MN, Sack MN, Moutsatsos G, Lu DY, Katz P, McCown R, Breall JA, Rackley CE. Autoantibody titers to oxidised low-density lipoprotein in patients with coronary atherosclerosis. *Am Heart J* 1996; 131: 663-667.

Carew TE, Schwenke DC, Steinberg D. Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: Evidence that antioxidants *in vivo* can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbit. *Proc Natl Acad Sci USA* 1987; 84: 7725-7729.

Carpenter KLH, Taylor SE, Ballantine JA, Fussell B, Halliwell B, Mitchinson MJ. Lipids and oxidised lipids in human atheroma and normal aorta. *Biochem Biophys Acta* 1993; 1167: 121-130.

Cassidy A, Bingham S, Setchell KDR. Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women. *Am J Clin Nutr* 1994; 60: 333-340.

Castelli WP, Doyle JT, Gordon T, Hames CG, Hjortland MC, Hulley SB, Kagan A, Zukel WJ. Alcohol and blood lipids. The cooperative phenotyping study. *Lancet* 1977; 2: 153-155.

Castelli WP. Epidemiology of coronary heart disease: the Framingham study. Am J Med 1984; 76: 4-12.

Cathcart MK, Morel DW, Chisolm GM. Monocytes and neutrophils oxidise low density lipoprotein making it cytotoxic. *J Leukocyte Biol* 1985; 38: 341-350.

Chen LY, Metha P, Metha JL. Oxidised LDL decreases *L*-arginine uptake and nitric oxide synthase protein expression in human platelets: relevance of the effect of oxidised LDL on platelet function. *Circ* 1996; 93: 1740-1746.

Chin JPF, Dart AM. Therapeutic restoration of endothelial function in hypercholesterolemic subjects: Effects of fish oils. *Clin Exp Pharmacol Physiol* 1994; 21: 749-756.

Chisaka T, Matsuda H, Kubomura Y, Mochizuki M, Yamahara J, Fujimura H. The effect of crude drugs on experimental hypercholesterolemia: Mode of action of (-)-epigallocatechin gallate in tea leaves. *Chem Pharm Bull* 1988; 36: 227-233.

Chisolm GM, Ma G, Irwin KC, Martin LL, Gunderson KG, Linberg LF, Morel DW, DiCorleto PE. 7β-Hydroperoxycholest-5-en-3β-ol, a component of human atherosclerotic lesions, is the primary cytotoxin of oxidised human low density lipoprotein. *Proc Natl Acad Sci USA* 1994; 91: 11452-11456.

Chung BH, Segrest JP, Ray MJ, Brunzell JD, Hokanson JE, Krauss RM, Beaudrie K, Cone JT. Single vertical spin gradient ultracentrifugation. *Methods in Enzymology* 1986; 128: 181-209.

Clevidence BA, Reichman ME, Judd JT, Muesing RA, Schatzkin A, Schaefer EJ, Li Z, Jenner J, Sunkin, *et al.* Effects of alcohol consumption on lipoproteins of premenopausal women. A controlled diet study. *Arterioscler Thromb Vasc Biol* 1995; 15: 179-184.

Clifton PM, Chang L, Mackinnon AM. Development of an automated Lowry protein assay for the Cobas-Bio centrifugal analyser. *Anal Biochem* 1988; 172: 165-168.

Colf B, Verheyden J. Electrophoresis and sudan black staining of lipoproteins on gelatinised cellulose acetate. *Clin Chim Acta* 1967; 18: 325.

Colsher PL, Wallace RB. Is modest alcohol consumption better than none at all? An epidemiological assessment. *Ann Rev Public Health* 1989; 10:203-219.

Cominacini L, Garbin U, Pastorino AM, Davoli A, Campagnola M, De Santis A, Pasini C, Faccini GB, Trevisan MT, Bertozzo *et al.* Predisposition to LDL oxidation in patients with and without angiographically established coronary artery disease. *Atherosclerosis* 1993; 99: 63-70.

Cook NC, Samman S. Flavonoids - Chemistry, metabolism, cardioprotective effects, and dietary sources. *J Nutr Biochem* 1996; 7: 66-76.

Cooke JP, Andon NA, Girerd XJ, Hirsch AT, Creager MA. Arginine restores cholinergic relaxation of hypercholesterolemic rabbit aorta. *Circulation* 1991; 83: 1057-1062.

Criqui MH, Ringel BL. Does diet or alcohol explain the French paradox? *Lancet* 1994; 344: 1719-1723.

Croft KD, Williams P, Dimmitt S, Abu-Amsha R , Beilin LJ. Oxidation of low density lipoproteins: effect of antioxidant content, fatty acid composition and intrinsic phospholipase activity on susceptibility to metal ion-induced oxidation. Biochem Biophys Acta 1995; 1254: 250-256.

Croft KD, Puddey IB, Rakic V, Abu-Amsha R, Dimmitt S, Beilin LJ. Oxidative susceptibilty of low-density lipoproteins - influence of regular alcohol use. *Alcohol Clin Exp Res* 1996; 20: 980-984.

Cushing SD, Berliner JA, Valente AJ, Territo MC, Narab M, Parhami F, Gerrity R, Schwartz CJ, Fogelman AM. Minimally modified LDL induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc Natl Acad Sci USA* 1990; 87: 5134-5138.

Daley SJ, Herderick EE, Cornhill JF, Rogers KA. Cholesterol-fed and casein-fed rabbit models of atherosclerosis. Part 1: Differing lesion area and volume despite equal plasma cholesterol levels. *Arterioscler Thromb* 1994a; 14: 95-104.

Daley SJ, Klemp KF, Guyton JR, Rogers KA. Cholesterol-fed and casein-fed rabbit models of atherosclerosis. Part 2: Differing morphological severity of atherogenesis despite matched plasma cholesterol levels. *Arterioscler Thromb* 1994b; 14: 105-114.

Darley-Usmar VA, Hogg N, O'Leary VJ, Wilson MT, Moncada S. The simultaneous generation of superoxide and nitric oxide can initiate lipid peroxidation in human low density lipoprotein. *Free Radic Res Commun*. 1992; 17:9-20.

Darley-Usmar VA, Halliwell B. Blood radicals: reactive nitrogen species, reactive oxygen species, transition metal ions, and the vascular system. *Pharm Res* 1996; 13: 649-662.

Daugherty A, Zweifel B, Schonfeld G. Probucol attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits. *Br J Pharmacol* 1989; 98: 612-618.

Daugherty A, Zweifel B, Schonfeld G. The effects of probucol on the progression of atherosclerosis in mature Watanabe heritable hyperlipidemic rabbits. *Br J Pharmacol* 1991; 103: 1013-1018.

de Rijke YB, Vogelezang CJ, van Berkel TJ, Princen HM, Verwey HF, van der Laarse A, Bruschke AV. Susceptibility of low-density lipoproteins to oxidation in coronary bypass patients. *Lancet* 1992; 340: 858-859.

de Rijke YB, Demacker PNM, Assen NA, Sloots LM, Katan MB, Stalenhoef AFH. Red wine consumption does not affect oxidizability of low density lipoproteins in volunteers. *Am J Clin Nutr* 1996; 63: 329-334.

De Whalley CV, Rankin SM, Hoult RS, Jessup W, Leake DS. Flavanoids inhibit the oxidative modification of low density lipoproteins by macrophages. *Biochem Pharm* 1990; 39: 1743-1750.

Del Rio M, Chuila T, Merchen-Perez A, Remezal M, Valor S, Gonzalez J, Gutierrez JA, Contreras JA, Lasuncion MA, Tejerina T. Effects of indapamide on atherosclerosis development in cholesterol-fed rabbits. *J Cardiovasc Pharmacol* 1995; 25: 973-978.

Dieber-Rotheneder M, Puhl H, Waeg G, Striegl G, Esterbauer H. Effect of oral supplementation with D-α-tocopherol on the vitamin E content of human low density lipoproteins and resistance to oxidation. *J Lipid Res* 1991; 32: 1325-1332.

Di Rosa M, Ialenti A, Ianaro A, Sautebin L. Interaction between nitric oxide and cyclooxygenase pathways. *Prost, Leuk and Ess Fatty Acids* 1996; 54: 229-238.

Dreosti IE. In: Trace elements, micronutrients and free radicals. Free radical pathology and the genome. Ed: Dreosti IE, Humana Press, NJ, USA, 1991; 149-170.

