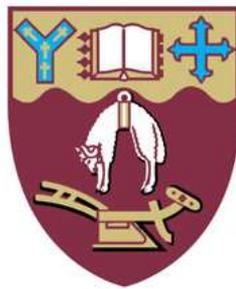


Toxicity of 7-Ketocholesterol
as a Mechanism of
Oxidised Low Density
Lipoprotein-Induced Cellular Death



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ABBREVIATIONS

7 α OH	7 α -hydroxycholesterol
7 β OH.....	7 β -hydroxycholesterol
7OOH.....	7-hydroperoxycholesterol
7,8-NP	7,8-dihydroneopterin
7KC.....	7-ketocholesterol
7KC-acLDL	7-ketocholesterol-loaded acetylated low density lipoprotein
7KC-oxLDL.....	7-ketocholesterol-loaded oxidised low density lipoprotein
7OH.....	7-hydroxycholesterol
24OH.....	24-hydroxycholesterol
25OH.....	25-hydroxycholesterol
27OH.....	27-hydroxycholesterol
AAPH	2,2-azobis(2-amidinopropane) dihydrochloride
ACAT	acyl coenzyme A: cholesterol transferase
AcLDL.....	acetylated LDL
ACN.....	acetonitrile
AgLDL.....	aggregated LDL
Apo A-1	apolipoprotein A-1
Apo B-100	apolipoprotein B-100
ANOVA.....	analysis of variance
BCA	bicinchoninic acid
BHT	butylated hydroxytoluene
BSA.....	bovine serum albumin
C ₂ H ₃ NaO ₂	sodium acetate
CD36.....	cluster of differentiation 36 scavenger receptor
CO ₂	carbon dioxide
CO ₃ ^{•-}	carbonate radical
CuCl ₂	copper chloride
DMSO.....	dimethyl sulphoxide
DNA.....	deoxyribonucleic acid
DOPA	3,4-dihydroxyphenylalanine

ECendothelial cell
EDTAethylenediaminetetraacetic acid
FBSfoetal bovine serum
GSHglutathione
H ₂ Owater
H ₃ PO ₄phosphoric acid
HClhydrochloric acid
HDLhigh density lipoprotein
HI-FBSheat-inactivated foetal bovine serum
HMGCoA-3-hydroxy-3-methyl-glutaryl-CoA-
HOClhypochlorous acid
HPLChigh performance liquid chromatography
KBrpotassium bromide
KOHpotassium bromide
L-lipid
L [•]lipid radical
LCATlecithin: cholesterol acyltransferase
LD ₅₀median lethal dose
LHpolyunsaturated fatty acyl group
LDLlow density lipoprotein
LOO [•]lipid peroxy radical
LOOHlipid hydroperoxide
lysoPtd-Cholysophosphatidylcholine
MBBmonobromobimane
MDAmalondialdehyde
MmLDLminimally oxidised low density lipoprotein
MOPS4-morpholine-propanesulfonic acid
MTT3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
NaClsodium chloride
NaHCO ₃sodium hydrogen carbonate
NaOClsodium hypochlorite
NaOHsodium hydroxide
NADPHreduced nicotinamide adenine dinucleotide phosphate

NaH ₂ PO ₄ ·H ₂ O	sodium dihydrogen phosphate monohydrate
nCEH	neutral cholesterol ester hydrolases
NO	nitric oxide
NO ₂ [•]	nitrogen dioxide
ONOO ⁻	peroxynitrite radical
O ₂	molecular oxygen
OH [•]	hydroxyl radical
OxLDL	oxidised low density lipoprotein
P-	protein-
PES	polyethersulfone
PBS	phosphate buffered saline
PMA	phorbol-12 myristate 13-acetate
Psi	pounds per square inch
PUFA	polyunsaturated fatty acid
REM	relative electrophoretic mobility
RO [•]	alkoxyl radical
RO ₂ [•]	peroxyl radical
ROS	reactive oxygen species
Rpm	revolutions/minute
RPMI-1640	Roswell Park Memorial Institute cell medium
SEM	standard error of the mean
SDS	sodium dodecyl sulfate
SMC	smooth muscle cell
SR-A	scavenger receptor type A
TBA	2-thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TCA	trichloroacetic acid
TMP	1,1,3,3-tetramethoxypropane
Trpc-1	transient receptor potential calcium channel-1
vLDL	very low density lipoprotein
^v / _v	volume/volume
R [•]	free radical
^w / _v	weight/volume

ABSTRACT

Atherosclerosis is a complex inflammatory disease involving the deposition of cholesterol-loaded macrophage cells within the artery wall. Progression of the initial fatty streak to an advanced atherosclerotic plaque is characterised by the development of a necrotic core region containing cholesterol and dead cells. It is well established that the formation of oxidised low-density lipoprotein (oxLDL) and the resulting toxicity to macrophage cells is a key driver in the development of the necrotic core. OxLDL contains the oxysterol, 7-ketocholesterol, which is the predominant oxysterol found within advanced atherosclerotic plaques. Numerous research groups have demonstrated the toxicity of 7-ketocholesterol to various cell types, but the route of 7-ketocholesterol delivery is important to its cytotoxicity. 7-Ketocholesterol is almost entirely lipoprotein-associated *in vivo*. The aim of this study was to use a more physiologically relevant model to assess the toxicity of 7-ketocholesterol to U937 human monocyte cells. U937 cells were found to be very sensitive to both oxLDL and 7-ketocholesterol. Yet incorporation of 7-ketocholesterol into high-uptake acetylated LDL greatly reduced the oxysterol cytotoxicity, when compared to an equivalent amount of 7-ketocholesterol added directly to U937 cell culture medium. While low intracellular concentrations of 7-ketocholesterol correlated with very high oxLDL toxicity, comparatively large intracellular 7-ketocholesterol content from the uptake of 7KC-acLDL caused only a small viability loss. Enrichment of oxLDL with 7-ketocholesterol did not significantly enhance lipoprotein toxicity. Collectively, these findings indicate that 7-ketocholesterol is not the toxic agent within oxLDL and have implications for the mechanism of oxLDL-induced cytotoxicity involved in atherosclerotic plaque necrotic core development.

1. INTRODUCTION

1.1 Overview

Atherosclerosis is a condition of the major arteries in which progressive occlusion of the vessel occurs and is the result of an ongoing inflammatory process (Lusis, 2000). The involvement of low density lipoproteins (LDL) in the pathogenesis of atherosclerosis is widely accepted. According to the oxidative modification hypothesis of atherogenesis, LDL within the artery wall undergoes oxidative modification, forming oxidised LDL (oxLDL) which is then taken up by macrophage cells in an unregulated manner, triggering their transformation into lipid laden foam cells (Libby *et al.*, 2002).

Oxysterols are products of cholesterol oxidation that are present in atherosclerotic plaque and may be involved in their development (Brown and Jessup, 1999). Oxysterols, such as 7-ketocholesterol, are thought to be the main toxic component of oxLDL. *In vivo* studies have demonstrated that oxysterols, including 7-ketocholesterol, accumulate in human foam cells from atherosclerotic plaque (Mattsson-Hulten *et al.*, 1996). Oxysterols are thought to be involved in the cell death process that characterises that progression of the fatty streak to advanced atherosclerotic plaque. Although cytotoxicity of oxysterols to many cells types, including macrophages, has been reported (Clare *et al.*, 1995, Larsson *et al.*, 2006, Lizard *et al.*, 1998), such studies have involved direct incubation of oxysterols with cell cultures. As oxysterols are principally transported within lipoproteins *in vivo* (Addis *et al.*, 1989, Lin and Morel, 1996), the incorporation of oxysterols into high-uptake lipoprotein provides a more physiologically relevant model for examining the effect of the oxysterol of interest, in the mechanism of atherosclerosis.

There has been little work carried out describing the influence of oxysterol-loaded lipoprotein on cell systems. This thesis investigates the toxicity of 7-ketocholesterol-enriched acetylated LDL on the U937 human monocyte-like cell line, to test the hypothesis that the oxysterol, 7-ketocholesterol, is not the main toxic agent of oxLDL.

1.2 Atherosclerosis

Atherosclerosis is a complex multi-factorial process. The disease is characterised by a local thickening of the artery wall due to the deposition of lipid-laden macrophages, fibrous proteins, lipid deposits and debris from dead cells. Atherosclerosis and its complications (cardiovascular disease, cardiac infarction and stroke) are the leading causes of death in the developed countries (Halliwell & Gutteridge, 2007). This brief outline of atherosclerosis will follow the most widely accepted oxidative modification hypothesis describing initiation, progression and rupture, with a focus on the influence of LDL.

Lesion initiation is thought to occur with the accumulation of LDL and apolipoproteins in the sub-endothelial matrix. Trapped LDL is then thought to undergo modifications such as oxidation, proteolysis and aggregation, which may be caused in part by exposure to oxidants released by vascular cells (Lusis, 2000). The oxidation of LDL promotes its unregulated uptake by macrophages via scavenger receptors. Studies suggest that oxLDL is present in the intima before the appearance of monocytes (Rosenfeld, 1996) but there is also strong support for the entry of lipoproteins as a secondary event, consistent with the theory that endothelial injury allows more LDL to enter the vessel wall (Halliwell & Gutteridge, 2007). Atherosclerotic foam cells are generated *in vivo* from macrophages, of monocytic origin, as demonstrated using animal models, cell marker studies and ultra-structural and immunohistochemical techniques (Aqel *et al.*, 1984).

Monocyte and T-lymphocyte recruitment is facilitated by increased expression of various adhesion and pro-inflammatory molecules that are triggered by minimally oxidised LDL (mmLDL). In the presence of reactive oxygen species (ROS) produced by endothelial cells (ECs) and smooth muscle cells (SMCs), and several enzymes, mmLDL is further oxidised to generate oxLDL (Lusis, 2000). OxLDL has also been shown to directly attract monocytes (Steinberg *et al.*, 1989). OxLDL and other forms of modified LDL are taken up by macrophages via scavenger receptors, such as CD36 and SR-A, in an unregulated manner (Glass and Witztum, 2001, Steinberg *et al.*, 1989). However there is also evidence that LDL oxidation might occur only once inside the macrophages, and specifically, within lysosomes (Wen and Leake, 2007). The rapid uptake of oxidised (and otherwise modified) LDL by macrophages causes cholesterol esters to accumulate in droplets in the

macrophage cytoplasm, resulting in the formation of foam cells, which are the main constituent of the early stage atherosclerotic lesion, known as the fatty streak.

The formation of foam cells is likely to be encouraged and sustained by an imbalance in normal macrophage cholesterol homeostasis, a mechanism that normally prevents the toxic accumulation of cholesterol within cells (Warner *et al.*, 1995). Normal mechanisms of reverse cholesterol transport to extracellular acceptors such as high density lipoprotein (HDL) and apolipoprotein A-1 (Apo A-1) for the elimination of cholesterol is impaired in macrophages loaded with oxLDL (Kritharides *et al.*, 1995). In the absence of available extracellular acceptors, excess free cholesterol within the cell undergoes re-esterification to detoxify the cholesterol, before it is stored as cholesterol ester in the cytosol. The intracytoplasmic cholesterol ester accumulates as membrane-free lipid droplets, transforming macrophages into foam cells (Van Reyk and Jessup, 1999).

The development of a fatty streak into a fibrous plaque is characterised by further recruitment of inflammatory cells and the migration and proliferation of SMCs within the intima, in response to cytokines, growth factors and reactive species secreted by activated macrophages and T lymphocytes (Lusis, 2000). Cell death appears to be central to the formation of a necrotic core in advanced atherosclerotic plaques (Giese *et al.*, 2009a). Together with the wealth of experimental evidence that confirms the cytotoxicity of oxLDL (Baird *et al.*, 2004, Bjorkerud and Bjorkerud, 1996, Harada-Shiba *et al.*, 1998, Reid and Mitchinson, 1993) and its presence within atherosclerotic plaques (Brown *et al.*, 1997, Fu *et al.*, 1998, Ylaherttuala *et al.*, 1989), it is likely that oxLDL is a major driver of cell death *in vivo* and hence, of necrotic core development.

The advanced fibrous plaque has a fibrous cap which consists of SMCs and secreted extracellular matrix proteins (Glass and Witztum, 2001) that protects the lipid core from contact with the blood. Macrophages within the intima can secrete cytokines that amplify a local inflammatory response and matrix metalloproteinases that can degrade the fibrous cap, weakening the plaque (Halliwell & Gutteridge, 2007). Plaque rupture is more likely to occur in advanced plaques that have undergone calcification and neovascularisation (Glass and Witztum, 2001) and have large necrotic cores and thin fibrous caps (Lusis, 2000). Plaque rupture exposes oxidised plaque lipids and proteins and tissue factor to the blood,

initiating the coagulation cascade, platelet adherence and thrombosis (Glass and Witztum, 2001).

1.3 Low density lipoprotein

As the early atherosclerotic lesion consists of lipid-laden macrophages, this implies lipoprotein uptake by these cells during the early stages of atherogenesis (Meilhac *et al.*, 1999). LDL is the main carrier of cholesterol within the body and is crucial to the controlled transport and metabolism of lipids in the bloodstream. The serum LDL concentration in normolipidemic persons is approximately 3 mg/ml and is responsible for transporting approximately 60% of total plasma cholesterol (Esterbauer *et al.*, 1992). LDL is a spherical molecule consisting of protein (mainly apolipoprotein B-100) embedded in a monolayer surface of polar phospholipids and cholesterol, surrounding a core of neutral cholesterol esters and triglycerides. LDL may be characterised by its small size, with a diameter of 19-25 nm, relative molecular weight between 1.8 and 2.8 million and density of 1.063-1.090 g/ml. Based on calculations of the mean molecular weight as 2.5 million, each LDL particle core is estimated to contain 1600 molecules of cholesterol ester and 170 molecules of triglycerides. The surrounding monolayer is estimated to contain 700 phospholipid molecules and 600 molecules of free cholesterol (Esterbauer *et al.*, 1992).

LDL is clearly abundant in lipids, of which almost half are fatty acids. Of the fatty acids, approximately half are polyunsaturated fatty acids (PUFAs), depending on the donor, which makes LDL highly susceptible to free radical-mediated oxidation. The PUFAs in LDL are normally protected against free radical attack and oxidation by a range of antioxidants, of which α -tocopherol is the most significant. Antioxidants are present within both the core and phospholipid coat (Esterbauer *et al.*, 1992) and since the structure of LDL is quite fluid, the lipid soluble antioxidants are able to move freely between the core and outer layer (Schuster *et al.*, 1995).

LDL uptake by cells may occur either via a receptor-mediated pathway or by non-specific endocytosis. LDL interacts with the LDL receptor via ionic interactions between clusters of amino acids and the acidic amino acids of the receptor (Brown and Goldstein, 1979). Certain modifications to LDL (oxidation, acetylation, aggregation) cause blockage of the

α -amino group of the lysine residues of apolipoprotein B-100 (apoB-100), which results in a relative increase in the negative charge of apoB-100. Once lysines are blocked, the modified LDL does not recognise the LDL receptor (Esterbauer *et al.*, 1992). Modified LDL therefore enters cells via receptor-mediated endocytosis, not under feedback control, resulting in cellular cholesterol accumulation (Esterbauer *et al.*, 1992).

1.3.1 LDL oxidation

Despite extensive research, the mechanisms by which LDL becomes oxidised in atherosclerotic lesions remain uncertain. The oxidative modification hypothesis proposes that LDL oxidation takes place in the extracellular space of atherosclerotic lesions, in the interstitial fluid, before being taken up by macrophages. However, recent work proposes that oxidation occurs within macrophages, inside lysosomes, following the uptake of aggregated (non-oxidised) LDL by the cell (Wen and Leake, 2007).

LDL oxidation may be initiated *in vitro* by incubation with monocytes, macrophages, endothelial cells, SMCs, lymphocytes or in cell-free systems using pro-oxidants (Esterbauer *et al.*, 1993). All three of the major cell types present in the artery wall have the ability to convert LDL to a form that is recognised by scavenger receptors (Steinberg *et al.*, 1989). The most widely used procedure for preparing oxLDL for use in model systems involves incubation for 12 or more hours in either cell culture media or phosphate buffered saline supplemented with Cu^{2+} ions in the range of 5 to 100 μM .

The oxidation of LDL occurs via a lipid peroxidation chain reaction that is driven by the formation of lipid peroxy radicals. The oxidation is typically described in terms of its three consecutive time phases; the lag phase, propagation phase and decomposition phase. LDL oxidation begins when a C-H bond in a polyunsaturated fatty acyl group (LH) (in a phospholipid, cholesteryl ester or triacylglycerol molecule within LDL) is attacked by a free radical (R'), forming a lipid alkyl radical (*reaction i*) (Esterbauer *et al.*, 1990). The identity of the initiating radical in both *in vivo* and *in vitro* oxidation remains controversial. Once formed, the carbon-centered PUFA radical reacts quickly with molecular oxygen to create a lipid peroxy radical (*reaction ii*). The newly formed lipid peroxy radical then reacts with a hydrogen atom of an adjacent PUFA, forming a lipid hydroperoxide and a

new PUFA radical (*reaction iii*) (Esterbauer *et al.*, 1993), which again yields a lipid peroxy radical that will react with another PUFA molecule creating a chain reaction.



