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

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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 perox 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 commercially 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 $1.13 \pm 0.16 \, \mu g$ cholesterol/mg wt wgt compared to rabbits fed cholesterol alone ($0.65 \pm 0.03, \, P<0.01$), but this was not different to rabbits consuming cholesterol + ethanol ($1.11 \pm 0.2$).

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.
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

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<table>
<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>AcLDL</td>
<td>acetylated low density lipoprotein</td>
</tr>
<tr>
<td>Apo B</td>
<td>apolipoprotein B-100</td>
</tr>
<tr>
<td>CAD</td>
<td>coronary artery disease</td>
</tr>
<tr>
<td>CE</td>
<td>cholesteryl ester</td>
</tr>
<tr>
<td>d</td>
<td>density</td>
</tr>
<tr>
<td>DMEM</td>
<td>dulbecco's modified eagles media</td>
</tr>
<tr>
<td>ECD</td>
<td>electrochemical detection</td>
</tr>
<tr>
<td>EDRF</td>
<td>endothelium-dependent relaxing factor</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid disodium salt</td>
</tr>
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<td>g</td>
<td>grams</td>
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<td>GAE</td>
<td>gallic acid equivalents</td>
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<td>high density lipoprotein</td>
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<tr>
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<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IDL</td>
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</tr>
<tr>
<td>KPSS</td>
<td>potassium chloride physiological salt solution</td>
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<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>MDA</td>
<td>malondialdehyde</td>
</tr>
<tr>
<td>PBS</td>
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</tr>
<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acid</td>
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<tr>
<td>WHHL</td>
<td>Watanabe heritable hyperlipidemic rabbit</td>
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PUBLICATIONS ARISING FROM THIS THESIS

Full Publications


Abstracts


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.

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).

\[-LH + R \cdot \rightarrow -L \cdot + RH \quad (A)\]
\[-L \cdot + O_2 \rightarrow -LOO \cdot \quad (B)\]
\[-LOO \cdot + -LH \rightarrow -LOOH + -L \cdot \quad (C)\]

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 (Henriksson 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.
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 A₂ (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 Fcy-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 malondialdehyde-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 AHEROGENICITY 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 I₂) and endothelium-derived hyperpolarising factor. While the polypeptide, endothelin, and certain eicosanoid metabolites, thromboxane A₂ and prostaglandin H₂, 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 α1-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 \( \alpha \)-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 (\( \alpha \), \( \lambda \) and \( \beta \)-tocopherol), \( \beta \)-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 subendothelial 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 \( \alpha \)-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 Viellissement 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 \( \alpha \)-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 \( \alpha \)-tocopherol during \textit{in vitro} oxidation of LDL with low concentrations of thermolabile azo-compounds which produce slow fluxes of aqueous peroxo 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 peroxo radicals which result in elevated lipid hydroperoxide formation in the presence of \( \alpha \)-tocopherol (Bowry et al. 1992). During this azo-initiated oxidation, \( \alpha \)-tocopherol is oxidised by peroxo radicals forming \( \alpha \)-tocopheroxy radicals within the LDL particle. The \( \alpha \)-tocopheroxy radical in this situation can be either reduced back to \( \alpha \)-tocopherol by coantioxidants in LDL such as ubiquinol-10, or may react with bisallylic carbons within PUFA forming lipid peroxo radicals which initiate lipid peroxidation. It is via the latter mechanism that \( \alpha \)-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 \( \alpha \)-tocopherol.

These observations have been supported by Kontush et al. (1996a). Under strong oxidative conditions (high concentrations of copper or azo compounds), \( \alpha \)-tocopherol supplementation decreased LDL and plasma oxidation. Under mild oxidative conditions an antioxidant effect in \( \alpha \)-tocopherol supplemented plasma was observed only in the presence of coantioxidants.
such as ascorbate. A prooxidant effect of $\alpha$-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 $\alpha$-tocopherol’s antioxidant or prooxidant activity, since physiological levels of coantioxidants are required to reduce $\alpha$-tocopheroxyl radicals.

The discussion on antioxidants so far has focused mainly on the mechanisms involved in \textit{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 \textit{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 peroxyl or other free radicals. Although the mechanisms of \textit{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

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 (Kleinveit 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
(Yamamoto 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 \textit{in vitre} 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).

\textbf{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), ß-carotene (Keaney et al. 1993) and probucol (Simon et al. 1993, Keaney et al.
Studies employing \textit{in vivo} photoplethsmography measurements (Klemdale \textit{et al.} 1994) and \textit{in vitro} organ chamber experiments have demonstrated that dietary antioxidants such as vitamin E can restore endothelium-dependent relaxation in aorta (Keaney \textit{et al.} 1994), coronary circulation (Andersson \textit{et al.} 1994), ear microcirculation (Matz \textit{et al.} 1994) and carotid artery (Stewart-Lee \textit{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 \textit{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  Flavan-3-ols  Catechin
Myricetin  R = H, R₂ = OH
Rutin  R = D-Glucose-L-Rhamnose

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), peroxyl radicals (Torel et al. 1986), inhibiting lipoxygenase-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
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 (EC₅₀) 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 reisolated. 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 its 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 KH₂PO₄, 16 mmol/L Na₂HPO₄·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 ($\epsilon_{234} 29 500$).