Drexler H, Zeiher AM, Meinzer K, Just H. Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolemic patients by *L*-arginine. *Lancet* 1991; 338: 1546-1550.

Du ZY, Woodman OL. The effect of hypercholesterolemia and atherosclerosis on α-adrenoceptor-mediated vasoconstriction in conscious rabbits and rabbit aorta. *Europ J Pharm* 1992; 211: 149-156.

Dusting GJ. Nitric oxide in cardiovascular disorders. *J Vasc Res* 1995; 32: 143-161.

Endemann G, Stanton LW, Madden KS, Bryant CM, White RT, Protter AA. CD36 is a receptor for oxidised low density lipoprotein. *J Biol Chem* 1993; 268: 11811-11816.

Esterbauer H, Jurgens G, Quehenberger O, Koller E. Autoxidation of human LDL: loss of polyunsaturated fatty acids and vitamin E and generation of aldehydes. *J Lipid Res* 1987; 28: 495-509.

Esterbauer H, Striegl G, Puhl H, Rotheneder M. Continuous monitoring of *in vitro* oxidation of human low density lipoprotein. *Free Rad Res Commun* 1989a; 6:67-75.

Esterbauer H, Striegl G, Puhl H, Oberreither S, Rotheneder M, El-Saadani M, Jurgens G. The role of vitamin E and carotenoids in preventing oxidation of low density lipoproteins. *Ann NY Acad Sci* 1989b; 570: 254-267.

Esterbauer H, Puhl H, Dieber-Rotheneder M, Waeg G, Rabl H. Effect of antioxidants on oxidative modification of LDL. *Ann Med* 1991; 23: 573-581.

Esterbauer H, Gebicki J, Puhl H, Jurgens G. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Rad Biol Med* 1992; 13: 341-390.

Fitzpatrick DF, Hirschfield SL, Coffey RG. Endothelium-dependent vasorelaxing activity of wine and other grape products. *Am J Physiol* 1993; 265: H774-778.

Flavahan NA. Atherosclerosis or lipoprotein-induced endothelial dysfunction. *Circulation* 1992; 85: 1927-1938.

Fogelman AM, Shechter I, Seager J, Hokom M, Child JS, Edwards PA. Malondialdehyde alteration of low density lipoproteins lead to cholesteryl ester accumulation in human monocyte-macrophages. *Proc Natl Acad Sci USA* 1980; 77: 2214-2218.

Fogelman AM, Haberland ME, Seager J, Hokom M, Edwards PA. Factors regulating the activities of the low-density lipoprotein receptor and the scavenger receptor on human monocyte-macrophage. *J Lipid Res* 1981; 22: 1131-1141.

Folch J, Lees M. A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 1957; 226: 494-509.

Fong LG, Parthasarathy S, Witztum JL, Steinberg D. Non-enzymatic cleavage of peptide bonds in apoprotein B-100. *J Lipid Res* 1987; 28: 1466-1477.

Fotsis T, Pepper M, Adlercreutz H, Fleischmann G, Hase T, Montesano R, Schweigerer L. Genistein, a dietary inhibitor of *in vitro* angiogenesis. *Proc Natl Acad Sci USA* 1993; 90: 2690-2694.

Fowler S, Shio H, Haley NJ. Characterisation of lipid-laden aortic cells from cholesterol-fed rabbits. IV. Investigation of macrophage-like properties of aortic cell populations. *Lab Invest* 1979; 41: 372-378.

Frankel EN, Kanner J, German JB, Parks E, Kinsella JE. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *Lancet* 1993a; 341: 454-457.

Frankel EN, Waterhouse AL, Kinsella JE. Inhibition of human LDL oxidation by resveratrol. *Lancet* 1993b; 341: 1103-1104.

Frankel EN, Waterhouse AL, Teissedre PL. Principal phenolic phytochemicals in selected California red wines and their antioxidant activity in inhibiting oxidation of human low-density lipoproteins. *J Agric Food Chem* 1995; 43: 890-894.

Fredrickson DS, Goldstein JL, Brown MS. In: The metabolic basis of inherited disease. Ed: JB Stanbury, New York: McGraw Hill 4th Ed 1978.

Freeman M, Ashkenas J, Rees DJ, Kingsley DM, Copeland NG, Jenkins NA, Krieger M. An ancient, highly conserved family of cysteine-rich protein domains revealed by cloning type I and II murine macrophage scavenger receptors. *Proc Natl Acad Sci USA* 1990; 87: 8810-8814.

Freeman M, Ekkel Y, Rohrer L, Penman M, Freedman NJ, Chisolm GM, Krieger M. Expression of type I and II bovine scavenger receptors in Chinese hamster ovary cells: Lipid droplet accumulation and nonreciprocal cross competition by acetylated and oxidised low density lipoprotein. *Proc Natl Acad Sci USA* 1991; 88: 4931-4935.

Frei B, Yamamoto Y, Niclas D, Ames BN. Evaluation of an isoluminol chemiluminescence assay for the detection of hydroperoxides in human blood plasma. *Anal Biochem* 1988; 175: 120-130.

Fuhrman B, Lavy A, Aviram M. Consumption of red wine with meals reduces the susceptibility of human plasma and low-density lipoprotein to lipid peroxidation. *Am J Clin Nutr* 1995; 61: 549-554.

Fujiwara T, Chiba S. Alterations of vascular alpha 1-adrenergic contractile responses in hypercholesterolemic rabbit common carotid arteries. *J Cardiovas Pharmacol* 1993; 22: 58-64.

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

Gaziano JM, Buring JE, Breslow JL, Goldhaber SZ, Rosner B, van Denburgh M, Willett W, Hennekens CH. Moderate alcohol intake, increased levels of high-density lipoprotein and its subfractions, and decreased risk of myocardial infarction. *N Engl J Med* 1993; 329: 1829-1834.

Gerrity RG. The role of the monocyte in atherogenesis. I. Transition of blood-borne monocytes into foam cells in fatty lesions. *Am J Pathol* 1981a; 103: 181-190.

Gerrity RG. The role of the monocyte in atherogenesis. II. Migration of foam cells from atherosclerotic lesions. *Am J Pathol* 1981b; 103: 191-200.

Gey KF, Puska P, Jordan P, Moser UK. Inverse correlation between plasma vitamin E and mortality from ischemic heart disease in cross-cultural epidemiology. *Am J Clin Nutr* 1991; 53: 326S-334S.

Gey KF, Stahelin HB, Eichholzaer M. Poor plasma status of carotene and vitamin C is associated with higher mortality from ischemic heart disease and stroke: Basel Prospective Study. *Clin Invest* 1993; 71: 3-6.

Giry C, Giroux LM, Roy M, Davignon J, Minnich A. Characterisation of an inherited scavenger receptor over expression and abnormal macrophage phenotype in a normolipidemic subject with planar xanthomas. *J Lipid Res* 1996; 37: 1422-1435.

Goldstein JL, Brown MS. Familial hypercholesterolemia. A genetic regulator defect in cholesterol metabolism. *Am J Med* 1975; 58: 147-150.

Goldstein JL, Brown MS. The low-density lipoprotein pathway and its relation to atherosclerosis. *Ann Rev Biochem* 1977; 46: 897-930.

Goldstein JL, Ho YK, Basu SK, Brown MS. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc Natl Acad Sci USA* 1979; 76: 333-337.

Gooderham MJ, Adlercreutz H, Ojala ST, Wahala K, Holub BJ. A soy protein isolate rich in genistein and daidzein and its effect on plasma isoflavone concentrations, platelet aggregation, blood lipids and fatty acid composition of plasma phospholipid in normal men. *Human and Clin Nutr* 1996; 126: 2000-2006.

Graham HN. Green tea composition, consumption, and polyphenol chemistry. *Prev Med* 1992; 21: 334-350.

Grønbaek M, Deis A, Sørensen TIA, Becker U, Schnohr P, Jensen G. Mortality associated with moderate intakes of wine, beer, or spirits. *BMJ* 1995; 310: 1165-169.

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

Haberland ME, Fogelman AM, Edwards PA. Specificity of receptor-mediated recognition of malondialdehyde-modified low density lipoprotein. *Proc Natl Acad Sci USA* 1982; 79: 1712-1716.

Haberland ME, Olch CL, Fogelman AM. Role of lysines in mediating interaction of modified low density lipoproteins with the scavenger receptor of human monocyte macrophages. *J Biol Chem* 1984; 259: 11305-11311.