The antioxidants within LDL initially compete with these chain propagating reactions by scavenging lipid peroxy radicals (*reaction iv*) so that minimal lipid peroxidation occurs. This initial phase is aptly named the lag phase (Esterbauer *et al.*, 1993). Once the LDL has become depleted of endogenous antioxidants, the propagation phase commences and the PUFAs within LDL are thought to be rapidly oxidised to lipid hydroperoxides at a rate that increases exponentially. The decomposition phase involves the decomposition of lipid hydroperoxides to yield a wide range of products including aldehydes, hydrocarbon gases, epoxides and alcohols. This decomposition has been shown to occur spontaneously at a slow rate but is greatly accelerated by the presence of transition metal ions, due to the catalysed conversion of lipid hydroperoxides to lipid alkoxy radicals (Cheeseman and Slater, 1993). The lipid alkoxy radicals and peroxy radicals cause secondary oxidative damage to cholesterol, generating a range of oxysterols. The breakdown of lipid hydroperoxides to peroxy and alkoxy radicals generates isoprostanes and short chain aldehydes, such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA). These lipid-derived aldehydes bind lysine residues of amino acid side chains of apoB-100 (Gerry *et al.*, 2008). The subsequently modified apoB-100 has a greater net negative charge and is no longer recognised by the LDL receptor. The modified LDL is instead recognised by scavenger receptors and taken up rapidly by macrophages.

The sequence of events described for LDL oxidation has been demonstrated in both copper- and macrophage-mediated oxidation and is thought to be common to all methods of LDL oxidation (Esterbauer *et al.*, 1992). Both copper- and cell-mediated oxidation of LDL yield oxLDL that contains significant quantities of cholesterol products. The LDL-associated cholesterol is oxidised predominantly at the 7th position, and 7-ketocholesterol comprises one half to two thirds of the total cholesterol.

Major characteristics of native and oxidised LDL

In contrast to the single long chain polypeptides of apoB-100 in native LDL, oxLDL has many proteolytic fragments derived from apoB-100. The fragmented protein is proposed to have been covalently modified by lipid peroxidation products such as aldehydes, possibly derived from esterified lipid. Compared to native LDL, oxLDL contains depleted levels of PUFAs and antioxidants, massively increased levels of lipid peroxides and their degradation products, increased amounts of oxidised cholesterol products and enzyme break-down products such as lysophosphatidylcholine (lysoPtd-Cho) and various protein oxidation species (Parthasarathy *et al.*, 1999).

OxLDL contains a number of oxidised protein products that may be of importance to its mechanism of cytotoxicity. The destruction of tryptophan residues on apoB-100 is an early and potentially important event in the copper-mediated oxidation of LDL (Esterbauer *et al.*, 1995) and it has been shown that LDL oxidation mediated by the macrophage myeloperoxidase product, hypochlorous acid, preferentially modifies the protein component of lipoproteins (Gerry *et al.*, 2008). In addition, it has been shown that protein hydroperoxides are generated at relatively high concentrations on apoB-100 during copper-, AAPH-generated peroxy radical- and cell-mediated LDL oxidation (Giese *et al.*, 2003). The oxidation of the LDL protein moiety causes the loss of structural integrity of the apoB-100 protein, but also results in the loss of select amino acids, carbonyl formation, protein fragmentation, and aggregation via protein cross-linking (Giese *et al.*, 2003).

Different types of oxLDL can be produced depending on the oxidation conditions. Of influence are the type and concentration of oxidant, level of antioxidants and modification of apoB-100. A novel method was recently described for the copper-mediated oxidation of LDL that yields either hydroperoxide-rich or oxysterol-rich oxLDL, depending on the incubation temperature (Gerry *et al.*, 2008). It is important to note that LDL oxidation is a progressive process that leads to the formation of a continuum of oxLDL (from minimally to extensively oxidised LDL), containing a variety of oxidised components in variable proportions. Since oxLDL is heterogeneous in composition, it is difficult to define the role of each potentially toxic oxidised component in the overall toxic effect of oxLDL (Salvayre *et al.*, 2002).

1.3.2 Oxidised LDL and atherogenesis

OxLDL is suggested to have a plethora of pro-atherogenic effects that previously have been attributed predominantly to its oxidised lipid components (Parthasarathy *et al.*, 1999). The oxidative modification hypothesis of atherogenesis is based upon four potentially atherogenic effects of oxLDL. These effects are chemotactic activity that facilitates the recruitment of circulating monocytes to the endothelium; the inhibition of macrophage migration from within the artery back to the plasma compartment; enhanced uptake of LDL by macrophages via scavenger receptors contributing to the formation of foam cells; and cytotoxicity (Steinberg *et al.*, 1989), which is the focus of this study.

A selection of the large range of studied effects of oxLDL, thought account for its atherogenic properties, are described. OxLDL has been shown to act as a chemoattractant for monocytes and induce endothelial cells to secrete monocyte chemoattractants and monocyte adhesion molecules (Colles *et al.*, 2001). OxLDL can promote foam cell formation by binding macrophage scavenger receptors, alter the expression and secretion of various growth factors and cytokines from vascular SMCs, induce migration and proliferation of SMCs, impede endothelial cell migration, interfere with endothelium-mediated relaxation and promote pro-coagulant properties of vascular cells such as enhancement of tissue factor expression in endothelial cells, SMCs and macrophages (Colles *et al.*, 2001). For some of the above effects, the specific mechanism and actively involved moieties of oxLDL have been identified through *in vitro* and *ex vivo* studies, although mechanisms *in vivo* remain unclear.

1.3.3 Acetylated LDL

Aside from oxidation, other LDL modifications yield lipoproteins that are commonly used and useful models for research, such as acetylated LDL (acLDL) and aggregated LDL (agLDL). While agLDL occurs *in vivo*, acLDL is an artificial particle that may be used to model the uptake of oxLDL by cells. Previous studies have shown that oxLDL and acLDL are both recognised by the scavenger receptor class A types I and II in macrophages (Ling *et al.*, 1997) and to some extent the class B scavenger receptors, such as CD36 (Martin *et al.*, 2007). Studies on the uptake of oxLDL and acLDL revealed no difference between the two types of modified lipoprotein uptake in either THP-1 cells or mouse peritoneal

macrophages (Yancey *et al.*, 2002). Much like oxidation, acetylation of ϵ -amino groups of lysine residues on apoB-100 increases net electronegativity of the particle and prevents recognition by the LDL receptor. Uptake into the macrophage via scavenger receptors, a pathway that is not regulated by intracellular cholesterol levels, leads to intracellular accumulation of cholesterol esters (Brown and Goldstein, 1983). AcLDL provides an effective model for oxLDL in foam cell systems since they are both high-uptake ligands for scavenger receptors, although importantly, acLDL is not cytotoxic and neither of its lipid or protein components have been oxidised.

1.3.4 Antioxidant defences

Normally, cells are able to survive in the presence of a wide range of oxidants because they possess antioxidant defences that enable them to combat the oxidant's potential to cause cellular damage and alter the redox-state of the cell. Typical mechanisms of antioxidant action include a) agents that catalytically remove ROS such as superoxide dismutase, catalase and peroxidase enzymes, b) agents that decrease ROS formation including proteins that decrease the availability of pro-oxidants such as transition metal ions, c) proteins that protect biomolecules against oxidative damage by other mechanisms and d) the physical scavenging of ROS that neutralises the radical (Halliwell and Gutteridge, 2007).

Glutathione (GSH) is the most significant low molecular weight antioxidant synthesised in cells and exists primarily in the thiol-reduced form (Meister, 1988). GSH plays a crucial role in a multitude of cellular processes such as cell differentiation, proliferation and apoptosis and so disturbances in GSH homeostasis are implicated in the progression of many human diseases including atherosclerosis (Ballatori *et al.*, 2009). The primary function of GSH lies in reducing oxidative stress and maintaining the thiol-redox status of the cell (Forman *et al.*, 2009). GSH exerts its antioxidant properties by reacting with a wide range of oxidants including OH^\bullet , HOCl , ONOO^- , RO^\bullet , RO_2^\bullet , $\text{CO}_3^{\bullet-}$, NO_2^\bullet and O_2 (Halliwell and Gutteridge, 2007). GSH deficiency or a decrease in the GSH/glutathione disulfide ratio appears to result in increased susceptibility to oxidative stress or low cellular antioxidant capacity (Ballatori *et al.*, 2009).

Another molecule with antioxidant capacity that is relevant to this research is 7,8-dihydroneopterin (7,8-NP). 7,8-NP has been shown to exert an antioxidant effect by inhibiting oxidative damage to a variety of substrates including U937 cells (Giesege *et al.*, 2001). Specifically, 7,8-NP prevents oxLDL-induced intracellular GSH loss in U937 cells by scavenging oxLDL-induced intracellular oxidants (Baird *et al.*, 2004). The mechanism of inhibition of metal ion- and peroxy radical-mediated LDL oxidation appear to involve scavenging of the lipid peroxy radical, even though 7,8-NP is a water soluble antioxidant.

1.4 Oxysterols

Oxysterols are the 27-carbon products of cholesterol oxidation and have been widely implicated in the development of atherosclerosis. It is well established that the formation of oxLDL and the resulting cytotoxicity to macrophage cells is a key driver in the development of the necrotic core of the atherosclerotic lesion (Giesege *et al.*, 2009a). During the copper-mediated oxidation of LDL *in vitro*, it has been shown that up to 50% of the cholesterol is converted to oxysterols (Brown *et al.*, 1996). The high oxysterol content of oxLDL has been cited by numerous research groups as a major source of oxLDL cytotoxicity (Brown and Jessup, 2009, Clare *et al.*, 1995, Colles *et al.*, 2001, Hessler *et al.*, 1983, Hughes *et al.*, 1994, Larsson *et al.*, 2006, Parthasarathy *et al.*, 1999). However, contrary to this predominating opinion in the literature, recent work by Steffen *et al.* (2006) suggests that 7-ketocholesterol (7KC) may even serve as a defence mechanism against the progression of atherosclerosis by protecting the endothelium against 7 β -hydroxycholesterol-induced damage. Whether oxysterols are pro-atherogenic, anti-atherogenic or benign *in vivo* remains unresolved.

1.4.1 Sources of oxysterols

Generally, oxysterols in the circulation are short-lived and present at very low (nanomolar) concentrations due to their rapid metabolism. However, elevated levels of various oxysterols have been detected in atherogenic lipoproteins and atherosclerotic plaque. There are three potential sources of oxysterols in human tissue and fluids; dietary sources and *in vivo* formation via either enzymic processes or non-enzymic oxidation (Brown and Jessup, 1999).

The most common oxysterols detected in food are the major products of cholesterol autooxidation; 7-ketocholesterol, the 7-hydroxycholesterols (7 β OH and 7 α OH) and the 5,6- α/β -cholesterol epoxides (Van Reyk *et al.*, 2006). The possibility exists that such oxysterols could be absorbed and transported to tissues and cells, resulting in an accumulation. But recent research has demonstrated little to no accumulation of oxysterols in host tissues injected with exogenous oxysterols. Whole animal rat models were used to trace the metabolism of radio-labelled 7-ketocholesterol esters relative to cholesterol, showing that unlike cholesterol, 7-ketocholesterol was rapidly metabolised and excreted by the liver (Lyons *et al.*, 1999). These results suggest that dietary oxysterols may not be a major source of oxysterols in atherosclerotic plaques.

$^{18}\text{O}_2$ enrichment of oxysterols in rats exposed to $^{18}\text{O}_2$ by inhalation has provided evidence for the *in vivo* formation of 7 α -hydroxycholesterol (7 α OH), 7 β -hydroxycholesterol (7 β OH), 7-ketocholesterol, 24-hydroxycholesterol (24OH), 25-hydroxycholesterol (25OH) and 27-hydroxycholesterol (27OH) in plasma, but does not distinguish between an enzymic versus non-enzymic origin (Breuer and Bjorkhem, 1995). Oxysterols may be produced non-enzymically by direct radical attack on cholesterol, yielding varying proportions of oxysterols as determined by the attacking radical. In lipoproteins, peroxy radicals derived from PUFA oxidation are likely to be a major source of such radicals (Van Reyk and Jessup, 1999). Evidence supporting non-enzymic oxidative oxysterol generation has come from various studies that examine the reduction of oxysterol levels in plasma or tissue in response to antioxidant supplementation (Brown and Jessup, 1999).

Some of the most abundant oxysterols found *in vivo* are the enzymic products of cholesterol break-down. For example, sterol 27-hydroxylase is a mitochondrial P450 enzyme responsible for the hydroxylation of 27OH, which is one of the most abundant oxysterols in human atherosclerotic lesions and macrophage-derived foam cells (Brown and Jessup, 1999). The enzymic routes of oxysterol generation are largely involved in the disposal of cholesterol and involve the formation of more easily excreted, water soluble bile acids and occur mainly in the liver (Jessup and Brown, 2005).

1.4.2 Oxysterols and atherogenesis

Oxysterols have been tested in numerous *in vitro* studies for their effects on cellular metabolism. Although oxysterols are very stable and do not generally react to form oxidants and free radicals, their uptake directly into cells is known to result in cell death. It is important to note that the majority of the studies cited have employed unphysiologically high concentrations and modes of delivery of oxysterols. The majority of studies have used oxysterols dissolved in ethanol or dimethyl sulfoxide (DMSO) which is subsequently added directly to cultured cells at relatively high concentrations. In most cases, the cellular oxysterol levels achieved have not been measured or compared with the physiological level found *in vivo* (Brown and Jessup, 1999). However, the application of atheroma-relevant proportions of oxysterols to U937 cells revealed a synergistic effect of oxysterol cytotoxicity and confirmed previous observations of toxicity (Larsson *et al.*, 2006). In addition, studies have shown that the concentration of oxysterol required to achieve detectable cytotoxicity is higher in the presence of serum or lipoproteins than in serum-free media (Clare *et al.*, 1995). Such findings raise questions about the relevance of studies that involve direct application of single oxysterol solutions to cell culture, especially when it has been demonstrated that oxysterols are predominantly lipoprotein-associated in plasma, *in vivo* (Addis *et al.*, 1989, Javitt *et al.*, 1981, Lin and Morel, 1996, Peng *et al.*, 1982).

When considering the biological relevance of *in vitro* studies that explore the cellular effects of oxysterols, there are several factors to assess. These are a) the conditions under which the oxysterols are supplied (in terms of appropriate concentrations of oxysterols and in the presence of an excess of cholesterol) b) the means of delivery of oxysterol and c) the cellular oxysterol content achieved and its relevance to *in vivo* levels (Van Reyk *et al.*, 2006). Since oxysterols are contained within lipoproteins in plasma *in vivo* (Addis *et al.*, 1989, Lin and Morel, 1996), the following research will involve the selective incorporation of the oxysterol of interest into lipoprotein before examining its effect on cell cultures. However, the possibility remains that oxysterols may dissociate from their associated lipoprotein within the unique environment of the atherosclerotic plaque and so this section will provide a brief review of the current literature concerning the potential role of oxysterols in atherogenesis.

For cells *in vivo*, the usually low ratio of oxysterols relative to cholesterol means that oxysterols have little impact on membrane structure and function. However, in some experimental conditions, where the proportion of oxysterol is much greater, the effects of oxysterols on membrane structure can become significant (Brown and Jessup, 2009) *and is discussed in the following section*. At present there are very few reports on *in vivo* cellular levels of oxysterols, but based on estimates, oxysterols constitute only a small amount of the total sterol in most tissues. For example, levels of the three major oxysterols in normal plasma are at sub-micromolar concentrations (Van Reyk *et al.*, 2006). In subjects with arterial disease, the levels of plasma oxysterols were significantly higher but were 0.01% or less than that for the native sterol (Prunet *et al.*, 2006). A study of oxidative stress status in healthy and atherosclerotic artery reported an average plaque 7-ketocholesterol level in healthy subjects of 334 ± 200 nmol/mmol cholesterol compared to 950 ± 180 nmol 7KC/mmol cholesterol in atherosclerotic plaque (Iuliano *et al.*, 2003). Analysis of plaque material combined with time-course investigations again demonstrated elevated levels of oxysterol in atherosclerotic plaque and suggested that hydroperoxycholesterol is the principal oxysterol early in atherosclerosis, while 7-ketocholesterol and 7-hydroxycholesterol are the major later stage products (Brown *et al.*, 1997, Jessup and Kritharides, 2000).