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 ($\epsilon_{335} 1.56 \times 10^5$).
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 \( \varepsilon_{292} = 75,800 \) (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 were 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% H₂SO₄ 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 contaminatees 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) Na₂CO₃ 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, 5α-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 autooxidation 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 peroxo 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 KH₂PO₄ 4 mmol/L, Na₂HPO₄·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 C₁₈ 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 [3H]cholesterol ester (CE)-LDL
The LDL was labelled with [3H]cholesterol as described by the method of Barter and Jones (1979). Briefly, 50 µl of [3H]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 [3H] 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% CO₂ in air. For uptake experiments macrophages were trypsinised, resuspended in DMEM + 10% FCS and plated into 6 well culture plates containing 5 x 10⁵ cells/well. After 2 days of culture, the macrophage monolayers were washed twice with PBS and incubated with 10 µg (protein) of either native [3H]CE-LDL or copper-oxidised [3H]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).
<table>
<thead>
<tr>
<th></th>
<th>undialysed</th>
<th>dialysed</th>
<th>gel filtered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag time (min)</td>
<td>nd</td>
<td>47.3 ± 2.5</td>
<td>47.0 ± 3.2</td>
</tr>
<tr>
<td>Oxidation rate</td>
<td>nd</td>
<td>18.30 ± 0.91</td>
<td>17.05 ± 0.95</td>
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<tr>
<td>(nmol diene/mg LDL</td>
<td></td>
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<tr>
<td>protein/min)</td>
<td></td>
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<tr>
<td>LDL α-tocopherol</td>
<td>6.53 ± 0.27</td>
<td>6.37 ± 0.14</td>
<td>6.54 ± 1.12</td>
</tr>
<tr>
<td>(μg α-tocopherol/mg LDL protein)</td>
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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 [3H]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 [3H]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 peroxo 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 peroxo 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 preincubation 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 [3H]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 peroxo 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 peroxo 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.
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 ε₂₈₄ (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 <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.
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 \( \mu \)mol/L) and ECD (0.05 to 0.5 mg/L or 0.2 to 2 \( \mu \)mol/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 \( \mu \)mol/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 \( \mu \)mol/L and 100 \( \mu \)mol/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).
### 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).

<table>
<thead>
<tr>
<th>Plasma incubation with genistein (µmol/L)</th>
<th>25</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>plasma genistein (µmol/L)</td>
<td>27.7 ± 3.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44.8 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>90.5 ± 7.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>LDL genistein (nmol genistein/mg LDL protein)</td>
<td>0.7 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.1 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.7 ± 0.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>% incorporation of genistein into LDL</td>
<td>2.8 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.0 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.6 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
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 malondialdehyde 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).
The effect of genistein on malondialdehyde concentrations in azo-oxidised LDL.

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>4.3±0.9</td>
<td>6.6±0.9</td>
<td>7.8±0.3</td>
<td>10.1±0.1</td>
<td>13.8±1.3</td>
<td>15.1±1.2</td>
<td>15.5±2.3</td>
<td>16.0±2.1</td>
<td>14.2±1.8</td>
</tr>
<tr>
<td>50 µmol/L genistein</td>
<td>4.6±1.3</td>
<td>5.6±0.2</td>
<td>7.3±0.5</td>
<td>8.7±0.6</td>
<td>11.4±0.3</td>
<td>12.5±0.6</td>
<td>12.9±1.8</td>
<td>14.9±1.4</td>
<td>14.4±1.8</td>
</tr>
<tr>
<td>100 µmol/L genistein</td>
<td>4.8±0.9</td>
<td>5.0±0.9</td>
<td>7.0±0.3</td>
<td>8.2±0.1</td>
<td>10.0±0.3</td>
<td>11.1±0.4</td>
<td>11.7±1.4</td>
<td>13.5±2.1</td>
<td>12.8±2.3</td>
</tr>
<tr>
<td>200 µmol/L genistein</td>
<td>6.2±2.1</td>
<td>5.9±0.3</td>
<td>6.1±0.7</td>
<td>6.9±0.2</td>
<td>7.8±0.7</td>
<td>8.2±0.1</td>
<td>9.0±0.6</td>
<td>10.0±1.2</td>
<td>9.0±0.6</td>
</tr>
</tbody>
</table>

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 FeCl₃/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-desmethylandolensin 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, Clevéndence 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

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

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).
### 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.