Haberland ME, Fong D, Cheng L. Malondialdehyde-altered protein occurs in atheroma of Watanabe heritable hyperlipidemic rabbits. *Science* 1988; 241: 215-218.

Habib J, Bossaller C, Wells S, Williams C, Morrisett J, Henry PD. Preservation of endothelium-dependent vascular relaxation in cholesterol-fed rabbit by treatment with the calcium blocker PN 200110. *Cir Res* 1986; 58: 305-309.

Halliwell B. How to characterise a biological antioxidant. *Free Rad Res Comm* 1990; 19: 1-32.

Hanasaki Y, Ogawa S, Fukui S. The correlation between active oxygen scavenging and antioxidative effects of flavanoids. *Free Rad Biol Med* 1994; 16: 845-850.

Hatake K, Wakabayashi I, Hishida S. Mechanism of inhibitory action of ethanol on endothelium-dependent relaxation in rat aorta. *Eur J Pharmacol* 1993; 238: 441-444.

Head RJ, Loghurst PA, Panek RL, Stitzel RE. A contrasting effect of diabetic state upon the contractile responses of aortic preparations from rat and rabbit. *Br J Phramacol* 1987; 91: 275-286.

Hegsted DM, Ausman LM. Diet, alcohol and coronary heart disease in man. *J Nutr* 1988; 118:1184-1189.

Heinecke JW, Rosen H, Chait A. Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture. *J Clin Invest* 1984; 74: 1890-1894.

Heinecke JW, Baker L, Rosen H, Chait A. Superoxide-mediated modification of low density lipoprotein by arterial smooth muscle cells. *J Clin Invest* 1986; 77: 757-761.

Henriksen T, Evensen SA, Carlander B. Injury to endothelial cells in culture induced by low-density lipoproteins. *Scand J Clin Lab Invest* 1979; 39: 361-368.

Henriksonn T, Mahoney EM, Steinberg D. Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: Recognition by receptors for acetylated low density lipoproteins. *Proc Natl Acad Sci USA* 1981; 78: 6499-6503.

Herrmann RA, Malinauskas RA, Truskey GA. Characterization of sites with elevated LDL permeability at intercostal, celiac, and iliac branches of the normal rabbit aorta. *Arterioscler Thromb* 1994; 14: 313-323.

Hertog MG, Feskens EJ, Hollman PCH, Katan MB, Kromhout D. Dietary antioxidant flavanoids and risk of coronary heart disease: the Zutphen elderly study. *Lancet* 1993; 342: 1001-1111.

Hessler JR, Robertson AL, Chisolm GM. LDL induced cytotoxicity and its inhibition by HDL in human vascular smooth muscle and endothelial cells in culture. *Atherosclerosis* 1979; 32: 213-229.

Hiramatsu K, Rosen H, Heinecke JW, Wolfbauer G, Chait A. Superoxide initiates oxidation of low density lipoprotein by human monocytes. *Arterioscler* 1987; 7: 55-60.

Hodgson JM, Croft KD, Puddey IB, Mori TA, Beilin LJ. Soybean isoflavonoids and their metabolic products inhibit in vitro lipoprotein oxidation in serum. *J Nutr Biochem* 1996; 7: 664-669.

Hogg N, Darley-Usmar VM, Wilson MT, Moncada S. Production of hydroxyl radicals from the simultaneous generation of superoxide and nitric oxide. *Biochem J* 1992; 281: 419-424.

Hogg N, Kalyanaraman B, Joseph J, Struck A, Parthasarathy S. Inhibition of low-density lipoprotein oxidation by nitric oxide. Potential role in atherogenesis. *FEBS Lett* 1993; 334: 170-174.

Hojnacki JL. Alcohol's influence on plasma lipoproteins: A nonhuman primate model. *Nutr Res* 1994; 14: 1241-1295.

Holvoet P, Perez G, Zhao Z, Brouwers E, Bernar H, Collen D. Malondialdehyde-modified low density lipoproteins in patients with atherosclerotic disease. *J Clin Invest* 1995; 95: 2611-2619.

Hulley SB, Gordon S. Alcohol and high-density lipoprotein cholesterol: causal inference from diverse study designs. *Circ* 1981; 64: 57-63.

Hunt JV, Smith CCT, Wolff SP. Autoxidation glycosylation and possible involvement of peroxides and free radicals in LDL modification by glucose. *Diabetes* 1990; 39: 1420-1424.

Husain SR, Cillard J, Cillard P. Hydroxyl radical scavenging activity of flavanoids. *Phytochem* 1987; 26; 2489-2491.

Ikeda I, Imasato Y, Sasaki E, Nakayama M, Nagao H, Takeo T, Yayabe F, Sugano. Tea catechins decrease micellar solubility and intestinal absorption of cholesterol in rats. *Biochem Biophys Acta* 1992; 1127: 141-146.

Jacobs M, Plane F, Bruckdorfer KR. Native and oxidised low-density lipoproteins have different inhibitory effects on the endothelium-derived relaxing factor in the rabbit aorta. *Br J Pharm* 1990; 100: 21-26.

Jaworski AW, Lee CY. Fractionation and HPLC determination of grape phenols. *J Agric Food Chem* 1987; 35: 257-259.

Jayakody L, Senaratne M, Thomson A, Kappagoda T. Endothelium-dependent relaxation in experimental atherosclerosis in rabbits. *Cir Res* 1987; 60: 251-264.

Jessup W, Rankin SM, De Whalley CV, Hoult JRS, Scott J, Leake DS. α-Tocopherol consumption during low-density lipoprotein oxidation. *Biochem J* 1990; 265: 399-405.

Jessup W, Mohr D, Gieseg SP, Dean RT, Stocker R. The participation of nitric oxide in cell free- and its restriction of macrophage-mediated oxidation of low-density lipoprotein. *Biochem Biophys Acta* 1992; 1180: 73-82.

Jha HC, von Recklinghausen G, Zilliken F. Inhibition of *in vitro* microsomal lipid peroxidation by isoflavanoids. *Biochem Pharmacol* 1985; 34: 1367-1369

Jialal I, Vega GL, Grundy SM. Physiological levels of ascorbate inhibit the oxidative modification of LDL. *Atherosclerosis* 1990; 82: 185-191.

Jialal I, Grundy S. Effect of dietary supplement with alpha-tocopherol on the oxidative modification of LDL. *J Lipid Res* 1992; 33: 899-906.

Kalant N, McCormick S, Parniak MA. Effects of copper and histidine on oxidative modification of low-density lipoprotein and its subsequent binding to collagen. *Arterioscler Thromb* 1991; 11: 1322-1329.

Kannel WB, Castelli WP, Gordon T. Cholesterol in the prediction of atherosclerotic disease. New perspectives based on the Framingham study. *Ann Intern Med* 1977; 90: 85-91.

Kannel WB. Alcohol and cardiovascular disease. *Proceed Nutr Soc* 1988; 47: 99-110.

Kanner J, Frankel E, Granit R, German B, Kinsella JE. Natural antioxidants in grapes and wines. *J Agric Food Chem* 1994; 42: 64-69.

Keaney JF Jr, Gaziano JM, Xu A, Frei B, Curran-Celentano J, Shwaery GT, Loscalzo J, Vita JA. Dietary antioxidants preserve endothelium-dependent vessel relaxation in cholesterol-fed rabbits. *Proc Natl Acad Sci USA* 1993; 90: 11880-11884.

Keaney JF Jr, Gaziano JM, Frei B, Curran-Celentano J, Shwaery GT, Loscalzo J, Vita JA. Low dose alpha-tocopherol improves and high dose alpha-tocopherol worsens endothelial vasodilator function in cholesterol-fed rabbits. *J Clin Invest* 1994; 93: 844-851.

Keaney JF Jr, Cunningham D, Jackson T, Frei B, Vita JA. Dietary probucol preserves endothelial function in cholesterol-fed rabbits by limiting vascular stress and superoxide generation. *J Clin Invest* 1995; 95: 2520-2529.

Kerrigan M. The effect of extended post-fermentation skin contact on the phenolic composition of Cabernet Sauvignon wine. Thesis, Dept Hort, Vitic & Oenol, The University of Adelaide 1995.

Kiener PA, Rankin BM, Davis PM, Yocum SA, Warr GA, Grove RI. Immune complexes of LDL induce atherogenic responses in human monocytic cells. *Arterioscler Thromb Vasc Biol* 1991; 15: 990-999.