An exploration of the lipid composition of copper-oxidised LDL and of a macrophage foam-cell model generated by the uptake of oxLDL demonstrated that when LDL undergoes oxidation *in vitro* a number of changes in lipid composition occur, including the substantial loss of free and esterified cholesterol and the generation of oxysterols (Brown *et al.*, 1996). Consistent with previous studies, it was reported that 7-ketocholesterol is the major oxysterol present in copper-oxidised LDL (Brown *et al.*, 1996).

1.4.3 7-Ketocholesterol

7-Ketocholesterol (5-cholesten-3 β -ol-7-one) differs from cholesterol by a single ketone group present at the 7- position. Free radical attack of cholesterol produces 7-hydroperoxycholesterols, which decompose creating 7-hydroxycholesterol and 7-ketocholesterol (Figure 1.1).

A possible mechanism for the formation of 7-oxygenated sterols has been proposed by Brown *et al.*, (1997). Fatty acid radicals are thought to abstract a hydrogen atom from the susceptible 7-allylic position of cholesterol, then reaction with molecular oxygen and subsequent hydrogen atom abstraction leads to the formation of 7-hydroperoxycholesterol (7OOH), the precursor of non-enzymically formed 7-ketocholesterol. The decomposition of 7OOH to 7KC and 7OH is evident in kinetic studies of *in vitro* systems, suggesting that this is a likely mechanism for 7-oxygenated sterol formation in atherosclerotic plaque (Brown *et al.*, 1997).

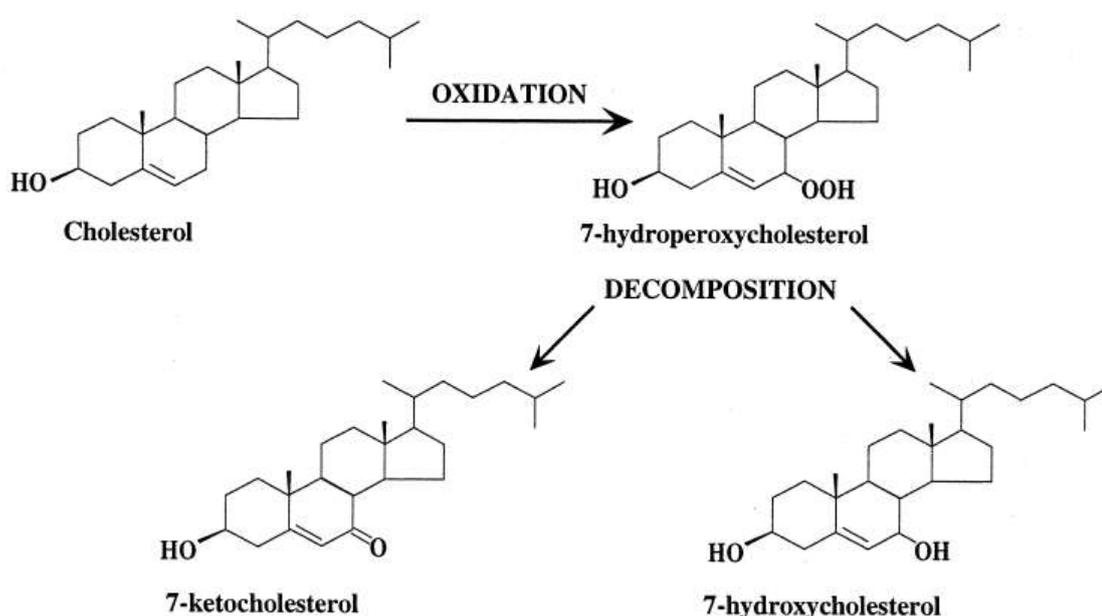


Figure 1.1 The *in vivo* synthesis of 7-ketocholesterol.

The major products of free radical attack of cholesterol are the 7-hydroperoxycholesterols. These molecules are labile and are rapidly converted to the 7-hydroxycholesterols and 7-ketocholesterol. Figure adapted from Lyons and Brown (1999).

The non-enzymic precursor of 7-ketocholesterol, 7-hydroperoxycholesterol, has been detected in human atherosclerotic plaque (Brown *et al.*, 1997). The current thinking is that the bulk of 7-ketocholesterol in atherosclerotic lesions is formed non-enzymically by the above mechanism. Therefore, the uptake of oxLDL by macrophage cells is likely the primary route of entry of 7-ketocholesterol into atherosclerotic lesions (Lyons *et al.*, 1999). Furthermore, it is generally accepted that oxysterols, including 7-ketocholesterol, are primarily lipoprotein-associated (Addis *et al.*, 1989). Incubation of plasma with 7-

ketocholesterol results in a similar distribution to cholesterol; with less than 10% found in the lipoprotein-free serum fraction (Gelissen *et al.*, 1996). 7-ketocholesterol esters were also detected in the above study, comprising up to 80% of the total 7-ketocholesterol detected (Brown *et al.*, 1997). 7-Ketocholesterol is thought to become esterified by the action of acyl coenzyme A: cholesterol acyltransferase (ACAT) within the cell, lecithin: cholesterol acyltransferase (LCAT) in plasma or by the oxidation of the sterol moiety of cholesteryl esters (Lyons *et al.*, 1999).

7KC-induced cellular death

7-Ketocholesterol, when given directly to cell cultures, is cytotoxic and has been shown to induce apoptosis in cultured SMCs (Nishio *et al.*, 1996), vascular endothelial cells (Lizard *et al.*, 1997), lymphocytes (Christ *et al.*, 1993) and macrophages (Clare *et al.*, 1995). Mechanisms of 7-ketocholesterol (and general oxysterol) toxicity are not entirely clear, although suggestions include reduced cholesterol availability through inhibition of HMGCoA reductase, oxysterol replacement of cholesterol in membranes causing perturbations to vital properties, stimulation of apoptosis (Brown and Jessup, 1999), inhibition of DNA and cholesterol synthesis, immune response modulation (Clare *et al.*, 1995), inhibition of calmodulin (a calcium-binding protein involved in numerous cellular processes) (Tipton *et al.*, 1987) and inhibition of nitric oxide release in human vascular endothelial cells (Lyons *et al.*, 1999).

The effects of oxysterols on cell membrane properties have been well studied. Oxysterols such as 7-ketocholesterol may be important regulators of the biophysical properties of plasma membrane microdomains called lipid rafts (Massey and Pownall, 2005). 7-Ketocholesterol is less effective than cholesterol in forming phospholipid-sterol complexes that support raft formation and dynamics, and 7-ketocholesterol also affects the number or properties of phase boundaries that determine membrane-protein interactions (Massey and Pownall, 2005). In monocytes, such disruption of lipid rafts by 7-ketocholesterol induces apoptosis by the mitochondrial death pathway which involves a sustained increase in cytosolic free calcium ions and the corresponding calcium-dependent activation of pro-apoptotic pathways (Berthier *et al.*, 2005). The role of calcium ion influx in 7-ketocholesterol-induced apoptosis was further confirmed by Sasaki *et al.*, (2007) whose work showed that treatment with the calcium channel blocker, nifedipine, prevented

apoptosis in vascular SMCs. Within THP-1 cells, the calcium ions that drive this apoptotic pathway enter cells via the store operated Ca^{2+} entry channel (SOC). Accumulation of 7-ketocholesterol within lipid raft domains causes translocation of the transient receptor potential calcium channel-1 (Trpc-1) protein to lipid rafts to form SOCs that permit calcium ion influx into the cytosol (Berthier *et al.*, 2004). It is possible that many of the initial oxysterol-mediated effects are mediated by lipid raft-associated receptors and channels within the plasma membrane (Giesege *et al.*, 2009a).

Another possible mechanism for 7-ketocholesterol-induced cellular death is via intracellular ROS overproduction. Recent work that delivered physiological concentrations of 7-ketocholesterol to murine J774.1A cells demonstrated the involvement of nicotinamide adenine dinucleotide- (NADPH) oxidase and intracellular ROS overproduction in the observed cell death (Leonarduzzi *et al.*, 2006). The near total protection provided to cells by two selective-inhibitors of NADPH-oxidase indicates the ability of 7-ketocholesterol to induce an oxidative burst by activating this enzyme (Leonarduzzi *et al.*, 2006).

7KC-induced foam cell formation

A widely investigated mechanism by which 7-ketocholesterol is thought to contribute to atherogenesis is via the disruption of reverse sterol transport. Reverse sterol transport is the process by which excess cholesterol is returned to the liver for catabolism and excretion via HDL, thereby preventing an accumulation of excess cholesterol within the cell. Cholesterol homeostasis requires a delicate balance between its uptake and endogenous synthesis compared to its metabolism and export.

Acyl coenzyme A: cholesterol acyltransferase (ACAT) is an important enzyme in the regulation of cholesterol metabolism since it is responsible for the formation of cholesteryl esters from cholesterol and long-chain fatty acids, in turn regulating the free cholesterol content of cell membranes to within the necessary narrow limits. A number of studies have explored the potential regulation of ACAT by oxysterols, including 7-ketocholesterol. In cell extracts, very high concentrations of 7-ketocholesterol caused a slight stimulation of ACAT (Cheng *et al.*, 1995) and in another study, 7-ketocholesterol stimulated basal cholesterol esterification in mouse macrophages, while acLDL-induced accelerated

cholesteryl esterification was further stimulated by 7-ketocholesterol (Zhang *et al.*, 1990). However, it was subsequently found that 7-ketocholesterol-enriched acLDL did not have any effect on macrophage cholesterol esterification (Gelissen *et al.*, 1996). This study investigated whether the impairment of normal cholesterol efflux from oxLDL-loaded macrophages could be attributed to the presence of oxysterols in the cells. For this purpose, 7-ketocholesterol was selectively incorporated into native LDL, which was then acetylated to produce a high uptake form (7KC-acLDL). Cholesterol efflux from macrophages loaded with 7KC-acLDL (using ApoA-I as a sterol acceptor) was significantly impaired compared to cells loaded with oxysterol-free acetylated LDL (Gelissen *et al.*, 1996). It is plausible that the direct influence of 7-ketocholesterol on the plasma membrane may account for its observed effects on cholesterol homeostasis, since in model membrane systems 7-ketocholesterol has been shown to have a membrane-condensing effect caused by altered cholesterol packing within the bilayer. Alternatively it may be caused by impaired phospholipid desorption (Brown and Jessup, 1999).

7-Ketocholesterol has also been implicated in the transformation of monocytes to macrophages within the artery wall. THP-1 monocyte-like cells, when treated with 7-ketocholesterol over 7 days, demonstrate enhanced adherence, morphological changes and expression of cellular markers that are characteristic of mature macrophages (Hayden *et al.*, 2002). Importantly, incubation with 7-ketocholesterol-loaded LDL also promoted THP-1 differentiation into mature macrophages. The aforementioned studies that have explored the effect of oxysterol-loaded LDL indicate that 7-ketocholesterol may play an important role in the formation of lipid-laden foam cells within atherosclerotic plaque, but do not implicate the oxysterol in oxLDL-induced cell death.

1.5 Objectives of research

Numerous studies have examined the toxicity of oxysterols when added directly to cell cultures (Brown and Jessup, 1999, Lizard *et al.*, 1998). This observed toxicity has been correlated with the high oxysterol content of oxLDL and therefore oxysterols have been cited as a major source of toxicity caused by oxLDL in the mechanism of atherosclerotic plaque progression, from fatty streak to advanced plaque.

Since oxysterols are principally transported within lipoprotein *in vivo* (Addis *et al.*, 1989, Lin and Morel, 1996), the incorporation of oxysterols into high-uptake lipoprotein provides a more physiologically relevant model for examining the effect of the oxysterol of interest. A previous study has therefore used the approach of generating foam cells using LDL selectively enriched with a single oxysterol (7KC-acLDL) before examining its effect on sterol trafficking in mouse macrophages. It was shown that cholesterol efflux, using apoA-1 as a sterol acceptor, from mouse macrophages loaded with 7KC-acLDL was impaired, suggesting a role for 7-ketocholesterol in foam cell formation (Gelissen *et al.*, 1996). Using this model of 7-ketocholesterol-enriched acetylated LDL, the following research will examine the effect of 7-ketocholesterol on cell death in human monocyte-like U937 cells. 7-Ketocholesterol is the major oxysterol of oxLDL, oxLDL-loaded cells and of advanced atherosclerotic plaque (Brown *et al.*, 1996).

To test the hypothesis that 7-ketocholesterol is not the toxic component of oxLDL in the mechanism of advanced plaque development, this research will examine the uptake and toxicity of both 7-ketocholesterol-loaded acetylated LDL and 7-ketocholesterol-loaded oxidised LDL to the U937 human monocyte cell line.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents

All reagents used were of analytical grade or better. All solutions were prepared using ion-exchanged ultra filtered water, which was produced using a NANOpure ultrapure water system from Barnstead/Thermolyne (IA/USA).

1,1,3,3-Tetramethoxypropane (TMP)	Sigma Chemical Co., Missouri, USA
2-Thiobarbituric acid (TBA)	Sigma-Aldrich Chemical Co., Steinheim, Germany
3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT)	Sigma Chemical Co., Missouri, USA
4-Morpholine-propanesulfonic acid (MOPS)	Sigma Chemical Co., Missouri, USA
7,8-Dihydroneopterin (7,8-NP)	Schirck Laboratories, Switzerland
7-Ketocholesterol (7KC)	Sigma-Aldrich Chemical Co., Missouri, USA
Acetic acid (glacial)	J. T. Baker, USA
Acetic anhydride	Fluka, Sigma-Aldrich Chemical Co., Steinheim, Germany
Acetonitrile (ACN)	Merck, Darmstadt, Germany
Argon gas	BOC Gases, Auckland, New Zealand
Bicinchoninic acid (BCA) protein determination kit	Pierce, Illinois, USA
Bovine serum albumin (BSA)	Gibco Invitrogen Corporation, Auckland, New Zealand
Butylated hydroxytoluene (BHT)	Sigma Chemical Co., Missouri, USA
Chelex 100 resin	Bio-Rad Laboratories, California, USA
Copper chloride (CuCl ₂)	Sigma Chemical Co., Missouri, USA
Diethyl ether	Merck, Darmstadt, Germany
Dimethyl sulphoxide (DMSO)	AnalaR, Poole, England
Ethanol	Merck, Darmstadt, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma Chemical Co., Missouri, USA
Glutathione (reduced form)	Sigma Chemical Co., Missouri, USA
Hexane	Mallinckrodt Chemicals, New Jersey, USA
Hydrochloric acid, fuming 37 % (HCl)	Merck, Darmstadt, Germany
Isopropanol	Mallinckrodt Chemicals, New Jersey, USA
Lipoprotein Electrophoresis Kit	Beckman Coulter, USA

Methanol	Merck, Darmstadt, Germany
Monobromobimane (MBB)	Fluka Analytical, Switzerland
Nitrogen gas	BOC Gases, Auckland, New Zealand
Oil Red-O (ORO)	Sigma Chemical Co., Missouri, USA
Paraformaldehyde	Sigma Chemical Co., Missouri, USA
Phosphoric acid (H ₃ PO ₄)	Merck, Darmstadt, Germany
Potassium bromide (KBr)	Merck, Darmstadt, Germany
Potassium hydroxide (KOH)	Merck, Darmstadt, Germany
Sodium acetate (C ₂ H ₃ NaO ₂)	Merck, Darmstadt, Germany
Sodium chloride (NaCl)	Merck, Darmstadt, Germany
Sodium dihydrogen phosphate monohydrate (NaH ₂ PO ₄ ·H ₂ O)	Scharlau Chemie, Italy
Sodium dodecyl sulphate (SDS)	Sigma Chemical Co., Missouri, USA
Sodium hydrogen carbonate (NaHCO ₃)	Merck, Darmstadt, Germany
Sodium hydroxide (NaOH)	Scharlau Chemie, Italy
Sodium hypochlorite (NaOCl)	Jasol Dynawhite, Christchurch, New Zealand
Trichloroacetic acid (TCA)	Merck, Darmstadt, Germany
Trypan blue solution (0.4%)	Sigma Chemical Co., Missouri, USA

2.1.2 Media

Foetal bovine serum (FBS)	Invitrogen, California, USA
Penicillin/Streptomycin (10000 units/ml penicillin G and 10000 µg/ml streptomycin)	Gibco Invitrogen, Auckland, New Zealand
Roswell Park Memorial Institute (RPMI) -1640 media, with phenol red	Sigma-Aldrich Chemical Co., Missouri, USA
Roswell Park Memorial Institute (RPMI) -1640 media, without phenol red	Sigma-Aldrich Chemical Co., Missouri, USA

2.1.3 General solutions, media and buffers

2.1.3.1 Phosphate buffered saline (PBS)

PBS (150mM sodium chloride and 10mM sodium dihydrogen orthophosphate, pH 7.4) was stirred with 1g of washed Chelex-100 resin for at least 2 hours to remove transition metal ions present in the buffer, followed by vacuum filtration using a 0.45 µm Phenex filter membrane (Phenomenex).