<table>
<thead>
<tr>
<th>VLDL + IDL fraction (d 1.006 to 1.019 g/ml)</th>
<th>control</th>
<th>cholesterol</th>
<th>cholesterol + red wine</th>
<th>cholesterol + ethanol</th>
<th>ANOVA</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>cholesterol (mmol/L)</td>
<td>0.09 ± 0.03 (^{a})</td>
<td>13.61 ± 2.53 (^{b})</td>
<td>17.14 ± 5.63 (^{b})</td>
<td>10.65 ± 2.39 (^{b})</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>triglyceride (mmol/L)</td>
<td>0.38 ± 0.08 (^{a})</td>
<td>1.55 ± 0.32 (^{b})</td>
<td>1.54 ± 0.43 (^{b})</td>
<td>1.22 ± 0.17 (^{b})</td>
<td>0.0013</td>
<td></td>
</tr>
<tr>
<td>protein (g/L)</td>
<td>0.12 ± 0.02 (^{a})</td>
<td>1.95 ± 0.35 (^{b})</td>
<td>2.14 ± 0.84 (^{b})</td>
<td>1.59 ± 0.42 (^{b})</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>LDL fraction (d 1.019 to 1.063 g/ml)</th>
<th>control</th>
<th>cholesterol</th>
<th>cholesterol + red wine</th>
<th>cholesterol + ethanol</th>
<th>ANOVA P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>cholesterol (mmol/L)</td>
<td>0.05 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.18 ± 0.35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.61 ± 0.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.53 ± 0.24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>triglyceride (μmol/L)</td>
<td>21.4 ± 2.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>63.9 ± 9.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>80.2 ± 23.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>82.5 ± 8.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0012</td>
</tr>
<tr>
<td>protein (mg/L)</td>
<td>27.8 ± 8.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>397 ± 36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>430 ± 84&lt;sup&gt;b&lt;/sup&gt;</td>
<td>313 ± 50&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

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.

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

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.
<table>
<thead>
<tr>
<th>Ratio of cholesterol in lipoprotein fractions</th>
<th>control</th>
<th>cholesterol</th>
<th>cholesterol + red wine</th>
<th>cholesterol + ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL + IDL: HDL-cholesterol</td>
<td>0.5 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.7 ± 4.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.0 ± 6.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.6 ± 5.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>LDL: HDL-cholesterol</td>
<td>0.2 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.1 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.0 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.8 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>VLDL + IDL + LDL: HDL-cholesterol</td>
<td>0.7 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.8 ± 5.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.0 ± 6.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.4 ± 5.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**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
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.
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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 wt or red wine consisting of 0.5 g ethanol /d/kg body wt. 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-Gesellschaft, 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 \( \alpha \)-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.

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

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.

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

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.

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>cholesterol</th>
<th>cholesterol + red wine</th>
<th>cholesterol + ethanol</th>
<th>ANOVA P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>aortic arch fatty streak</td>
<td>0.06 ± 0.04 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.3 ± 7.0 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.1 ± 4.1 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.9 ± 6.1 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0034</td>
</tr>
<tr>
<td>(percentage total surface area with lipophilic stain)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>thoracic aortic cholesterol</td>
<td>0.54 ± 0.03 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.65 ± 0.03 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.13 ± 0.16 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.11 ± 0.20 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0011</td>
</tr>
<tr>
<td>(µg cholesterol/mg wet wgt)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

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.
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 anomalous 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 (Abby 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 hypercholesterolemia 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 \textit{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-supplemented 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 KH₂PO₄, 1.2 mmol/L MgSO₄·7H₂O, 0.6 mmol/L ascorbic acid, 11.2 mmol/L glucose and 2.5 mmol/L CaCl₂). 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% O₂ and 5% CO₂ 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 Linearorder 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 α₁-contractile agonist, phenylephrine was performed. Relaxation to acetylcholine (1 to 1000 nmol/L) was studies in rings precontracted with a half-maximal concentration (EC₅₀) 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 (EC₃₀, EC₅₀ and EC₇₀) 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 EC₃₀, EC₅₀ and EC₇₀ 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 EC₅₀ values for phenylephrine contraction were approximately -6.4 to -6.5 log M irrespective of dietary treatment (Table 7.1). Although this EC₅₀ 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 EC₃₀, EC₅₀ and EC₇₀. 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 (EC₅₀) 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.
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.
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). EC₃₀, EC₅₀ and EC₇₀ 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 (EC50) 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.
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, Klembsdale 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 $\alpha_1$-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 $\alpha$-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 be 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 NaH₂PO₄ 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 NaH₂PO₄, 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.
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 8.1](image)

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

![Figure 8.2](image)

**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 phosphotungstic 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 \textit{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 \textit{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 \textit{in vitro}. Genistein may not be a good antioxidant \textit{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 \textit{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 \textit{in vitro} catalysed oxidation would strengthen the claim that red wine is an effective \textit{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 \textit{et al.} 1995, Kondo \textit{et al.} 1994, Maxwell \textit{et al.} 1994, Whitehead \textit{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 \textit{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 \textit{et al.} 1979, Renaud \textit{et al.} 1992a).

An anomaly exists between the \textit{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 \textit{in vivo} prooxidant effects of ethanol. Croft \textit{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.


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