King R, Broadbent JL, Head R. Absorption and excretion of the soy isoflavone genistein in rats. *J Nutr* 1996; 126: 176-182.

King RA, Bursill DB. Plasma and urinary kinetics of the isoflavones daidzein and genistein following a single soy meal in humans (personal communication).

Kinsella JE, Frankel E, German B, Kanner J. Possible mechanisms for the protective role of antioxidants in wine and plant foods. *Food Technology* 1993: 85-89.

Kinter M, Robinson CS, Grimminger LC, Gillies PJ, Shimshick EJ, Ayers C. Whole blood and plasma concentrations of 4-hydroxy-nonenal in Watanabe heritable hyperlipidemic rabbits. *Biochem Biophys Res Comm* 1994; 199: 671-675.

Kita T, Nagano Y, Yokode M, Ishii K, Kume N, Ooshima A, Yoshida H, Kawai C. Probucol prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbit, an animal model for familial hypercholesterolemia. *Proc Natl Acad Sci USA* 1987; 84: 5928-5931.

Kivits GAA, van der Sman FJP, Tjiburg LBM. Analysis of catechins in plasma. A specific and sensitive colorimetric assay of total catechins in biological fluids. *Inter J Food Sci Nutr* 1997; 48: 387-392.

Kleinveld HA, Hak-Lemmers HLM, Stalenhoef AFH, Demacker PNM. Improved measurements of low density lipoprotein suspectibility to copper-induced oxidation: application of a short procedure for isolating low-density lipoprotein. *Clin Chem* 1992; 38: 2066-2072.

Kleinveld HA, Demacker PNM, Stalenhoef AFH. Comparative study on the effect of low dose vitamin E and probucol on the susceptibility of LDL to oxidation and the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbits. *Arterioscler Thromb Vasc Biol* 1994; 14: 1386-1391.

Kleinveld HA, Hak-Lemmers HLM, Hectors MPC, de Fouw NJ, Demacker PNM, Stalenhoef AFH. Vitamin E and fatty acid intervention does not attenuate the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbits. *Arterioscler Thromb Vasc Biol* 1995; 15: 290-297.

Klemsdale TO, Andersson TLG, Matz J, Ferns GAA, Gjesdal K, Anggard EE. Vitamin E restores endothelium dependent vasodilation in cholesterol-fed rabbits: in vivo measurements by photoplethsmography. *Cardiovas Res* 1994; 28: 1397-1402.

Klurfeld DM, Tepper SA, Mueller MA, Kritchevsky D. In: Metabolic effects of alcohol. Influences of alcohol from different sources on cholesterol metabolism in rats. Ed: Avogaro P, Sirtori CR, Tremoli. 1979: 187-196.

Klurfeld DM, Kritchevsky D. Differential effects of alcoholic beverages on experimental atherosclerosis in rabbits. *Expt and Mol Path* 1984; 34: 62-71.

Kodama T, Reddy P, Kishimoto C, Krieger M. Purification and characterization of a bovine acetyl low density lipoprotein receptor. *Proc Natl Acad Sci USA* 1988; 85: 9238-9242.

Kodama T, Freeman M, Rohrer L, Zabrecky J, Matsudaira P, Krieger M. Type I macrophage scavenger receptor contains α-helical and collagen-like coiled coils. *Nature* 343; 1990: 531-535.

Kolodgie FD, Virmani R, Rice HE, Mergner WJ. Vascular reactivity during the progression of atherosclerotic plaque. A study in Watanabe heritable hyperlipidemic rabbits. *Cir Res* 1990; 66: 1112-1126.

Kolodgie FD, Katocs AS, Largis EE, Wrenn SM, Cornhill JF, Herderick EE, Lee SJ, Virmani R. Hypercholesterolemia in the rabbit induced by feeding graded amounts of low-level cholesterol. *Arterioscler Thromb Vasc Biol* 1996; 16: 1454-1464.

Kondo K, Matsumoto A, Kurata H, Tanahashi H, Koda H, Amachi T, Itakura H. Inhibition of oxidation of low-density lipoprotein with red wine. *Lancet* 1994; 344: 1152.

Kontush A, Finckh B, Karten B, Kohlschutter A, Beisiegel U. Antioxidant and prooxidant activity of alpha-tocopherol in human plasma and low density lipoprotein. *J Lipid Res* 1996a; 37: 1436-1448.

Kontush A, Meyer S, Finckh B, Kohlschutter A, Beisiegel U. Alpha-tocopherol as a reductant for Cu(II) in human lipoproteins. Triggering role in the initiation of lipoprotein oxidation. *J Biol Chem* 1996b; 271: 11106-11112.

Kovanen PT, Brown MS, Basu SK, Bilheimer DW, Goldstein JL. Saturation and suppression of hepatic lipoprotein receptors: a mechanism for the hypercholesterolemia of cholesterol-fed rabbits. *Proc Natl Acad Sci USA* 1981; 78: 1396-1400.

Krieger M, Acton S, Ashkenas A, Penman M, Resnick D. Molecular flypaper, host defense, and atherosclerosis. *J Biol Chem* 1993; 268: 4569-4572.

Kritchevsky SB, Shimakawa T, Tell GS, Dennis B, Carpenter M, Eckfeldt JH, Peacher-Ryan H, Heiss G. Dietary antioxidants and carotid artery wall thickness. The ARIC Study. *Circ* 1995; 92: 2142-2150.

Kroon PA, Thompson GM, Chao Y. β-VLDL in cholesterol-fed rabbits are of hepatic origin. *Atherosclerosis* 1985; 56: 323-329.

Ku G, Thomas CE, Akeson AL, Jackson RL. Induction of interleukin 1 beta expression from human peripheral blood monocyte-derived macrophages by 9-hydroxyoctadecadienoic acid. *J Biol Chem* 1992; 267: 14183-14188.

Kugiyama K, Kerns SA, Morrisett JD, Roberts R, Henry PD. Impairment of endothelium-dependent arterial relaxation by lysolecithin in modified low-density lipoproteins. *Nature* 1990; 344: 160-162.

Kuhn H, Belkner J, Wiesner R, Schewe T, Lamkin VZ, Tikhaze AK. Structure elucidation of oxygenated lipids in human atherosclerotic lesions. *Eicosanoids* 1992; 5: 17-22.

Kuhn H, Belkner J, Zaiss S, Fahrenklemper T, Wohlfeil S. Involvement of 15-lipoxygenase in early stages of atherogenesis. *J Exp Med* 1994; 179: 1903-1911.

Kume N, Cybulsky MI, Gimbrone M. Lysophosphatidylcholine, a component of atherogenic lipoproteins, includes mononuclear leukocyte adhesion molecules in cultured human and rabbit endothelial cells. *J Clin Invest* 1992; 90: 1138-1144.

Lanninghamfoster L, Chen C, Chance DS, Loo G. Grape extract inhibits lipid peroxidation of human low density lipoprotein. *Biol Pharm Bull* 1995; 18: 1347-1351.

Lavy A, Fuhrman B, Markel A, Dankner G, Ben-Amotz A, Presser D, Aviram M. Effect of dietary supplementation of red or white wine on human blood chemistry, hematology and coagulation: Favourable effect of red wine on plasma high-density lipoprotein. *Ann Nutr Metab* 1994; 38: 287-294.

Lecomte E, Herbeth B, Paille F, Steinmetz J, Artur Y, Siest G. Changes in serum apolipoprotein and lipoprotein profile induced by chronic alcohol consumption and withdrawal: determinant effect on heart disease? *Clin Chem* 1996; 42: 1666-1675.

Lee M, Wang Z, Li H, Chen L, Sun Y, Gobbo S, Balentine DA, Yang CS. Analysis of plasma and urinary tea polyphenols in human subjects. *Cancer Epidemiol Biomark Prevent* 1995; 4: 393-399.

Lee YJ, Daida H, Yokoi H, Miyano H, Takaya J, Sakurai H, Mokuno H, Yamaguchi H. Effectiveness of probucol in preventing restenosis after percutaneous transluminal coronary angioplasty. *Japan Heart J* 1996; 37: 327-332.

Leeuwenburgh C, Hardy MM, Hazen SL, Wagner P, Oh-ishi S, Steinbrecher UP, Heinecke JW. Reactive nitrogen intermediates promote low density lipoprotein oxidation in human atherosclerotic intima. *J Biol Chem* 1997; 272: 1433-1436..

Lehtonen JY, Adlercreutz, Kinnunen PK. Binding of daidzein to liposomes. *Biochim Biophys Acta* 1996; 1285: 91-100.