2.1.3.2 Roswell Park Memorial Institute (RPMI)-1640 media (with and without phenol red)

Media was prepared as per manufacturer's instructions. Powdered RPMI (with or without phenol red) was dissolved in nanopure water, followed by addition of sodium bicarbonate and pH adjustment to 7.4 using 1 M HCl or 1 M NaOH. Media was filter-sterilised using a peristaltic pump (CP-600, Life Technologies) and a 0.20 µm Sartolab[®]-P20 filter (Sartorius AG, Goettingen, Germany) into sterile bottles. Media was stored at 4°C and warmed to 37°C in a water-bath before use.

2.1.3.3 7,8-Dihydroneopterin (7,8-NP) solution

A 2 mM stock of 7,8-NP (MW 255.2 g/mol) was prepared fresh, immediately before each experiment. 7,8-NP was dissolved in degassed ice cold RPMI-1640 medium during a 5 minute sonication. 7,8-NP solution was then filter-sterilised using a 0.22 µm MS[®] PES syringe filter (Membrane Solutions, USA) and diluted to working concentrations in warm RPMI-1640 media.

2.1.3.4 7-Ketocholesterol (7KC) solution

A 100 µM stock of 7KC (5-cholesten-3β-ol-7-one, MW 400.67 g/mol) was prepared fresh, immediately before each experiment. 7KC was dissolved in absolute ethanol during a 10 minute sonication, before dilution to working concentrations in warm RPMI-1640 media and addition to culture wells, so that the final concentration of ethanol in the culture medium was below 0.5% (v/v).

2.2 Methods

2.2.1 Cell culture

All cell experiments were carried out under aseptic conditions in a class II biological safety cabinet (Clyde-Apex BH 200). All instruments and equipment used were either sterile plasticware (Falcon, Nunc, Terumo, Unomedical and Greiner Bio-one) or had been sterilised by autoclave (15 minutes, 121°C, 15 psi). All equipment and tissue culture items were sprayed thoroughly with 70% (v/v) ethanol before being transferred to the class II biological safety cabinet.

Cells were maintained in an incubator at 37°C in a humidified atmosphere containing 5% CO₂ (Sanyo CO₂ Incubator). Viable cells were counted using a haemocytometer and a light microscope after staining with trypan blue at a ratio of 1:1.

2.2.1.1 Cell culture media

RPMI-1640 medium (with phenol red) was supplemented with approximately 100 units/ml penicillin G and 100 µg/ml streptomycin. For simplicity, this solution is referred to as RPMI-1640 medium throughout this thesis. The RPMI-1640 medium was then combined with heat-inactivated foetal bovine serum to a final concentration of 5% (v/v) during normal maintenance and in some experiments, where indicated.

2.2.1.2 Preparation of U937 cell line

The U937 cell line was originally developed from the pleural fluid of a 37-year old man with generalised lymphoma (Sundstrom and Nilsson, 1976). Our U937 cells were a gift from the Haematology Research Laboratory at the Christchurch School of Medicine, University of Otago. A 1 ml vial containing 20×10^6 cells/ml was removed from liquid nitrogen storage and defrosted in a 37°C water-bath until almost completely thawed. The concentrated cell suspension was poured into 30 ml of RPMI-1640 medium in a 50 ml centrifuge tube and centrifuged at 500 g for 5 minutes to separate the DMSO freezing medium and cells. The resulting cell pellet was re-suspended in 10 ml of RPMI-1640 medium in a 25 cm² tissue culture flask. Cell density was maintained at $0.3-1.5 \times 10^6$ cells/ml by passaging in Cellstar® 75 cm² tissue culture flasks (Greiner Bio-one) every 2-3 days.

2.2.1.3 Cell experiment procedures

Cell experiments were generally performed using Cellstar® 12-well suspension culture plates (Greiner Bio-one) which were coated with 8 µl of 5% bovine serum albumin (BSA) per well, to prevent cells adhering to the plastic in the absence of HI-FBS. Five percent BSA solution was made up in RPMI-1640 (without phenol red) immediately before each experiment. Viable cells were counted using a haemocytometer and a light microscope after staining with trypan blue at a ratio of 1:1. The required quantity of cells was pelleted by centrifugation at 500 g for 5 minutes at room temperature and re-suspended in RPMI-1640 (with or without phenol red) at 37 °C, to a concentration of 1×10^6 cells/ml. The cell

suspension was then aliquoted into wells containing RPMI-1640 (with or without phenol red) and any reagents specific to the experiment, to give a final concentration of 5×10^5 cells/ml.

2.2.2 Blood collection and plasma preparation

Written consent was first obtained from all blood donors. Human blood was collected from healthy volunteers following an overnight fast (ethics approval from Upper South A Ethics Committee, CTY/01/04/036). Blood was drawn by venipuncture using a 15G needle, line and 50 ml syringe (Terumo, USA), which was then transferred directly to 50 ml centrifuge tubes (Greiner Bio-one) containing 0.5 ml of 100 mg/ml EDTA, pH 7.4.

Whole blood was centrifuged at 4100 g for 20 minutes at 4°C to separate red blood cells and plasma. Plasma was transferred to SS34 rotor centrifuge tubes and centrifuged at 11,000 g for 30 minutes at 4°C, with slow acceleration/deceleration, to remove remaining cellular debris. Plasma from individual donors (5-7 donors) was then pooled, to minimise inter-individual variation, before storage at -80°C in 32 ml aliquots. Plasma was stored for no longer than 6 months before use.

2.2.3 LDL preparation

2.2.3.1 Extraction of LDL from plasma

This method of LDL isolation utilises a Beckman Near Vertical Rotor and employs the method of Chung *et al.* (1980) with modifications described by Giesege and Esterbauer, (1994) which was directly adapted from Dr. Wendy Jessup (Heart Research Institute Ltd, Sydney) for LDL preparation in a vertical rotor. The protocol requires a single gradient that redistributes during ultracentrifugation to form a density gradient, which separates lipoproteins.

A 32 ml tube of frozen human plasma was defrosted under cold running water and centrifuged at 4700 rpm for 10 minutes at 4°C to pellet precipitated fibrinogen. The supernatant was decanted into a beaker and placed immediately on ice. Potassium bromide (KBr) was gradually dissolved in plasma, to a final concentration of 382 mg KBr/ml plasma, altering the plasma density to 1.24 g/ml. Plasma was stirred gently to prevent foam

formation, which is an indication of LDL denaturation. Plasma was maintained on ice and under argon gas until ultracentrifugation.

Eight millilitres of 1 mg/ml EDTA (pH 7.4) was added to each of 8 OptiSeal™ polyallomer centrifuge tubes (Beckman Coulter, USA) before under-layering with 4 ml of KBr-plasma, using a needle attached to a 5 ml syringe. Ultracentrifuge tubes were transferred to the NVTi-65 rotor and centrifuged at 60,000 rpm for 2 hours at 10°C with slow acceleration/deceleration using an Optima™ L-90K Preparative Ultracentrifuge (Beckman Coulter, Inc., Fullerton, California). Following centrifugation, a yellow/orange coloured band of LDL in the density range of 1.019 – 1.063 g/ml was collected using a 20 ml syringe attached to a 90°-bend needle (Figure 2.1).

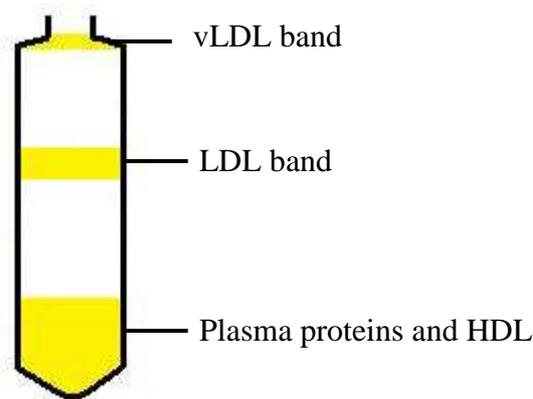


Figure 2.1 Location of lipoprotein fractions following ultracentrifugation

2.2.3.2 LDL cholesterol content determination

The cholesterol content of LDL was determined by enzymatic cholesterol assay using a CHOL kit (Roche Diagnostic). Ten microlitres of LDL was incubated with 1 ml of cholesterol reagent at room temperature for 10 minutes. The absorbance was read at 500 nm against a blank containing only cholesterol reagent.

LDL concentration was then calculated from the absorbance, based on the estimate that cholesterol accounts for 31.69% of the entire LDL particle, by weight, and that LDL has a molecular weight of 2.5 kDa (Giese and Esterbauer, 1994).

Calculation: $\text{absorbance} \times 14.9 = [\text{cholesterol}] \text{ (nM)}$
 $[\text{cholesterol}] \text{ (M)} \times 386.64 \text{ g/mol} = [\text{cholesterol}] \text{ (g/l)}$
 $[\text{cholesterol}] \text{ (M)} \times 100/31.69 = \text{g/l LDL or [LDL] (mg/ml)}$

2.2.3.3 LDL washing and concentration

The LDL was adjusted to a final concentration of 10 mg/ml (total mass) before any subsequent manipulations or use in experiments. LDL was concentrated using Amicon® Ultra-15 filter tubes (Millipore, USA). LDL was transferred to two filter tubes. Each was made up to 15 ml with chelex-treated PBS and centrifuged at 3000 g for 30 minutes at 10°C. This step was then repeated twice. The duration of the third centrifugation was adjusted according to the desired final volume/concentration of LDL, as determined by cholesterol assay (above). If no further manipulations were to be made to the LDL, it was filter-sterilised using a 0.22 µm syringe filter and stored at 4°C in the dark, under argon gas.

2.2.4 LDL oxidation

LDL at 10 mg/ml (total mass) was transferred to 12-14 kDa dialysis tubing (Medicell International, UK) secured at one end with a double knot and a weighted closure at the other. Fifty millimolar copper chloride (CuCl₂) solution was combined with the LDL inside the dialysis tubing to give a final concentration of 0.5 mM CuCl₂. The LDL-containing dialysis tubing was placed in a large bottle containing 1 L PBS/50 mg LDL, plus CuCl₂ at a final concentration of 0.5 mM CuCl₂. LDL was dialysed against 0.5 mM CuCl₂ in PBS for 24 hours at 37°C in a heated shaker.

The dialysis bag containing oxLDL was then transferred to a fresh bottle containing 1L of PBS and stirred with 1 g of washed chelex-100 at 4°C for two hours. This was repeated twice, with the final incubation taking place overnight. OxLDL was filter-sterilised using a 0.22 µm syringe filter and stored at 4°C.

2.2.5 Incorporation of 7KC into LDL

A centrifuge tube containing 32 ml of frozen human plasma was defrosted under cold running water and centrifuged at 4700 rpm for 10 minutes at 4°C to pellet precipitated fibrinogen. The supernatant was decanted into a glass bottle. Thirty milligrams of 7KC

(MW 400.65 g/mol) was dissolved in ethanol, with sonication, to a concentration of 75 mM. Dissolved 7KC was then added to human plasma to give a final concentration of approximately 1.0 mg 7KC/ml plasma and incubated at 37°C in the dark, with gentle shaking, under argon gas for 6 hours before being stored at 4°C overnight. 7KC-loaded LDL was then isolated from plasma by density gradient ultracentrifugation, via the method described above.

2.2.6 LDL acetylation

LDL or 7KC-LDL at 10 mg/ml (total mass) was gently stirred with an equal volume of saturated sodium acetate solution (added gradually) at 4°C. Two microlitres of acetic anhydride was added to the lipoprotein at 8-10 minute intervals to a final concentration of 6 µl acetic anhydride per mg LDL protein. The acetylation was performed at 4°C, under argon gas when possible and stirring was kept to a minimum to prevent precipitation of LDL. Excess reagents were removed from acetylated LDL using Amicon® Ultra-15 filter tubes. Acetylated LDL was transferred to two filter tubes. Each was made up to 15 ml with PBS and centrifuged at 3000 g for 30 minutes at 10°C. This step was then repeated twice. The duration of the third centrifugation was adjusted according to the desired final volume/concentration of LDL, as determined by cholesterol assay (described above). Acetylated LDL was filter-sterilised using a 0.22 µm syringe filter and stored at 4°C in the dark, under argon gas.

2.2.7 Lipoprotein gel electrophoresis

Relative electrophoretic mobility was determined using a Beckman Paragon® native gel electrophoresis kit and apparatus. Lipoprotein gel electrophoresis solutions were prepared as per manufacturer's instructions. B-2 barbital buffer (pH 8.6) was made by dissolving 3.64 g of buffer powder in 300 ml of nanopure water. The lipoprotein working stain was made by combining 3 ml of stain concentrate with 165 ml of ethanol and 135 ml of nanopure water. The fixative solution was made by combining 225 ml ethanol, 45 ml nanopure water and 15 ml glacial acetic acid. The destain solution was made by mixing 450 ml of ethanol with 550 ml of nanopure water. All solutions were kept at room temperature.

Three 2 μ l applications of LDL (native or modified) were applied to each lane, allowing 5 minutes between applications for absorption. The gel was electrophoresed for 35 minutes at 100 volts in B-2 barbital buffer before being placed in fixative solution for 5 minutes and dried in a cool oven overnight. Once dry, the gel was stained for 5 minutes in lipoprotein working stain and destained by dipping in destain solution followed by immersion for 5 minutes. It was then rinsed in nanopure water and dried overnight (or until dry) in a cool oven before evaluating the gel visually and photographing.

2.2.8 The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay

The MTT reduction assay is a widely used method for measuring cell viability. This laboratory has previously shown that results produced by this method agree well with results obtained by the trypan blue exclusion assay (Baird, 2003). The yellow MTT compound is reduced by metabolically active cells, via the action of NADPH dehydrogenase enzymes, to give an insoluble purple formazan product. The purple product can be solubilised and quantified spectrophotometrically so that colour intensity provides a measure of both the concentration of cells and their metabolic activity (Mosmann, 1983).

MTT powder was dissolved in RPMI-1640 medium (without phenol red) to a concentration of 5 mg/ml and filter-sterilised using a 0.22 μ m MS[®] PES syringe filter (Membrane Solutions, USA). MTT solution was stored at -20°C in the dark until use. A 0.01 M hydrochloric acid (HCl) solution was prepared from 11.44 M HCl and nanopure water and stirred slowly with sodium dodecyl sulphate (SDS) to give a final concentration of 10% (^w/_v) SDS.

Following experimental treatment, U937 cells from each well were washed with warm PBS and then incubated with 1 ml of RPMI-1640 medium (without phenol red) containing 0.5 mg/ml MTT for 2 hours. The purple formazan product was then dissolved by the addition of 1 ml of 10% (^w/_v) SDS in 0.01 M HCl to each well with thorough mixing. Absorbance was read at 570 nm, against a blank that contained all reagents but was without cells.

2.2.9 Oil red-O staining

Oil Red-O is a dye used for staining neutral triglycerides and lipids and the method used was adapted from Davies *et al.* (2005). Two grams of paraformaldehyde were added to 40 ml of PBS and heated on a heating block, at a temperature below 60°C. Several drops of 10 M NaOH were added gradually to help the paraformaldehyde to dissolve. Once the solution had cooled to room temperature it was made up to 50 ml with nanopure water, followed by pH adjustment to 7.4 using 11.44 M HCl. The solution was kept at 4°C for 2-3 weeks.

Following experimental treatment, cells were washed twice with warm PBS and then fixed in 4% paraformaldehyde for 20 minutes at room temperature. Paraformaldehyde having been removed, cells were washed with water and then stained by incubation with 0.5 ml of 0.05% (^w/_v) oil red-O in isopropanol:water (3:2) for 1 hour at room temperature. Cells were washed twice in water and viewed *in situ* using an inverted microscope. The images were taken using a Leica DFC290 C-Mount camera (Leica Microsystems Ltd., Germany) and processed using Leica Applications Suite Software.

2.2.10 High performance liquid chromatography (HPLC) analyses

HPLC, a system for the separation, identification and quantification of compounds, was used for a number of analyses. The HPLC system used (Shimadzu Corporation, Japan) comprised a controller (LC-20AD), a fluorescence detector (RF-10AXL), a UV-Vis detector (SPD-M20A), an auto sampler (SIL-20AC HT), a column oven (CTO-20A), inline vacuum degasser (DGU-20A₅) and a communication bus module (CBM-20A).

Before use in the HPLC system, all mobile phases were degassed by sonication (Alphatech Systems Ltd & Co., Auckland) for 10 minutes. Chromatogram results consisting of peak areas were quantified using Shimadzu LCSolution software package (version 1.22 SP1, 2002-2006). Analytes in samples were standardised against the correlating pure standards of known concentrations for their quantification.