Leung WH, Lau CP, Wong CK. Beneficial effect of cholesterol-lowering therapy on coronary endothelium-dependent relaxation in hypercholesterolemic patients. *Lancet* 1993; 341: 1496-1500.

Liao JK, Shin WS, Lee WY, Clark SL. Oxidised low-density lipoprotein decreases the expression of endothelial nitric oxide synthase. *J Biol Chem* 1995; 270; 310-324.

Liu KZ, Cuddy TE, Pierce GN. Oxidative status of lipoproteins in coronary disease patients. *Am Heart J* 1992; 123: 285-290.

Lowenstein CJ, Dinerman JL, Snyder SH. Nitric oxide: a physiologic messenger. *Ann Inter Med* 1994; 120: 227-237.

Lowry OH, Rosenburg NJ, Farr AL, Randall RJ. Protein measurements with the folin phenol reagent. *J Biol Chem* 1951; 193: 265-275.

MacKinnon AM, Savage J, Gibson RA, Barter PJ. Secretion of cholesteryl-ester rich very low density lipoproteins by the liver of cholesterol-fed rabbits. *Atherosclerosis* 1985; 54: 145-155.

Maclure M. Demonstration of deductive meta-analysis: ethanol intake and myocardial infarction. *Epidemiol Rev* 1993; 64: 743-750.

Mahley RW, Innerarity TL, Weisgraber KH, Oh SY. Altered metabolism (*in vivo* and *in vitro*) of plasma lipoproteins after selective modification of lysine residues of the apoproteins. *J Clin Invest* 1979; 64: 743-750.

Mahley RW, Innerarity TL, Brown MS, Ho YK, Goldstein JL. Cholesteryl ester synthesis in macrophages: stimulation by beta-very low density lipoproteins from cholesterol-fed animals of several species. *J Lipid Res* 1980; 21: 970-980.

Maiorino M, Zamburlini A, Roveri A, Ursini F. Copper-induced lipid peroxidation in liposomes, micelles, and LDL: Which is the role of vitamin E? *Free Rad Biol Med* 1995; 18: 67-74.

Manach C, Morand C, Texier O, Favier ML, Agullo G, Demigne C, Regerat F, Remesy C. Quercetin metabolites in plasma of rats fed diets containing rutin or quercetin. *J Nutr* 1995; 125: 1911-1922.

Mangiapane H, Thomson J, Salter A, Brown S, Bell GD, White DA. The inhibition of the oxidation of low density lipoprotein by (+)-catechin, a naturally occurring flavanoid. *Biochem Pharm* 1992; 43: 445-450.

Mao SJT, Yates MT, Parker RA, Chi EM, Jackson RL. Attenuation of atherosclerosis in a modified strain of hypercholesterolemic Watanabe rabbits with use of a probucol analogue (MDL 29,311) that does not lower serum cholesterol. *Arterioscler Thromb* 1991; 11: 1266-1275.

Matsumoto A, Naito M, Itakura H, Ikemoto S, Asaoka H, Hayakawa I, Kanamori H, Aburatani H, Takaku F, Suzuki H, *et al.* Human macrophage scavenger receptors: primary structure, expression and localisation in atherosclerotic lesions. *Proc Natl Acad Sci USA* 1990; 87: 9133-9137.

Matz J, Andersson TLG, Ferns GAA, Anggard EE. Dietary vitamin E increases the resistance to lipoprotein oxidation and attenuates endothelial dysfunction in the cholesterol-fed rabbit. *Atherosclerosis* 1994; 110: 241-249.

Maxwell S, Cruickshank A, Thorpe G. Red wine and antioxidant activity in serum. *Lancet* 1994; 344: 193-194.

Maxwell SRJ. Prospects for the use of antioxidant therapies. *Drugs* 1995; 49: 345-361.

McNally AK, Chisolm GM, Morel DW, Cathcart MK. Activated human monocytes oxidise low density lipoprotein by a lipoxygenase-dependent pathway. *J Immunol* 1990; 145: 254-259.

Messina MJ, Persky V, Setchell DR, Barnes S. Soy intake and cancer risk: A review of the *in vitro* and *in vivo* data. *Nutr Cancer* 1994; 21: 113-131.

Metha JL, Bryant JL, Metha P. Reduction of nitric oxide synthase activity in human neutrophils by oxidised low-density lipoprotein. *Biochem Pharmacol* 1995; 50: 1181-1185.

Michel T, Smith TW. Nitric oxide synthases and cardiovascular signaling *Am J Cardiol* 1993; 72: 33C-38C.

Miettinen TA, Tilvis RS, Kesaniemi YA. Serum plant sterols and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population. *Am J Epidemiol* 1990; 131: 20-31.

Minor RL, Myers PR, Guerra R, Bates JN, Harrison DG. Diet-induced atherosclerosis increases the release of nitrogen oxides from rabbit aorta. *J Clin Invest* 1990; 86: 2109-2116.

Moncada S, Radomski MW, Palmer RM. Endothelium-derived relaxing factor: Identification as nitric oxide and role in the control of vascular tone and platelet function. *Biochem Pharmacol* 1988; 37: 2495-2501.

Morel DW, Hessler JR, Chisolm GM. Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipid. *J Lipid Res* 1983; 24: 1070-1076.

Morel DW, DiCorleto PE, Chisolm GM. Endothelial and smooth muscle cells alter low density lipoprotein *in vitro* by free radical oxidation. *Arterioscler* 1984; 4: 357-364.

Morel DW, Llera-Moya M, Friday KE. Treatment of cholesterol-fed rabbits with dietary vitamins E and C inhibits lipoprotein oxidation but not development of atherosclerosis. *J Nutr* 1994; 124: 2123-2130.

Morton MS, Wilcox G, Wahlqvist ML, Griffiths K. Determination of lignans and isoflavanoids in human female plasma following dietary supplementation. *Journal of Endocrinology* 1994; 142: 251-259.

Mugge A, Elwell J, Peterson T, Hofmeyer T, Heistad D, Harrison D. Chronic treatment with polyethylene-glycolated superoxide dismutase partially restores endothelium-dependent vascular relaxation in cholesterol rabbits. *Circ Res* 1991; 69: 1293-1300.

Nagano Y, Nakamura T, Matsuzawa Y, Cho M, Ueda Y, Kita T. Probucol and atherosclerosis in the Watanabe heritable hyperlipidemic rabbits - long term antiatherogenic effect and effects on established plaques. *Atherosclerosis* 1992; 92: 131-140.

Nardini M, D'Aquino M, Tomassi G, Gentili V, Di Felice M, Scaccini C. Inhibition of human low-density lipoprotein oxidation by caffeic and other hydroxycinnamic acid derivatives. *Free Rad Biol Med* 1995; 19: 541-552.

Nestel PJ, Yamashita T, Sasahara T, Pomeroy S, Dart A, Komesaroff P, Owen A, Abbey M. Soy isoflavonoids improve systemic arterial compliance but not plasma lipids in menopausal women. *Arterioscler Thromb Vasc Biol* (in press)

Newman HAI, Zilversmit DB. Quantitative aspects of cholesterol flux in rabbit atheromatous lesions. *J Biol Chem* 1962; 237: 2078-2083.

Nordestgaard BG, Nielsen LB. Atherosclerosis and arterial influx of lipoproteins. *Curr Opinion Lipid* 1994; 5: 252-257.

O'Brien KD, Alpers CE, Hokanson JE, Wang S, Chait A. Oxidation-specific epitopes in human coronary atherosclerosis are not limited to oxidised low-density lipoprotein. *Circulation* 1996; 94: 1216-1225.

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

Ohara Y, Petersen TE, Zheng B, Kuo JF, Harrison DG. Lysophospatidylcholine increases vascular superoxide anion production via protein kinase C activation. *Arterioscler Thromb* 1994; 14: 1007-1013.

Osborne JA, Lento PH, Siegfried MR, Stahl GL, Fusman B, Lefer AM. Cardiovascular effects of acute hypercholesterolemia in rabbits: Reversal with lovastatin treatment. *J Clin Invest* 1989; 83: 465-473.

Oszmianski J, Ramos T, Bouzeix M. Fractionation of phenolic compounds in red wine. *Am J Enol Vitic* 1988; 39: 259-262.