2.2.10.1 Intracellular glutathione (GSH) analysis

Monobromobimane (MBB) is a cell-permeable fluorescent dye that may be used for the detection of GSH. MBB alkylates thiol groups to form a GSH-MBB adduct that is then detected by the fluorescence detector after protein precipitation (Cotgreave and Moldeus,

1986). All procedures involving MBB were performed under minimum exposure of light since MBB is light sensitive.

A 40 mM stock of MBB was prepared by dissolving MBB (MW 271.1 g/mol) in acetonitrile, which was stored at 4°C in the dark for up to 2 weeks. Reduced GSH (MW 307.3 g/mol) was dissolved in cold PBS, to 5 and 10 µM concentrations, immediately prior to HPLC analysis for use as standards. Mobile phase A was 0.25% acetic acid and mobile phase B was 100% acetonitrile.

After removal of the incubation medium by centrifugation, cells were washed in PBS and re-suspended in 1.7 ml microtubes in 400 µl of warm PBS, 9 µl of 0.1 M NaOH (to increase pH to 8) and 10 µl of 40 mM MBB, in this order. Cells were then incubated with the MBB for 20 minutes in darkness, at room temperature, before 20 µl of 100% (^{w/v}) trichloroacetic acid (TCA) was added, to lyse cells. After thorough mixing, the cell lysate was centrifuged at 11000 rpm for 5 minutes at 4°C to pellet cellular proteins. Eighty microlitres of the resulting supernatant was transferred to an autosampling vial insert and 10 µl was injected onto the Phenosphere reverse phase C-18, 150 x 4.6 mm, 5 µm column (Phenomenex, Auckland, NZ) which was heated to 35°C.

GSH-MBB adducts were detected by the fluorescence detector with excitation and emission wavelengths set at 394 nm and 480 nm respectively. Mobile phases A and B were pumped through the column at a flow rate of 1.5 ml/minute with the following gradient program.

Time (minutes)	Mobile Phase A	Mobile Phase B
0	90 %	10 %
10	90 %	10 %
11	0 %	100 %
15	0 %	100 %
16	95 %	5 %
20	95 %	5 %

2.2.10.2 Free 7KC analysis

Detection of free 7KC was performed using a modification of the method described by Kritharides *et al.*, (1993). Mobile phase was ACN/isopropanol/H₂O in a ratio of 44:54:2. Following experimental treatment and subsequent removal of the incubation medium, cells were washed in PBS and re-suspended in 0.2 M NaOH at 4°C for 15 minutes for cell lysis to occur. Lipid extraction was carried out by the addition of 10 µl of 20 mg/ml BHT, 20 µl of 100 mg/ml EDTA, 500 µl ethanol and 2 ml of hexane, before vortexing for 60 seconds. Samples were then centrifuged at 200 g for 10 minutes at 4°C to obtain complete phase separation. Fourteen hundred microlitres of the top hexane layer was removed, transferred to a tapered glass test tube and dried completely under nitrogen gas.

The dried residue was re-solubilised in 100 µl of resolubilising phase (ACN/isopropanol in a ratio of 4:5). Eighty microlitres of the resulting supernatant was transferred to a glass autosampling vial insert and 20 µl was injected onto the Phenosphere reverse phase C-18, 250 x 4.6 mm, 5 µm column (Phenomenex, Auckland, NZ) which was heated to 35°C. Analysis of free 7KC was performed by detecting 234 nm absorbance. Detection and quantification of the oxysterol was carried out by comparison of peaks from known concentrations of the pure standard, prepared immediately prior.

For measurement of free 7KC in cell incubation medium, cell samples were pelleted and the supernatant collected, before the same lipid extraction and analysis procedure was carried out. Analysis of free 7KC in lipoprotein preparations was performed by dilution of samples with nanopure water, before lipid extraction and analysis were performed, as described above.

2.2.10.3 Esterified 7KC analysis

Esterified 7KC was quantified by measuring both free and total 7KC in a sample and calculating the difference. As above, detection of 7KC was performed using a modification of the method described by Kritharides *et al.*, (1993) and alkaline hydrolysis was used to release esterified 7KC before detection. The same method of lipid extraction as described above for free 7KC was carried out.

After evaporation with nitrogen gas, the remaining residue was resolubilised in 2 ml of 20% (w/v) KOH in methanol and 2.5 ml of diethyl ether. The sample was then flushed with argon gas and vortexed thoroughly. Samples were incubated on ice for 3 hours, vortexing every 30 minutes before the reaction was stopped by the addition of 2 ml of 20% acetic acid. Then 2.5 ml of hexane was added and samples were vortexed for 60 seconds before transferring 4 ml of the upper layer into a tapered glass test tube and evaporating completely under nitrogen gas.

The remaining residue was resolubilised in 100 µl of resolubilising phase (ACN/isopropanol in a ratio of 4:5). Eighty microlitres of the resulting supernatant was transferred to a glass autosampling vial insert and 20 µl was injected onto the Phenosphere reverse phase C-18, 250 x 4.6 mm, 5 µm column (Phenomenex, Auckland, NZ) which was heated to 35 °C. Analysis of total 7KC was performed by detecting 234 nm absorbance. Mobile phase was ACN/isopropanol/H₂O in a ratio of 44:54:2. Detection and quantification of the sterol was carried out by comparison of peaks from known concentrations of the pure standard, prepared immediately prior.

2.2.10.4 Thiobarbituric acid reactive species (TBARS) analysis

The TBARS assay provides a means of quantifying general lipid peroxidation. The method used is an adaptation of that described by Draper *et al.*, (1993). When combined, 2-thiobarbituric acid (TBA) and the maldondialdehyde (MDA) lipid hydroperoxide breakdown product readily react, forming the pink coloured TBA-MDA adduct which is fluorometrically detected by HPLC.

The MDA standard was prepared on the day of analysis by the dilution of 6.07 M 1,1,3,3-tetramethoxypropane (TMP) in ethanol/water (2:3), with subsequent dilution in water to the desired concentration. The mobile phase was 50 mM sodium dihydrogen phosphate, with the pH adjusted to 6.8 using sodium hydroxide, and combined with methanol in a ratio of 65:35. A 42 mM 2-thiobarbituric acid (TBA) solution was made fresh on the day of analysis by dissolving in nanopure water on a hot plate, ensuring that the temperature did not exceed 55°C.

The lipoprotein sample was diluted with PBS at a ratio of 1:3, before 200 μ l of this solution was transferred to a 1.7 ml microtube. To this, 100 μ l of 50 mM phosphoric acid and 20 μ l of 20 mg/ml BHT in methanol, were added and vortexed to mix. Then 100 μ l of the above TBA reagent was added before incubating on a shaking heater block (Eppendorf Thermomixer 5436) at 95°C for 30 minutes. Samples were then placed directly onto ice and allowed to cool before centrifugation at 10,000 rpm for 10 minutes at 4°C.

One hundred microlitres of the remaining supernatant was transferred to an autosampling vial insert and 20 μ l was injected onto the Phenosphere reverse phase C-18, 50 x 4.6 mm, 5 μ m column (Phenomenex, Auckland, NZ) which was heated to 30°C. TBARS were detected using excitation and emission wavelengths of 525 nm and 550 nm, respectively. The mobile phase was methanol and 50 mM sodium dihydrogen phosphate (pH 6.8) in a ratio of 45:55. The concentration of TBARS was quantified by comparison with the peak areas of 0 and 1 μ M MDA standards.

2.2.11 Statistical analysis

Results shown are the result of single experiments and representative of 3 separate experiments, which is indicated in individual figures. The mean and standard error of the mean (SEM) shown within each experiment were calculated from triplicate samples in every case. Data were graphed and analysed statistically using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, California, USA). Significance was confirmed via a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Asterisks indicate significant difference from the indicated control value when $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***) .

3. RESULTS

3.1 Toxicity of oxLDL

To investigate the potential role of 7KC in oxLDL-induced cytotoxicity, this research began with an examination of the independent effects of 7KC and oxLDL on the U937 human monocyte-like cell line. These initial experiments were preliminary to those that investigate the effect of oxysterol-loaded lipoprotein on U937 cells. Aside from confirming previous findings regarding their toxicity and comparing their relative influence on cell viability and intracellular glutathione content, the following set of experiments aimed to establish appropriate concentrations of oxLDL and 7KC for use in later experiments.

3.1.1 The effect of oxLDL and HI-FBS on U937 cell viability

The toxicity of oxLDL to U937 cells was examined by exposing U937 cells to increasing concentrations of oxLDL in RPMI-1640 cell medium for 24 hours. Cell viability was subsequently measured using the MTT reduction assay (Figure 3.1). U937 cells displayed a concentration-dependent decrease in cell viability following incubation with oxLDL. Final oxLDL concentrations of 0.05, 0.1, 0.2 and 0.5 mg/ml reduced cell viability by approximately 12, 15, 70 and 100% of the control, respectively. The precise toxicity of oxLDL to U937 cells was found to vary slightly between different batches prepared, with the median lethal dose (LD_{50}) between 0.1-0.2 and up to 0.5 mg/ml oxLDL. The LD_{50} of oxLDL is known to vary from one batch to another although the exact cause has not been identified (Gieseg *et al.*, 2009a). Therefore, the toxicity of each oxLDL preparation was tested and used to determine the oxLDL concentration used in subsequent experiments.

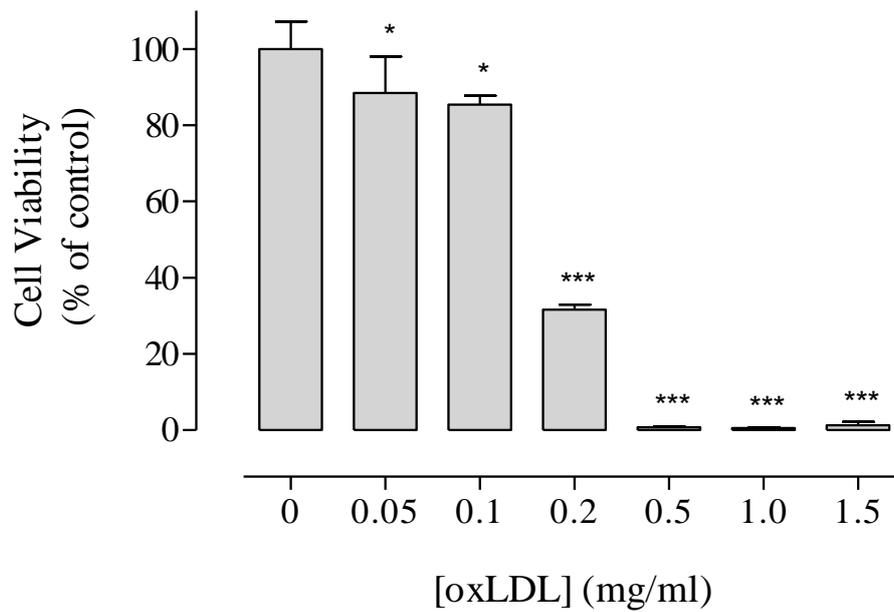


Figure 3.1 The effect of oxLDL on U937 cell viability.

U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 for 24 hours with increasing concentrations of oxLDL. Cell viability was analysed via MTT reduction assay. Results are expressed as a percentage of the 0 mg/ml oxLDL control data and significance is indicated from this control. Results displayed are the mean \pm SEM of triplicates from a single experiment and are representative of three separate experiments.

To examine the effect of heat-inactivated foetal bovine serum (HI-FBS) on oxLDL cytotoxicity, U937 cells were incubated with increasing concentrations of oxLDL in the presence and absence of 5% HI-FBS for 24 hours before measurement of cell viability (Figure 3.2). The presence of 5% HI-FBS significantly decreased the cytotoxicity of oxLDL. This effect was most pronounced at the concentration of 0.2 mg/ml oxLDL, where the presence of HI-FBS prevented an 88% decrease in cell viability relative to the equivalent non-HI-FBS treatment. At higher concentrations of oxLDL that caused total viability loss, the addition of HI-FBS did not prevent cell viability loss.

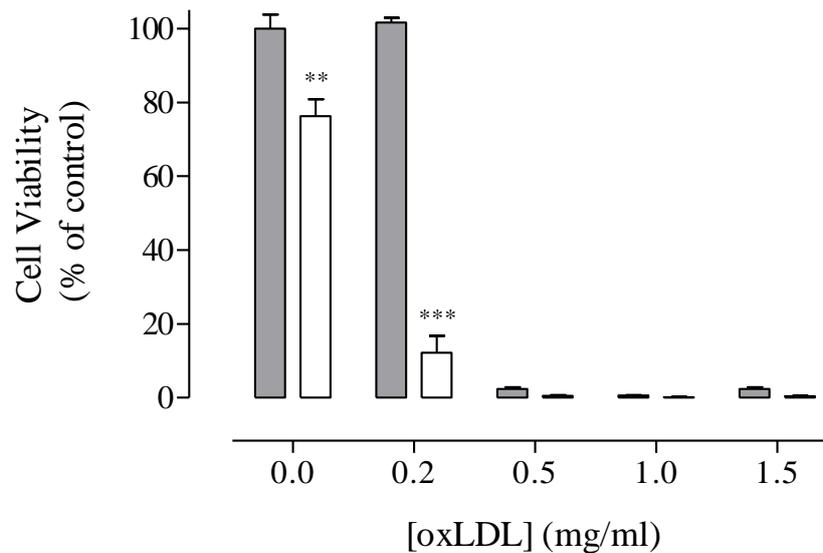


Figure 3.2 The effect of HI-FBS on oxLDL-induced cell viability loss in U937 cells.

U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 with increasing concentrations of oxLDL in the presence (grey bars) or absence (white bars) of 5% HI-FBS. Cell viability was measured using the MTT reduction assay. Data are expressed as a percentage of the 0 mg/ml oxLDL control (with 5% HI-FBS). Significance is indicated from the respective without-HI-FBS data at each concentration. Results shown are the mean \pm SEM of triplicates.

To examine the progression of oxLDL-induced death in U937 cells, the cells were incubated with 0.25 mg/ml oxLDL for 9 hours, during which time, samples were removed for analysis by the MTT reduction assay (Figure 3.3A). Following a 1.5 hour incubation with oxLDL, cell viability decreased by 10% compared to the control and continued to decrease rapidly over the next 2 hours. After 3 hours of incubation, cell viability decreased by 65% and continued to decline until total loss of cell viability occurred, following 6 hours incubation with oxLDL. Figures 3.3 B, C, D and E illustrate the morphological changes in U937 cells during their incubation with 0.25 mg/ml oxLDL, which appear to be consistent with necrosis. After 9 hours incubation with oxLDL (Figure 3.3E), cells appear to have undergone necrosis. This is evident from the fact that the majority of cells are swollen, have spilled their contents and that there is much cellular debris, which is consistent with previous work done by this laboratory (Baird, 2003).

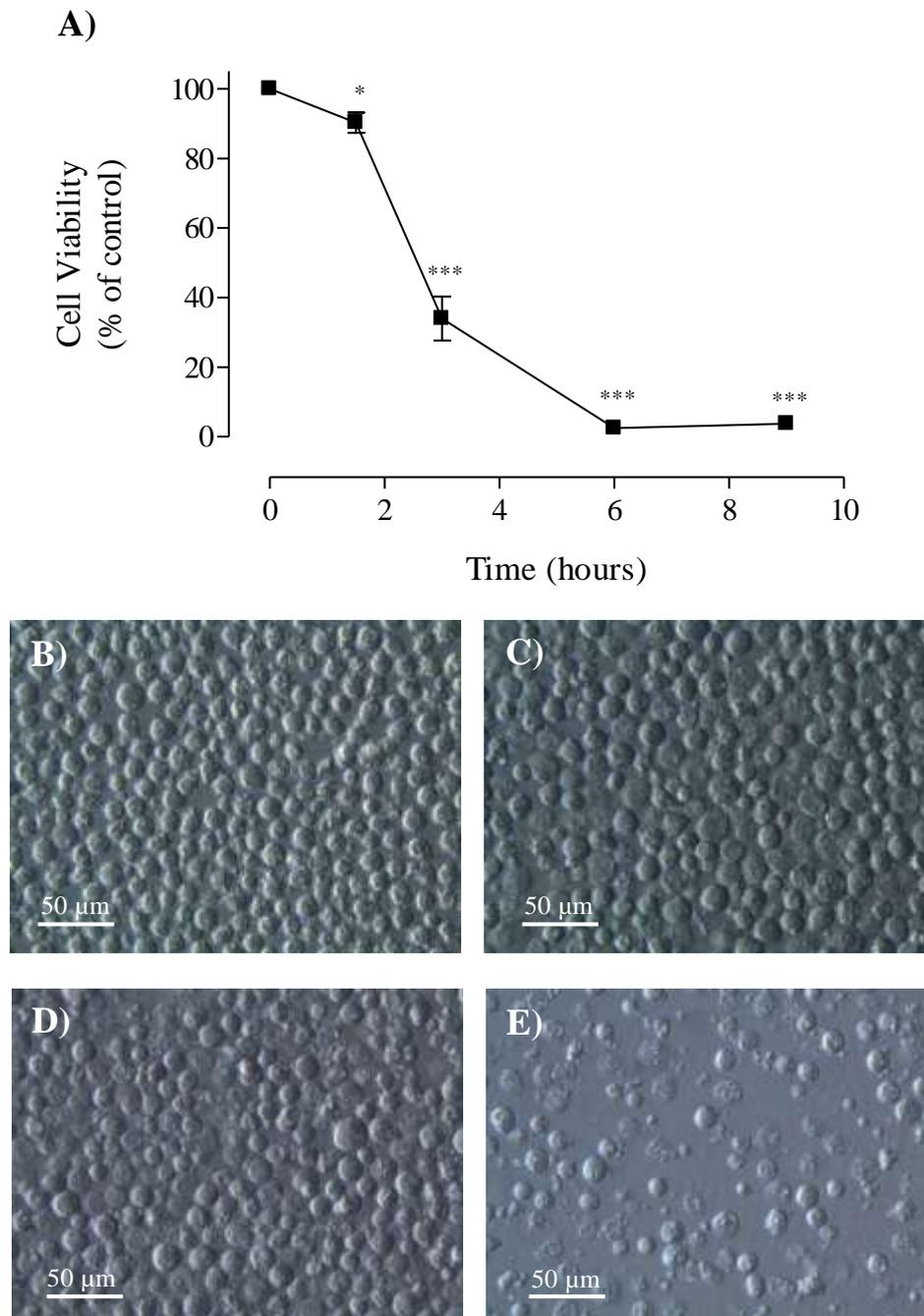


Figure 3.3 Time course study of oxLDL-induced cell viability loss in U937 cells.

U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 with 0.25 mg/ml oxLDL. A) At various times, cell samples were removed and cell viability was measured using the MTT reduction assay. Data are expressed as a percentage of cell viability at 0 hours and significance is indicated from 0 hour data. Results shown are the mean \pm SEM of triplicates. Cells were viewed *in situ* in tissue culture wells using an inverted microscope (400x magnification) after B) 0, C) 3, D) 6 and E) 9 hours. Images were taken using a Leica C-Mount camera and processed using Leica Applications Suite software.

3.1.2 The effect of oxLDL and HI-FBS on U937 cellular GSH content

Incubation of U937 cells with oxLDL caused a marked loss of intracellular glutathione (GSH) (Figure 3.4) closely reflecting the pattern of loss that was demonstrated for cell viability. In the absence of HI-FBS, GSH loss of more than 80% occurred following incubation with 0.2 mg/ml oxLDL and complete GSH loss occurred at concentrations of 0.5 mg/ml oxLDL and above. This experiment was performed in both the presence and absence of HI-FBS to examine its effect on GSH loss. As with cell viability, the presence of 5% HI-FBS appeared to dramatically prevent oxLDL-induced damage by way of GSH loss, and appeared to have the greatest effect at the lower end of oxLDL concentrations used. At 0.2 and 0.5 mg/ml oxLDL, the presence of HI-FBS caused increased cellular GSH content compared to the without-oxLDL control, preventing near-complete GSH loss that occurred in its absence, at these concentrations of oxLDL.

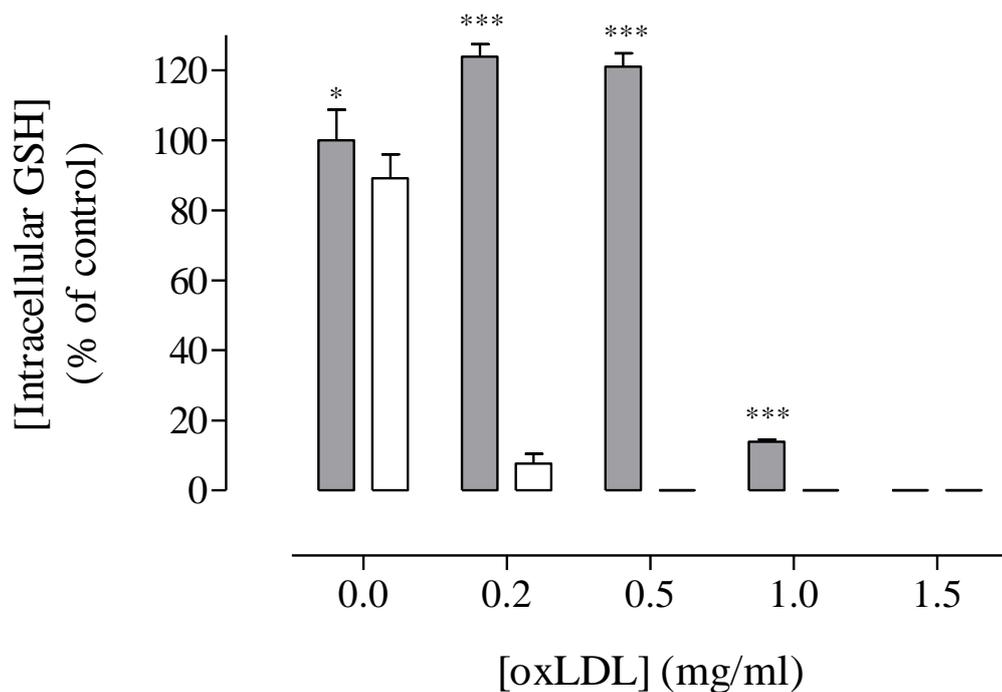


Figure 3.4 The effect of oxLDL on intracellular GSH level in U937 cells.

U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 with increasing concentrations of oxLDL for 12 hours in the presence (grey bars) and absence (white bars) of 5% HI-FBS. Intracellular GSH was measured by HPLC analysis. Data are expressed as a percentage of the 0 mg/ml oxLDL control (containing 5% HI-FBS). Significance is indicated from the respective without-serum values. Results shown are the mean \pm SEM of triplicates.

The pattern of oxLDL-induced GSH loss was studied over 9 hours using 0.25 mg/ml oxLDL (for comparison with the earlier results of oxLDL-induced cell viability loss) (Figure 3.5). This lethal concentration of oxLDL caused a rapid loss of intracellular GSH content of 80% within 6 hours of incubation and decreased to less than 10% of the initial intracellular GSH level after 9 hours. The pattern of intracellular GSH loss illustrated here closely mirrors the pattern of cell viability loss (Figure 3.3a).

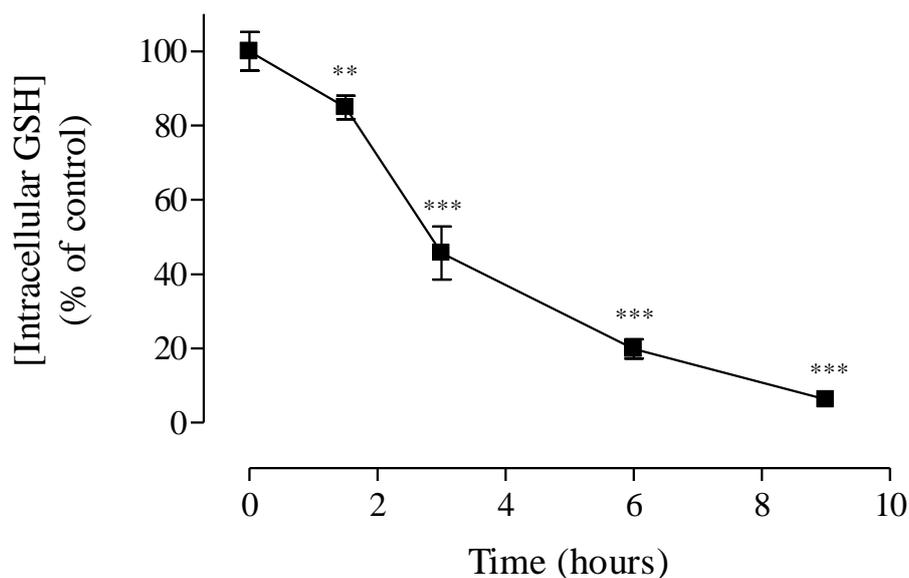


Figure 3.5 Time course study of oxLDL-induced GSH loss in U937 cells.

U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 with 0.25 mg/ml oxLDL. At various times, cell samples were removed and intracellular GSH was measured by HPLC analysis. Data are expressed as a percentage of the 0 hour data and significance is also indicated from this data. Results displayed are the mean \pm SEM of triplicates.

3.2 Toxicity of 7-Ketocholesterol

3.2.1 The effect of 7KC and HI-FBS on U937 cell viability

The toxicity of 7KC to U937 cells, when incubated directly with the cell culture, was tested. This set of experiments was conducted in parallel with the preceding set of experiments that aimed to test the toxicity of oxLDL to U937 cells. In this set of experiments, U937 cells were incubated with 7KC that was added directly to cell cultures as a simple ethanolic solution. A treatment containing the maximum concentration of ethanol used in the following experiments, 0.5% ethanol (v/v), was tested for cytotoxicity and none was detected (Figure 3.6). A final 7KC concentration of 10, 15, 20 and 30 μM in the cell culture medium caused cell viability loss of 24, 40, 54 and 85% of the control, respectively (Figure 3.6). Concentrations of 40 μM and above appeared to cause near/complete loss of cell viability. Micromolar concentrations of 7KC, when added directly to the cell culture medium as an ethanolic solution, are highly toxic to U937 cells.

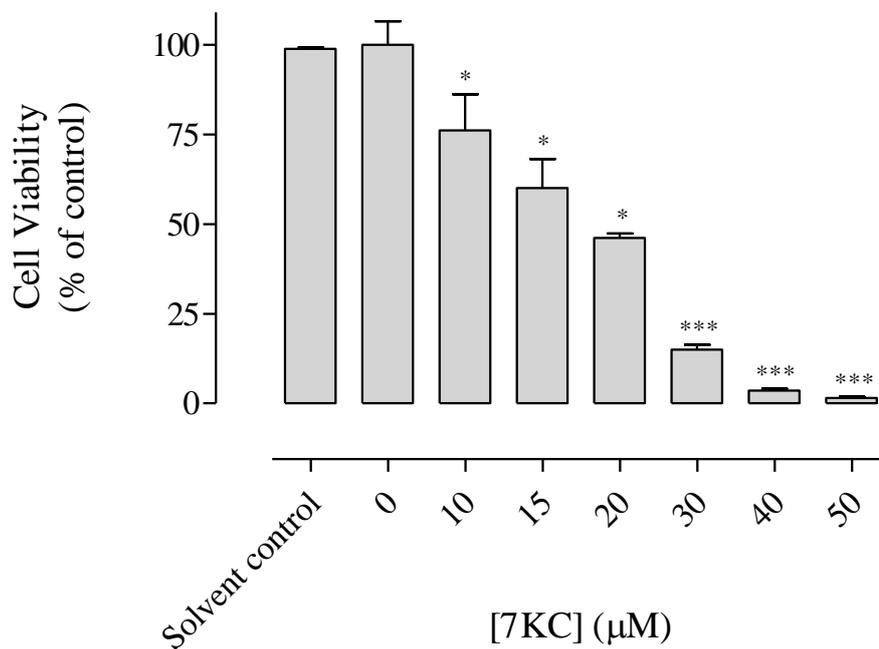


Figure 3.6 The effect of 7KC on U937 cell viability.

U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 containing increasing concentrations of 7KC for 24 hours. Cell viability was measured using the MTT reduction assay. Data are expressed as a percentage of the 0 μM 7KC control and significance also is indicated from this control. Results displayed are the mean \pm SEM of triplicates.

To study the effect of HI-FBS on 7KC-induced cytotoxicity, U937 cells were incubated with increasing concentrations of 7KC in the presence and absence of 5% HI-FBS (Figure 3.7). As with oxLDL-induced toxicity, HI-FBS displayed a protective effect to U937 cells at all 7KC concentrations tested, excluding those that induced near total viability loss. The presence of 5% HI-FBS in the cell culture medium maintained near total protection of viability even at concentrations up to 20 μM 7KC where cell viability in the absence of HI-FBS had decreased by 63% relative to the control. At higher 7KC concentrations, the addition of 5% HI-FBS displayed less protection, although it was still significant. At 30 and 40 μM 7KC, the HI-FBS treatment showed 36 and 16% higher viability than their non-HI-FBS equivalents, respectively.

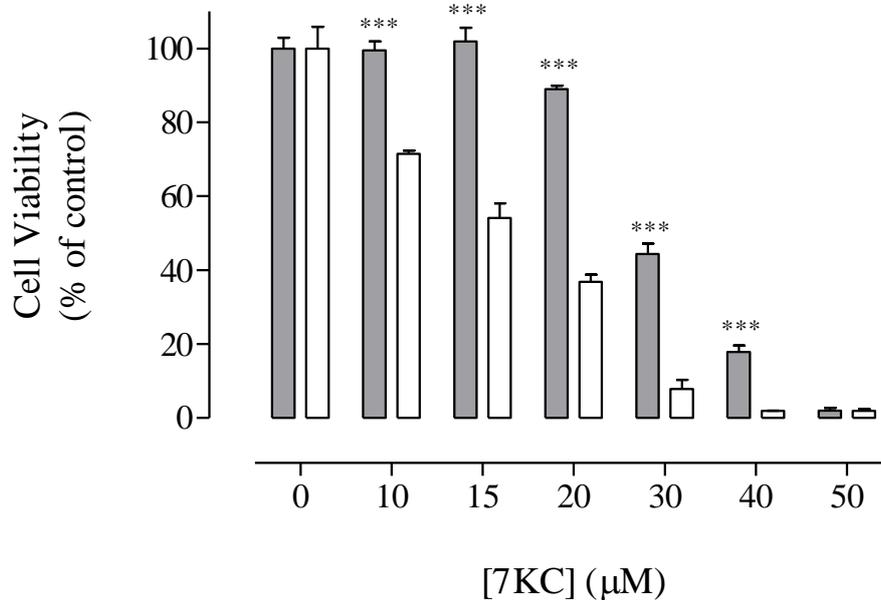


Figure 3.7 The effect of HI-FBS on 7KC-induced cell viability loss in U937 cells.

U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 containing increasing concentrations of 7KC for 24 hours in the presence (grey bars) and absence (white bars) of 5% HI-FBS. Cell viability was measured using the MTT reduction assay. Data are expressed as a percentage of the respective 0 μM 7KC controls and significance is indicated from the respective without-HI-FBS control data. Results displayed are the mean \pm SEM of triplicates.

U937 cells were incubated directly with a lethal concentration (30 μM) of 7KC (based on earlier viability results in Figure 3.6) for 28 hours, during which time, culture samples were tested for viability (Figure 3.8). During the first 9 hours of incubation, cells did not display any significant change in viability. Following 12 hours of incubation with 7KC, cell viability decreased by 17% compared to the control and continued to decrease at a constant rate over the next 16 hours until cell viability was just 20% of the control viability, after 28 hours. This time-course data for 7KC-induced cell death is very different to the parallel experiment performed with oxLDL, where a similar level of cell viability loss occurred after just 6 hours.

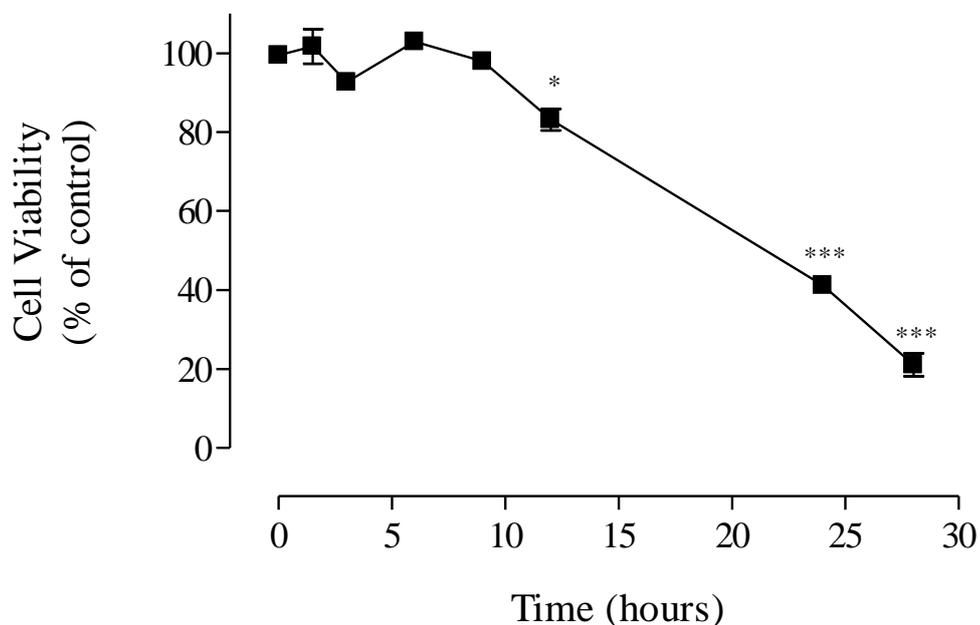


Figure 3.8 Time course study of 7KC-induced U937 cell viability loss.