Palinski W, Rosenfeld ME, Yla-Herttuala S, Gurtner GC, Socher SS, Butler SW, Parthasarathy S, Carew TE, Steinberg D, Witztum JL. Low density lipoprotein undergoes oxidative modification *in vivo*. *Proc Natl Acad Sci USA* 1989; 86: 1372-1376.

Palinski W, Ord VA, Plump AS, Breslow JL, Steinberg D, Witztum JL. Apo E-deficient mice are a model of lipoprotein oxidation in atherogenesis. Demonstration of oxidation-specific epitopes in lesions and high titers of autoantibodies to malondialdehyde-lysine in serum. *Arterioscler Thromb* 1994; 14: 605-616.

Palinski W, Tangirala RK, Miller E, Young SG, Witztum JL. Increased autoantibody titers against epitopes of oxidised LDL in LDL receptor-deficient mice with increased atherosclerosis. *Arterioscler Thromb Vasc Biol* 1995; 15: 1569-1576.

Parthasarathy S, Printz DJ, Boyd D, Joy L, Steinberg D. Macrophage oxidation of low density lipoprotein generates a modified form recognised by the scavenger receptor. *Arterioscler* 1986a; 6: 505-510.

Parthasarathy S, Young SG, Witztum JL, Pittman RC, Steinberg D. Probucol inhibits oxidative modification of low density lipoprotein. *J Clin Invest* 1986b; 77: 641-644.

Parthasarathy S, Fong LG, Otero D, Steinberg D. Recognition of solubilised apoproteins from delipidated, oxidised low-density lipoprotein (LDL) by the acetyl-LDL receptor. *Proc Natl Acad Sci USA* 1987; 84: 537-540.

Parthasarathy S, Wieland E, Steinberg D. The role for endothelial cell lipoxygenase in the oxidative modification of LDL. *Proc Natl Acad Sci USA* 1989a; 86: 1046-1050.

Parthasarathy S, Quinn MT, Schwenke DC, Carew TE, Steinberg D. Oxidative modification of beta-very low density lipoprotein. Potential role in monocyte recruitment and foam cell formation. *Arterioscler* 1989b; 9: 398-404.

Parthasarathy S Khoo JC, Miller E, Barnett J, Witztum JL, Steinberg D. Low density lipoprotein rich in oleic acid is protected against oxidative modification: implications for dietary prevention of atherosclerosis. *Proc Natl Acad Sci USA* 1990; 87: 3894-3898.

Parthasarathy S, Rankin SM. Role of oxidised LDL in atherogenesis. *Prog Lip Res* 1992; 31: 127-143.

Patel RP, Svistunenko D, Wilson MT, Darley-Usmar VM. Reduction of Cu(II) by lipid hydroperoxides: implications for the copper-dependent oxidation of low-density lipoprotein. *Biochem J* 1997; 322: 425-433.

Paterson JR, Rumley AG, Oldroyd TG, Tait GW, Smellie WS, Packard CJ, Shepherd J, Lorimer AR. Probucol reduces plasma lipid peroxides in man. *Atherosclerosis* 1992; 97; 63-66.

Pan HY, Liu DL, Xu PP, Lu ML. Determination of D-catechin plasma concentration and pharmacokinetic parameters using HPLC method. *Acta Pharmaceutica Sinica* 1991; 26: 371-374.

Pikkar NA, Wedel M, van der Beek EJ, van Dokkum W, Kempen HJM, Kluft C, Ockhuizen T, Hermus RJJ. Effects of moderate alcohol consumption on platelet aggregation, fibrinolysis and blood lipids. *Metab* 1987; 36:538-543.

Pinardi G, Brieva C, Vinet R, Penna M. Effects of chronic ethanol consumption on alpha-adrenergic induced contractions in rat thoracic aorta. *Gen Pharmacol* 1992; 23: 245-248.

Podet EJ, Shaffer DR, Gianturco SH, Bradley WA, Yang CY, Guyton JR. Interaction of low density lipoproteins with human aortic elastin. *Arterioscler & Thromb* 1991; 11: 116-122.

Pratt DE, Birac PM. Source of antioxidant activity of soybeans and soy products. *Journal of Food Science* 1979; 44: 1720-1722.

Puhl H, Waeg G, Esterbauer H. In: Methods in Enzymology, Vol. 233, Oxygen radicals in biological systems, Part C. Ed: Parker L, Academic Press, 1994; 425-441.

Quinn MT, Parthasarathy S, Steinberg D. Endothelial cell-derived chemotactic activity for mouse peritoneal macrophages and the effects of modified forms of LDL. *Proc Natl Acad Sci USA* 1985; 82: 5949-5953.

Quinn MT, Parthasarathy S, Fong LG, Steinberg D. Oxidatively modified low-density lipoproteins: A potential role in recruitment and retention of monocyte/macrophages during atherogenesis. *Proc Natl Acad Sci USA* 1987; 84: 2995-2998.

Quinn MT, Parthasarathy S, Steinberg D. Lysophosphatidylcholine: A chemotactic factor for human monocytes and its potential role in atherogenesis. *Proc Natl Acad Sci USA* 1988; 85: 2805-2809.

Rapola JM, Virtamo J, Haukka JK, Heinonen OP, Albanes D, Taylor PR, Huttunen JK. Effect of vitamin E and beta carotene on the incidence of angina pectoris. A randomised, double-blind, controlled trial. *JAMA* 1996; 275: 693-698.

Reaven PD, Khouw A, Beltz WF, Parthasarathy S, Witztum JL. Effect of dietary antioxidant combinations in humans. Protection of vitamin E but not of beta-carotene. *Arterioscler & Thromb* 1993; 13: 590-600.

Record IR, Dreosti IE, McInerney J. The antioxidant activity of genistein in vitro. *J Nutr Biochem* 1995; 6: 481-485.

Regnstrom J, Walldius G, Carlson LA, Nilsson J. Effect of probucol treatment on the susceptibility of LDL isolated from hypercholesterolemic patients to become oxidatively modified *in vitro*. *Atherosclerosis* 1990; 82: 43-51.

Regnstrom J, Nilsson J, Tornvall P, Landou C, Hamsten A. Susceptibility to low-density lipoprotein oxidation and coronary atherosclerosis in man. *Lancet* 1992; 339: 1183-1186.

Regnstrom J, Walldius G, Nilsson S, Elinder LS, Johansson J, Molgaard J, Holme J, Olsson AG, Nilsson J. The effect of probucol on low density lipoprotein oxidation and femoral atherosclerosis. *Atherosclerosis* 1996; 125: 217-229.

Renaud S, De Lorgeril M. Wine, alcohol, platelets and the French paradox for coronary heart disease. *Lancet* 1992a; 339: 1523-1526.

Renaud S, Beswick AD, Fehily AM, Sharp DS, Elwood PC. Alcohol and platelet aggregation: the Caerphilly prospective heart disease study. *Am J Clin Nutr* 1992b; 55: 1012-1017.

Rice-Evans CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Rad Biol Med* 1996; 20: 933-956.

Riemersma RA, Oliver M, Elton RA, Alfthan G, Vartiainen E, Salo M, Rubba P, Mancini M, Georgi H, Vuilleumier JP, *et al.* Plasma antioxidants and coronary heart disease: Vitamins C and E, and selenium. *Eur J Clin Nutr* 1990; 44: 143-150.

Rifici VA, Schneider SH, Khachadurian AK. Stimulation of low-density lipoprotein oxidation by insulin and insulin like growth factor I. *Atherosclerosis* 1994; 107: 99-108.

Rimm EB, Giovannucci EL, Willett WC, Colditz GA, Ascherio A, Rosner B, Stampfer MJ. Prospective study of alcohol consumption and risk of coronary heart disease in men. *Lancet* 1991; 338: 464-486.

Rimm EB, Stampfer MJ, Ascherio A, Giovannucci E, Colditz GA, Willett WC. Vitamin E consumption and the risk of coronary heart disease in men. *New Engl J Med* 1993; 328: 1450-1456.

Rimm EB, Klatsky A, GrobbeeD, Stampfer MJ. Review of moderate alcohol consumption and reduced risk of coronary heart disease: is the effect due to beer, wine or spirits. *BMJ* 1996; 312: 731-736.

Robak J, Gryglewski RJ. Flavanoids are scavengers of superoxide anions. *Biochem Pharm* 1988; 37: 837-841.

Roher L, Freeman M, Kodama M, Penman M, Krieger M. Coiled-coil fibrous domains mediate ligand binding by macrophage scavenger receptor type II. *Nature* 1990; 343: 570-572.