U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 containing 30 μM 7KC. At various times, cell samples were removed and cell viability was measured using the MTT reduction assay. Data are expressed as a percentage of a 0 μM 7KC control and significance is also indicated from this control. Results displayed are the mean \pm SEM of triplicates.

3.2.2 The effect of 7KC on U937 cellular GSH content

The pattern of intracellular GSH loss induced by the same concentration of 7KC (30 μ M) (Figure 3.9) very closely reflects the pattern of cell viability loss. It should be noted that the time course investigations of 7KC-induced cell viability loss and intracellular GSH loss displayed here were performed concurrently. As with cell viability loss, intracellular GSH remained stable during the first 9 hours of incubation. This lag period was followed by a constant rate of decrease of intracellular GSH over the next 19 hours so that the intracellular GSH content following 12, 24 and 28 hours of incubation was 89, 36 and 18% respectively, compared to the control data.

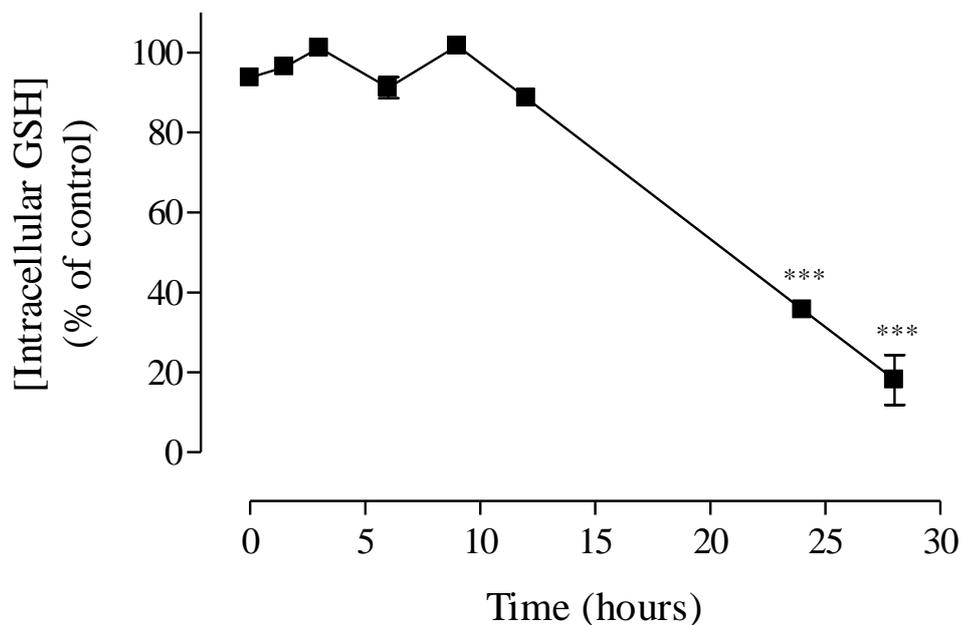


Figure 3.9 Time course study of 7KC-induced intracellular GSH loss in U937 cells.

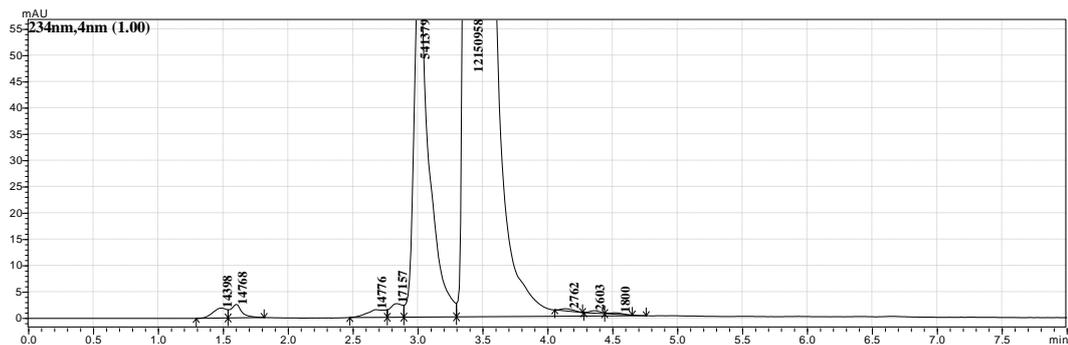
U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 with a final concentration of 30 μ M 7KC. At various times, cell samples were removed and intracellular GSH content measured by HPLC analysis. Data are expressed as a percentage of the 0 μ M 7KC GSH level and significance is also indicated from this data. Results displayed are the mean \pm SEM of triplicates.

3.3 7-Ketocholesterol Uptake

3.3.1 Measurement of U937 intracellular 7KC

To eliminate doubt concerning the uptake of modified lipoproteins by U937 cells, *intracellular* non-esterified 7-ketocholesterol concentrations were measured throughout the following set of experiments. Below is an example of the chromatograms obtained in the identification of 7KC by HPLC analysis (Figure 3.10). Part A) shows the result of a 24 hour incubation of U937 cells in RPMI-1640 medium only, followed by solvent extraction of lipids and then HPLC analysis. Part B) shows the result of the same procedure, although the incubation medium contained 20 μM 7KC. This HPLC method was adapted from Kritharides *et al.*, (1993) and produces a clear peak with a retention time of approximately 4.5 minutes.

A)



B)

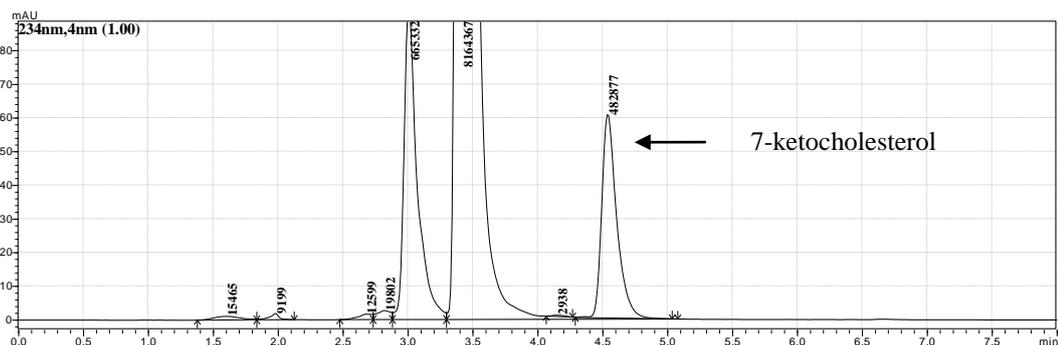
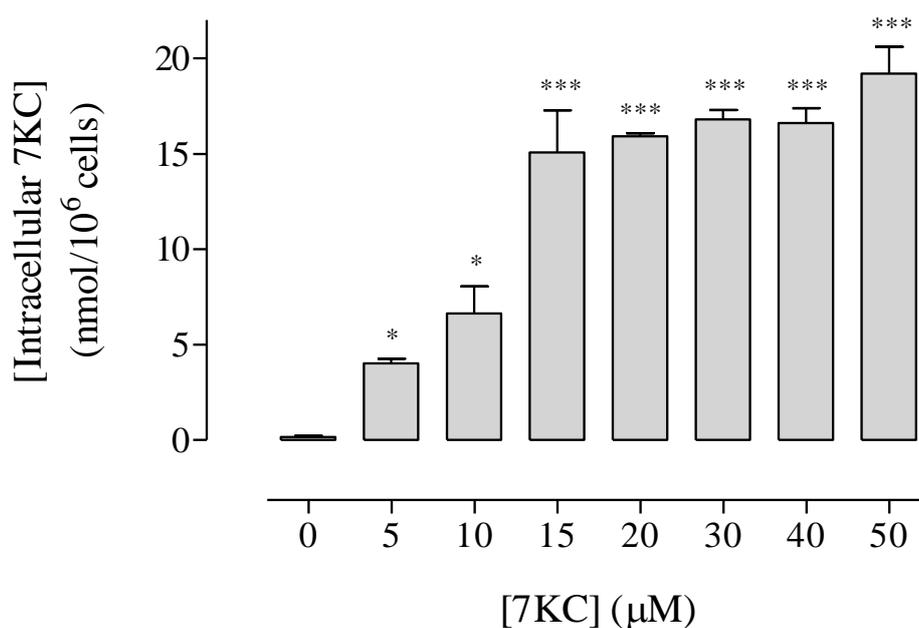


Figure 3.10 Identification of 7KC by HPLC analysis.

U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 containing a) no 7KC (control) or b) 20 μM 7KC for 24 hours. Following incubation, intracellular free 7KC was measured via HPLC analysis. Figure shows raw data as HPLC chromatograms. Peaks were identified using standards.

3.3.2 7KC uptake by U937 cells

U937 cells were incubated directly with increasing concentrations of 7KC (delivered as an ethanolic solution) for 24 hours, before solvent extraction of lipids was performed and intracellular 7KC measured using HPLC analysis (Figure 3.11). The uptake of 7KC occurred in a concentration-dependent manner. The greater the concentration of 7KC in the cell medium, the higher the resulting intracellular 7KC level. Minute quantities of non-esterified (free) 7KC were detected in the 0 μM 7KC control and 4.0, 6.6 and 15.1 nmol free 7KC/ 10^6 cells was measured in the 5, 10 and 15 μM 7KC treatments, before an apparent maximum was reached. There was little increase in 7KC uptake between the 15 and 40 μM 7KC treatments, but it is important to note that the corresponding cell viability at such 7KC concentrations was very low (see Figure 3.6) and cell lysis is likely to have occurred, releasing intracellular 7KC and resulting in the apparent saturation effect.



3.11 7KC uptake following incubation with 7KC as an ethanol solution.

U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 containing increasing concentrations of 7KC for 24 hours. Following incubation, lipid was extracted from cell samples and intracellular free 7KC was measured via HPLC analysis. Significance is indicated from the 0 μM 7KC control. Data shown are the mean \pm SEM of triplicates.

Similarly, the intracellular concentration of free 7KC was measured in response to 24 hour incubation with increasing concentrations of oxLDL (Figure 3.12) and showed a concentration-dependent increase in intracellular 7KC. Minute quantities of 7KC were detected in control (0 mg/ml oxLDL) cells. Intracellular 7KC increased with increasing concentrations of oxLDL in the cell medium until a maximum was reached at approximately 4.0 nmol 7KC/10⁶ cells, induced by the addition of 1.0 and 1.5 mg/ml oxLDL. As above, it is important to note that such concentrations of oxLDL caused almost complete loss of cell viability (Figure 3.6) and cell lysis is likely to have occurred, releasing intracellular 7KC and accounting for the observed saturation effect.

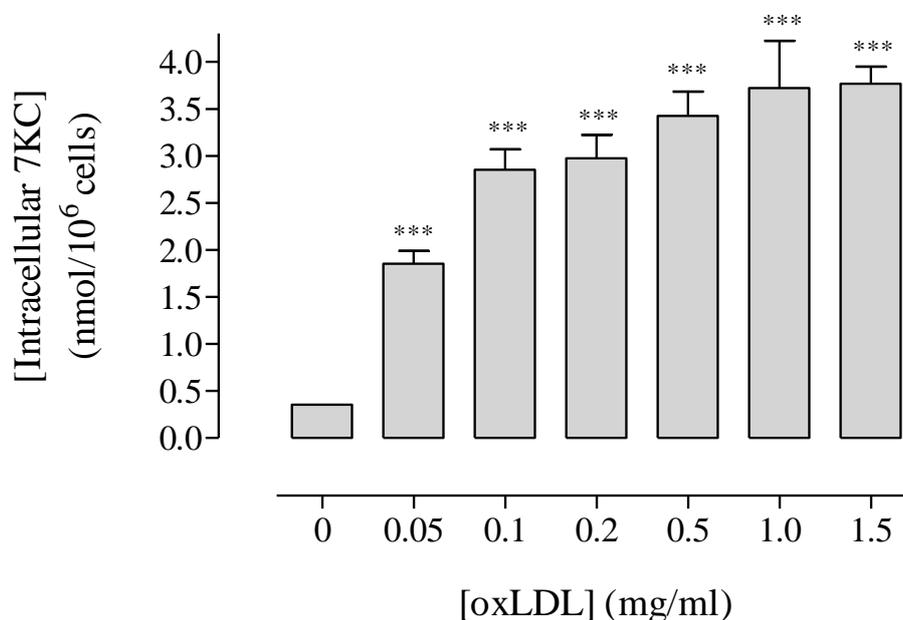


Figure 3.12 7KC uptake following incubation with oxidised LDL.

U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 containing increasing concentrations of oxLDL for 24 hours. Following incubation, lipid was extracted from cell samples and intracellular free 7KC was measured via HPLC analysis. Significance is indicated from the 0 μ M 7KC control. Data shown are the mean \pm SEM of triplicates.

A time course study of U937 intracellular non-esterified 7KC content over a 9 hour incubation with 0.2 mg/ml oxLDL was performed (Figure 3.13). A relatively constant rate of increase of intracellular 7KC occurred over the initial 9 hours, reaching an intracellular 7KC concentration of 2.1 nmol/10⁶ cells. The corresponding microscopy showed that a large degree of cell lysis occurred between 3 and 9 hours.

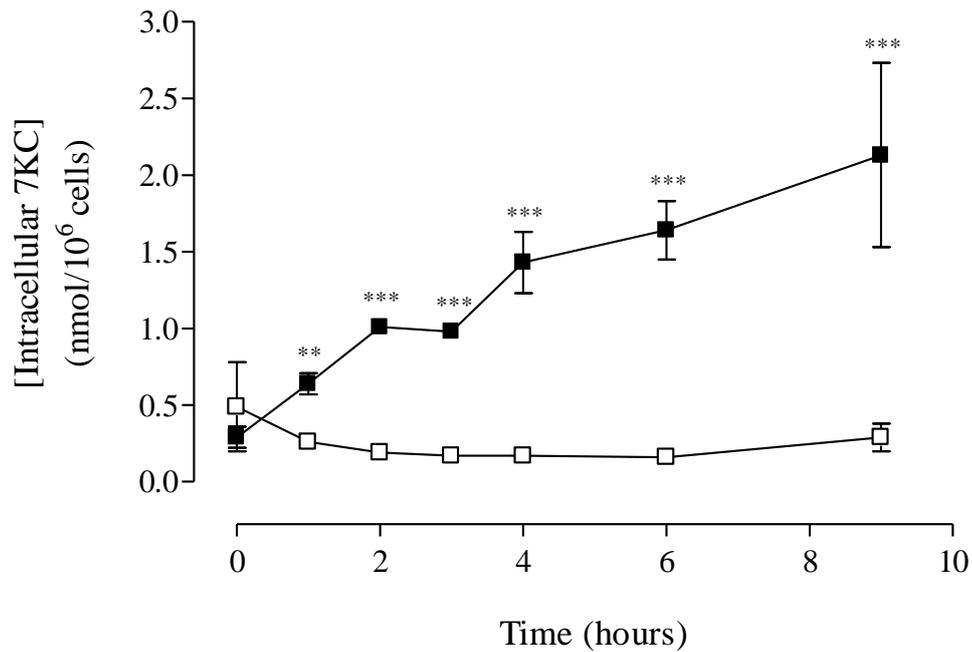


Figure 3.13 The effect of oxLDL on free 7KC content of U937 cells.

U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 with (■) and without (□) 0.2 mg/ml oxLDL. At various times, cell samples were removed and intracellular free 7KC was measured via HPLC analysis. Significance is indicated from the respective control data. Data shown are the mean \pm SEM of triplicates.

3.3.3 Mechanism of 7KC-induced U937 cell death

The water-soluble antioxidant compound 7,8-dihydroneopterin (7,8-NP), has been shown by this laboratory, to provide protection to U937 cells against oxLDL-induced cell death. Specifically, 7,8-NP was shown to prevent oxLDL-induced intracellular GSH loss by scavenging oxLDL-induced intracellular oxidants, independently of other antioxidants such as glutathione and α -tocopherol, and therefore maintain the intracellular redox environment (Baird *et al.*, 2004, Baird *et al.*, 2005). 7,8-NP was used here as a probe of the 7KC toxicity mechanism. U937 cells were treated with increasing concentrations of 7,8-NP prior to incubation with 15 μ M 7KC in the cell medium for 24 hours (Figure 3.14). Treatment with 200 μ M 7,8-NP caused a slight increase in cell viability compared to the cell-only control, which has been observed in previous experiments (Baird *et al.*, 2005). The presence of 15 μ M 7KC (without 7,8-NP treatment) caused a 43% decrease in viability. Treatment of cells with 50, 100 and 200 μ M 7,8-NP before incubation with 7KC resulted in cell viability loss of 65, 60 and 64% relative to the cell-only control. 7,8-NP did not protect U937 cells against 7KC-induced cell viability loss, and in fact, caused a further decrease in viability compared to cells treated only with 7KC.