Rosenfeld ME, Palinski W, Yla-Herttuala S, Butler S, Witztum JL. Distribution of oxidation specific lipid-protein adducts and apolipoprotein B in atherosclerotic lesions of varying severity from WHHL rabbits. *Arterioscler* 1990; 10: 336-349.

Rosenfeld ME, Khoo JC, Miller E, Parthasarathy S, Palinski W, Witztum JL. Macrophage-derived foam cells freshly isolated from rabbit atherosclerotic lesions degrade modified lipoproteins, promote oxidation of low-density lipoproteins, and contain oxidation-specific lipid-protein adducts. *J Clin Invest* 1991; 87: 90-99.

Ruf J, Berger J, Renaud S. Platelet rebound effect of alcohol withdrawal and wine drinking in rats. Relation to tannins and lipid peroxidation. *Arterioscler Thromb Vasc Biol* 1995; 15: 140-144

Salah N, Miller NJ, Paganga G, Tijburg L, Bolwell GP, Rice-Evans C. Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. *Arch Biochem Biophys* 1995; 322: 339-346.

Salonen JT, Yla-Herttuala S, Yamamoto R, Butler S, Korpela H, Salonen R, Nyyssonen K, Palinski W, Witztum JL. Autoantibody against oxidised LDL and progression of carotid atherosclerosis. *Lancet* 1992; 339: 883-887.

Savolainen MJ, Kesaniemi YA. Effects of alcohol on lipoproteins in relation to coronary heart disease. *Curr Opinion Lipid* 1995; 6: 243-250.

Schaffner T, Taylor K, Bartucci EJ, Fischer-Dzoga K, Beeson JH, Glagov S, Wissler RW. Arterial foam cells with distinctive immunomorphologic and histochemical features of macrophages. *Am J Pathol* 1980; 100: 77-80.

Scheek LM, Wiseman SA, Tijburg LBM, van Tol A. Dialysis of isolated low density lipoprotein induces a loss of lipophilic antioxidants and increases the susceptibility to oxidation in vitro. *Atherosclerosis* 1995; 117: 139-144.

Schmidt K, Graier WF, Kostner GM, Mayer B, Bohme E, Kukovetz WR. Stimulation of soluble guanylate cyclase by endothelium-derived relaxing factor is antagonised by oxidised low-density lipoprotein. *J Cardiovas Pharm* 1991; 17 (Suppl 3): S83-S88.

Seigneur M, Bonnet J, Dorian B, Benchimal D, Drouillet F, Gouverneur G, Larrue J, Crockett R, Boisseau MR, Ribereau-Gayon P, Bricaud H. Effect of the consumption of alcohol, white wine, and red wine on platelet function and serum lipids. *J Appl Cardiol* 1990; 5: 215-222.

Setchell KDR, Borriello SP, Kirk DN, Axelson M. Nonsteroidal estrogens of dietary origin: Possible role in hormone-dependent disease. *Am J Clin Nutr* 1984; 40: 569-578.

Shaish A, Pape M, Rea T, Srivastava RAK, Latour MA, Hopkins D, Schonfeld G. Alcohol increases plasma levels of cholesterol diet-induced atherogenic lipoproteins and aortic atherosclerosis in rabbits. *Arterioscler Thromb Vasc Biol* 1997; 17: 1091-1097.

Shechter I, Fogelman AM, Haberland ME, Seager J, Hokom M, Edwards PA. The metabolism of native and malondialdehyde-altered low density lipoprotein by human monocyte-macrophages. *J Lipid Res* 1981; 22: 63-71.

Shore VG, Shore B, Hart RG. Changes in apolipoproteins and properties of rabbit very low density lipoproteins on induction of cholesterolemia. *Biochemistry* 1974; 13: 1579-1584.

Simionescu M, Simionescu N. Proatherosclerotic events: pathobiochemical changes occurring in the arterial wall before monocyte migration. *FASEB J* 1993; 7: 1359-1366.

Simon BC, Haudenschild CC, Cohen RA. Preservation of endothelium-dependent relaxation in atherosclerotic rabbit aorta by probucol. *J Cardiovas Pharm* 1993; 21: 893-901.

Simonetti P, Brusamolino A, Pellegrini N, Viani P, Clemente G, Roggi C, Cestaro B. Evaluation of the effect of alcohol consumption on erythrocyte lipids and vitamins in a healthy population. *Alcohol Clin Exp Res* 1995; 19: 517-22.

Singh RB, Niaz MA, Agarwal P, Begom R, Rastogi SS. Effect of antioxidant-rich foods on plasma ascorbic acid, cardiac enzyme, and lipid peroxide levels in patients hospitalised with acute myocardial infarction. *J Am Diet Assoc* 1995; 95: 775-780.

Singh RB, Niaz MA, Rastogi SS, Rastogi S. Usefulness of antioxidant vitamins in suspected acute myocardial infarction (the Indian experiment of infarct survival-3). *Am J Cardiol* 1996; 77: 232-236.

Singleton VL. In: Wine Phenols. Modern Methods of Plant Analysis; Volume 6, Ed. Linskens HF and Jackson JF, Springer-Verlag. 1988: 173-218.

Sparrow CP, Parthasarathy S, Steinberg D. Enzymatic modification of LDL by purified lipooxygenase plus phospholipase A_2 mimics cell-mediated oxidative modification. *J Lipid Res* 1988; 29: 745-753

Sparrow CP, Parthasarathy S, Steinberg D. A macrophage receptor that recognises oxidised low-density lipoprotein but not acetylated low-density lipoprotein. *J Biol Chem* 1989; 264: 2599-2604.

Sparrow CP, Doebber TW, Olszewski J, Wu MS, Ventre J, Stevens KA, Chao Y. Low density lipoprotein is protected from oxidation and the progression of atherosclerosis is slowed in cholesterol-fed rabbits by the antioxidant *N, N'*-Diphenyl-Phenylenediamine. *J Clin Invest* 1992; 89: 1885-1891.

Sparrow CP, Oiszewski J. Cellular oxidation of LDL is caused by thiol production in media containing transition metal ions. *J Lipid Res* 1993; 34: 1219-1228.

St Leger AS, Cochrane AL, Moore F. Factors associated with cardiac mortality in developed countries with a particular reference to the consumption of wine. *Lancet* 1979; 1: 1017-1020.

Stampfer MJ, Hennekkens, CH, Manson JE, Colditz GA, Rosner B, Willett WC. Vitamin E consumption and the risk of coronary heart disease in women. *New Engl J Med* 1993; 328: 1444-1449.

Stanton LW, White RT, Bryant CM, Protter AA, Endemann G. A macrophage Fc receptor for IgG is also a receptor for oxidised low density lipoprotein. *J Biol Chem* 1992; 267: 22446-22451.

Stein Y, Stein O, Delplanque B, Fesmire JD, Lee DM, Alaupovic P. Lack of effect of probucol on atheroma formation in cholesterol-fed rabbits kept at a comparable plasma cholesterol level. *Atherosclerosis* 1989; 75: 145-155.

Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol: Modifications of LDL that increase its atherogenicity. *New Engl J Med* 1989; 320: 915-924.

Steinbrecher UP, Parthasarathy S, Leake DS, Witztum JL. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc Natl Acad Sci USA* 1984; 81: 3883-3887.

Stephens NG, Parsons A, Schofield PM, Kelly F, Cheeseman K, Mitchinson MJ. Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). *Lancet* 1996; 781-786.

Stewart-Lee AL, Forster LA, Nourooz-Zadeh J, Ferns GAA, Anggard EE. Vitamin E protects against impairment of endothelium-mediated relaxations in cholesterol-fed rabbits. *Arterioscler Thromb* 1994; 14: 494-499.

Stocker R. Lipoprotein oxidation: mechanistic aspects, methodological approaches and clinical relvance. *Curr Opinion in Lipid* 1994; 5: 422-433.

Struck M, Watkins T, Tomeo A, Halley J, Bierenbaum M. Effect of red and white wine on serum lipids, platelet aggregation, oxidation products and antioxidants: A preliminary report. *Nutr Res* 1994; 14: 1811-1819.

Stuehr DJ, Kwon NS, Nathan CF, Griffith OW, Feldman PL, Wiseman J. N omega-hydroxy-*L*-arginine is an intermediate in the biosynthesis of nitric oxide from *L*-arginine. *J Biol Chem* 1991; 266: 6259-6263

Suarna C, Dean RT, May J, Stocker R. Human atherosclerotic plaque contains both oxidised lipids and relatively large amounts of α-tocopherol and ascorbate. *Arterioscler Thromb Vasc Biol* 1995; 15: 1616-1624.

Suzukawa M, Abbey M, Clifton P, Nestel PJ. Effects of supplementing with vitamin E on the uptake of low density lipoprotein and the stimulation of cholesteryl ester formation in macrophages. *Atherosclerosis* 1994; 10: 77-86.

Takahama U. Inhibition of lipoxygenase-dependent lipid peroxidation by quercetin: Mechanism of antioxidative function. *Phytochem* 1985 ; 24: 1443-1446.

Tanner FC, Noll G, Boulanger CM, Luscher TF. Oxidised low density lipoproteins inhibit relaxations of porcine coronary arteries: role of scavenger receptor and endothelium-derived nitric oxide. *Circulation* 1991; 83: 2012-2020.

Teissedre PL, Frankel EN, Waterhouse AL, Peleg H, German JB. Inhibition of *in vitro* LDL oxidation by phenolic antioxidants from grapes and wines. *J Sci Food Agric* 1996; 70: 55-61.

Thompson KH, Zilversmit DB. Plasma very low density lipoprotein (VLDL) in cholesterol-fed rabbits: chylomicron remnants or liver lipoproteins? *J Nutr* 1983; 113: 2002-2010.

Tilvis RS, Miettinen TA. Serum plant sterols and their relation to cholesterol absorption. *Am J Clin Nutr* 1986; 43: 92-97.

Tomeo AC, Geller M, Watkins TR, Gapor A, Bierenbaum ML. Antioxidant effects of tocotrienols in patients with hyperlipidemia and carotid stenosis. *Lipids* 1995; 30: 1179-1183.

Torel J, Cillard J, Cillard P. Antioxidant activity of flavanoids and reactivity with peroxy radical. *Phytochem* 1986; 25: 383-385.

Treutter D. Chemical reaction detection of catechins and procyanidins with 4-dimethylaminocinnamaldehyde. *J Chrom* 1989; 185-193

Tsai EC, Chait A. Inhibition of low density lipoprotein oxidation by genistein. *J Invest Med* 1995; 43: 245A (Abstract).

Unno T, Kondo K, Itakura H, Takeo T. Analysis of (-)-epigallocatechin gallate in human serum obtained after ingesting green tea. *Biosci Biotech Biochem* 1996; 60: 2066-2068.

Uusitupa MI, Niskanen L, Luoma J, Vilja P, Mercuri M, Rauramaa R, Yla-Herttuala S. Autoantibodies against oxidised LDL do not predict atherosclerotic vascular disease in non-insulin dependent diabetes mellitus. *Arterioscler Thromb Vasc Biol* 1996; 16: 1236-1242.

Valimaki M, Taskinen MR, Ylikahri R, Roine R, Kuusi T, Nikkila EA. Comparison of the effects of two different doses of alcohol on serum lipoproteins, HDL-subfractions and apolipoproteins A-I and A-II: a controlled study. *Eur J Clin Invest* 1988; 18: 472-480.

van Acker SABE, Tromp MNJL, Haenen GRMM, van der Vijgh WJF, Bast A. Flavonoids as scavengers of nitric oxide radical. *Biochem Biophys Res Comm* 1995; 214: 755-759.

van Acker SABE, van den Berg D, Tromp MNJL, Griffioen DH, van Bennekom WP, van der Vijgh WJF, Bast A. Structural aspects of antioxidant activity of flavonoids. *Free Rad Biol Med* 1996; 20: 331-342.

Verbeuren TJ, Jordaens FH, Zonnekeyn LL, Van Hove CE, Coene M, Herman AG. Effect of hypercholesterolemia on vascular reactivity in the rabbit. *Circ Res* 1986; 58: 552-564.

Verhagen JV, Haenen GRMM, Bast A. Nitric oxide radical scavenging by wines. *J Agric Food Chem* 1996; 44: 3733-3734.

Vinson JA, Jang J, Dabbagh YA, Serry MM, Cai S. Plant polyphenols exhibit lipoprotein-bound antioxidant activity using an *in vitro* oxidation model for heart disease. *J Agric Food Chem* 1995; 43: 2798-2799.

Walldius G, Erikson U, Olsson AG, Bergstrand L, Hadell K, Johansson J, Kaijser L, Lassvik C, Molgaard J, Nilsson S, *et al.* The effect of probucol on femoral atherosclerosis: the probucol Quantitative Regression Swedish Trial (PQRST). *Am J Cardiol* 1994; 74: 875-883.

Walter ED. Genistin (an isoflavonoid glucoside) and its aglycone, genistein, from soybeans. *J Am Chem Soc* 1941; 63: 3273-3276.

Wang T, Powell WS. Increased levels of monohydroxy metabolites of arachidonic acid and linoleic acid in LDL and aorta from atherosclerotic rabbits. *Biochim Biophys Acta* 1991; 1084: 129-138.

Waterman PG, Mole S. In: Methods in Ecology. Analysis of phenolic plant metabolites. Blackwell Scientific Publications 1994.

Wei H, Wei L, Frenkel K, Bowen R, Barnes S. Inhibition of tumour promoter-induced hydrogen peroxide formation *in vitro* and *in vivo* by genistein. *Nutr Cancer* 1993; 20: 1-13.

Whitehead TP, Robinson D, Allaway S, Syms J, Hale A. Effect of red wine ingestion on the antioxidant capacity of serum. *Endocrin Metab* 1995; 41: 32-35.

Williams RJ, Motterim JM, Sharp CH, Gallagher PJ. Dietary vitamin E and the attenuation of early lesion development in modified Watanabe rabbits. *Atherosclerosis* 1992; 94: 153-159.

Wilson T, Knight TJ, Beitz DC, Lewis DS, Engen RL. Resveratrol promotes atherosclerosis in hypercholesterolemic rabbits. *Pharmacol Lett* 1996; 59: 15-21.

Wiseman SA, Van der Boom MA, De Fouw NJ, Wassink MG, Op den Kamp JA, Tijburg LB. Comparison of the effects of dietary vitamin E on in vivo and in vitro parameters of lipid peroxidation in the rabbit. *Free Rad Biol Med* 1995; 19: 617-626.

Wolthers BG, Walrecht HT, van der Molen JC, Nagel GT, Van Doormaal JJ, Wijnandts PN. Use of determinations of 7-lathosterol (5 alpha-cholest-7-en-3 beta-ol) and other cholesterol precursors in serum in the study and treatment of disturbances of sterol metabolism, particularly cerebrotendinous xanthomatosis. *J Lipid Res* 1991; 32: 603-612.

Yang CS, Lee MJ. Methodology of plasma retinol, tocopherol and carotenoid assays in cancer prevention studies. *J Nutr Growth Cancer* 1987; 4: 19-27.

Yang XC, Cai BL, Sciacca RR, Cannon PJ. Inhibition of inducible nitric oxide synthase in macrophages by oxidised low-density lipoprotein. *Cir Res* 1994; 74:318-328.

Yammato A, Matasuzawa Y, Kishino B, Hayashi R, Hirobe K, Kikkawa T. Effects of probucol on homozygous cases of familial hypercholesterolemia. *Atherosclerosis* 1983; 48: 157-66

Yap SC, Choo YM, Hew NF, Yap SF, Khor HT, Ong ASH, Goh SH. Oxidative susceptibility of low density lipoprotein from rabbits fed atherogenic diets containing coconut, palm, or soybean oils. *Lipids* 1995; 30: 1145-1150.

Yates MT, Lambert LE, Whitten JP, McDonald I, Mano M, Ku G, Mao SJ. A protective role for nitric oxide in the oxidative modification of low density lipoproteins by mouse macrophages. *FEBS Lett* 1992; 309: 135-138.

Yla-Herttuala S, Palinski W, Rosenfeld ME, Parthasarathy S, Carew TE, Butler S, Witztum JL, Steinberg D. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J Clin Invest* 1989; 84: 1086-1095.

Yokoyama M, Hirata K, Miyake R, Akita H, Ishikawa Y, Fukuzaki H. Lysophosphitadylcholine: essential role in the inhibition of endothelium-dependent vasorelaxation by low-density lipoprotein. *Biochem Biophys Res Commun* 1990; 168: 301-308.

Zieden B, Molgaard J, Olsson AG. Low density lipoprotein oxidation and coronary atherosclerosis. *Nature* 1992; 340: 727-728.