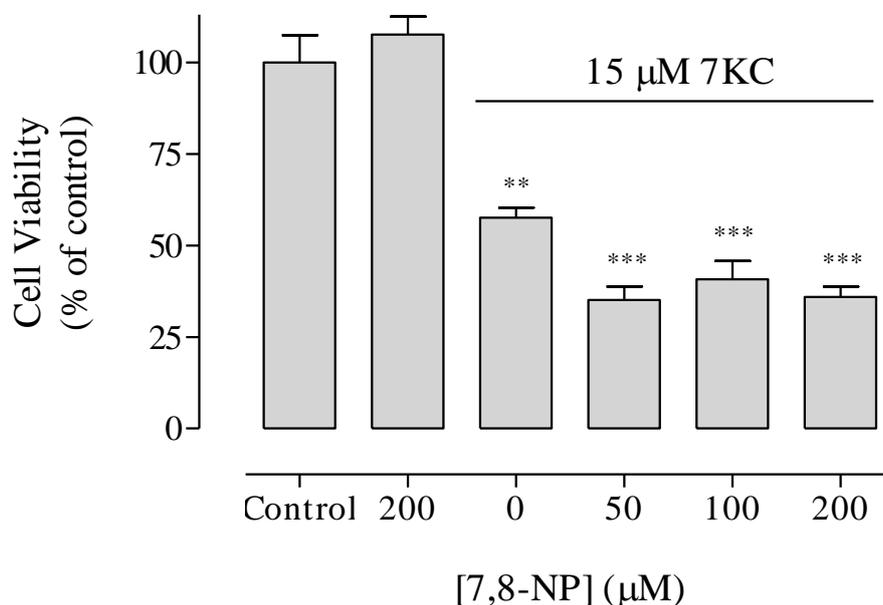


Figure 3.14 The effect of 7,8-NP treatment on 7KC-induced cell viability loss.

U937 cells at 5×10^5 cells/ml were treated with increasing concentrations of 7,8-NP for 10 minutes before incubation in RPMI-1640 containing 15 μ M 7KC for 24 hours. A treatment without either 7,8-NP or 7KC was prepared (Control). Cell viability was measured using the MTT reduction assay. Data are expressed as a percentage of the cell-only control and significance is also indicated from this control. Data shown are the mean \pm SEM of triplicates.

3.4 Properties of Native and Modified LDL

3.4.1 Characterisation of 7KC-acLDL

With the aim of creating a physiologically relevant model for testing the toxicity of 7KC to U937 cells, human plasma was incubated with 7KC over 6 hours, before isolation of 7KC-loaded LDL by ultracentrifugation and subsequent acetylation to create 7KC-acLDL, by the procedure described in section 2.2. Relative electrophoretic mobility (REM) and non-esterified (free) 7KC content were examined to confirm that acetylation had occurred and that 7KC had been successfully incorporated into the lipoprotein, respectively. The REM of native LDL, oxLDL, acLDL and 7KC-acLDL was compared using lipoprotein gel electrophoresis on a non-denaturing lipoprotein gel (Figure 3.15). The relative mobilities of native LDL, oxLDL, acLDL and 7KC-acLDL were 9, 40, 37 and 37 mm, respectively. Copper ion-mediated oxidation of LDL caused a 4.4-fold increase in mobility relative to that of the native LDL control, which indicates that oxidation was very extensive (Baird, 2003). The acetylation of LDL caused a similar 4-fold increase in REM relative to that of the control, indicating that modification of the lipoprotein had taken place. The enrichment of LDL with 7KC did not appear to cause any change in REM, indicating that there had been no major changes in the structure or charge of the LDL.

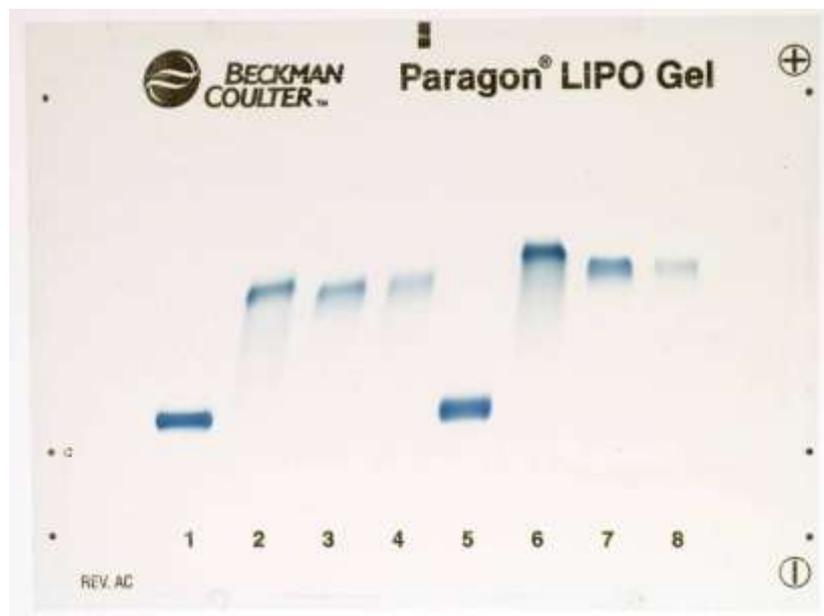


Figure 3.15 Relative electrophoretic mobility of native and modified LDL.

Samples of native and modified LDL at 2.0 mg/ml LDL protein were loaded into a non-denaturing Paragon lipoprotein gel. Lanes 1 and 5 are native LDL, lanes 2 and 6 are oxLDL, lanes 3 and 7 are acLDL and lanes 4 and 8 are 7KC-acLDL. This gel is representative of three separate experiments.

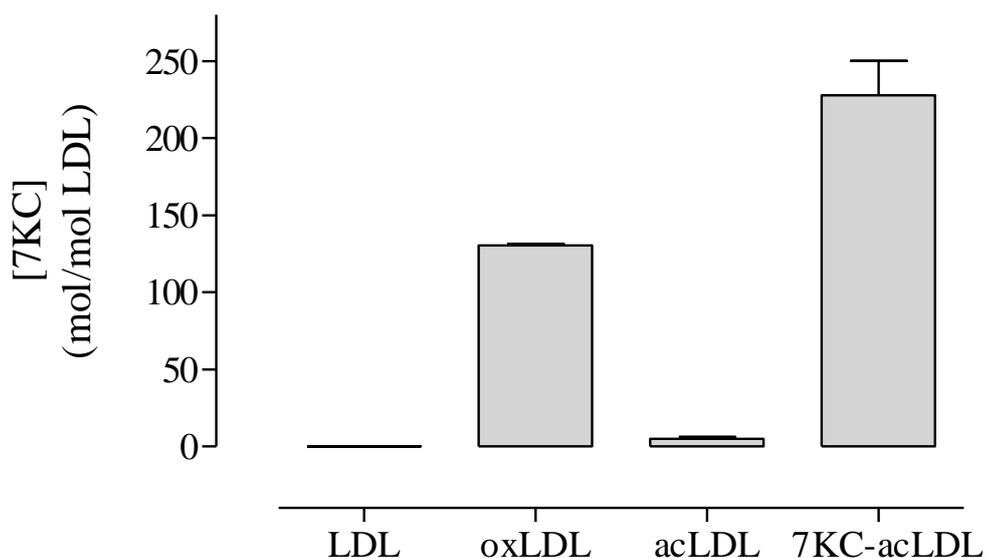


Figure 3.16 Non-esterified 7KC content of native and modified LDL.

The non-esterified 7KC content of native LDL, oxLDL, acLDL and 7KC-acLDL (at 4.0 μM LDL) was measured by HPLC analysis. Data shown are the mean \pm SEM of triplicates.

Measurement of the non-esterified 7KC content of native and modified LDL samples by HPLC analysis revealed that our method of copper ion-mediated LDL oxidation produces oxLDL with relatively high free 7KC content compared to that produced using other methods (Gerry *et al.*, 2008) and that the 7KC-acLDL contained approximately twice the concentration of non-esterified 7KC than did oxLDL (Figure 3.16). The free 7KC content of copper ion-oxLDL was 130 mol/mol LDL and that of 7KC-acLDL was 228 mol/mol LDL. These measurements also confirmed that the native LDL contains no detectable levels of free 7KC and that the acetylated LDL contains only minute quantities of non-esterified 7KC. This renders acLDL a good control for 7KC-acLDL and oxLDL since it is sufficiently modified to be a ligand for the high-uptake scavenger receptors but contains only minute quantities of non-esterified 7KC.

3.4.2 Uptake of native and modified LDL by U937 cells

U937 cells were incubated with 0.1 or 0.2 mg/ml native and modified LDL for 24 hours before intracellular free 7KC content was measured by HPLC analysis (Figure 3.17). The intracellular free 7KC content of LDL and acLDL-loaded cells were both very low, at less than 0.5 nmol/10⁶ cells. The intracellular 7KC content of oxLDL at 0.1 and 0.2 mg/ml oxLDL was approximately 1.4 nmol/10⁶ cells. Their similarity in value is likely to be due to release of intracellular 7KC caused by cell death and lysis occurring with 0.2 mg/ml oxLDL. Incubation with 0.1 and 0.2 mg/ml 7KC-acLDL caused U937 7KC uptake to concentrations of 3.1 and 5.8 nmol/10⁶ cells, which confirms that 7KC-acLDL was successfully internalised by U937 cells.

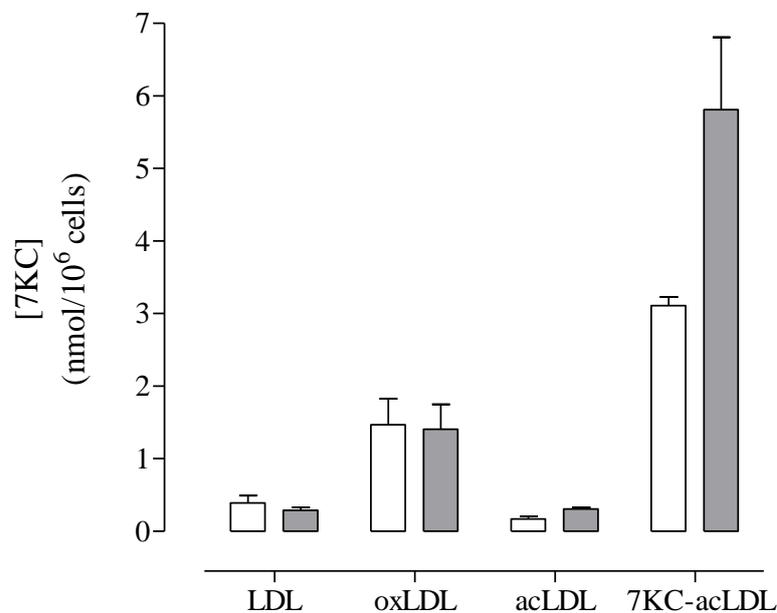


Figure 3.17 U937 intracellular free 7KC level following incubation with native and modified LDL. U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 containing 0.1 (white bars) or 0.2 (grey bars) mg/ml LDL, native or modified, for 24 hours. Following incubation, intracellular 7KC concentration was measured via HPLC analysis. Data shown are the mean \pm SEM of triplicates.

Lipid accumulation in U937 cells following incubation with native and modified LDL was examined microscopically following staining with oil red-O, which is lysochrome diazo dye typically used for staining neutral triglycerides and lipids (Figure 3.18). Incubation in the absence of LDL and with 0.5 mg/ml LDL resulted in a minimal amount of observable intracellular lipid staining, while incubation with 0.5 mg/ml acLDL and 7KC-acLDL did not result in any visible lipid staining. Overall, the procedure for intracellular lipid staining

using oil red-O dye, adapted from Davies *et al.*, (2005) was not found to be successful with U937 cells.

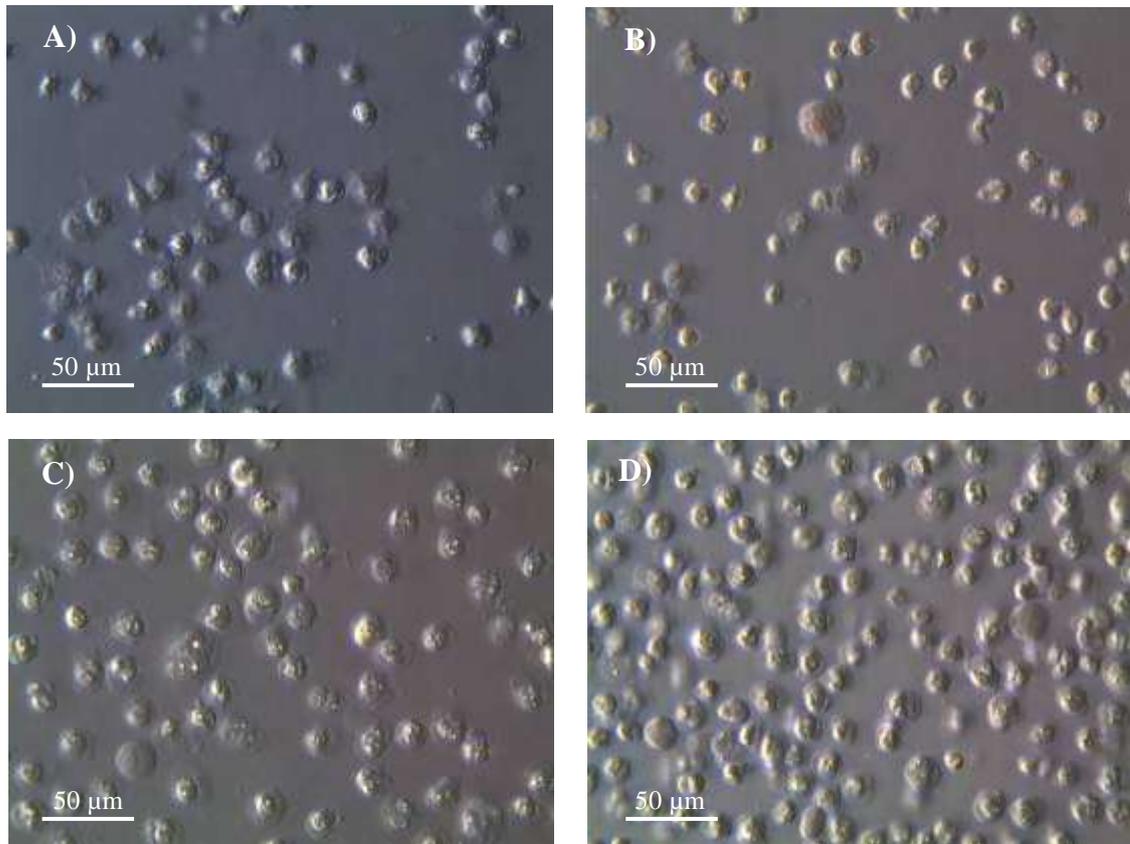


Figure 3.18 Oil red-O stained U937 cells.

U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 containing A) no treatment, B) 0.5 mg/ml LDL, C) 0.5 mg/ml acLDL and D) 0.5 mg/ml 7KCacLDL for 24 hours. Following incubation, cells were washed in PBS, fixed and stained with Oil Red-O dye. Cells were viewed *in situ* in tissue culture wells using an inverted microscope at 400x magnification. Images were taken using a Leica C-Mount camera and processed with Leica Applications Suite Software.

3.4.4 7KC formation during U937 cell death

As 7KC has been implicated in the death of lipoprotein-loaded foam cells within atherosclerotic plaques, an experiment was designed to mimic this death process. U937 cells were loaded with high-uptake acLDL before cell death was induced using a mechanism that was believed unlikely to interfere with the molecules of interest. According to the hypothesis that 7KC is responsible for oxLDL toxicity, we would have expected to detect a clear increase in intracellular 7KC content in acLDL-loaded U937 cells, relative to control cells. U937 cells were loaded with acLDL by incubation with 1.0 mg/ml acLDL in RPMI cell medium for 24 hours before being washed and re-suspended in RPMI cell medium for 120 hours in the absence of oxygen, to induce cell death. Hypoxia

was induced in cell cultures by flushing thoroughly with argon gas. Subsequent measurement of intracellular free and esterified 7KC revealed no significant difference between control and acLDL-loaded cell cultures (Figure 3.19).

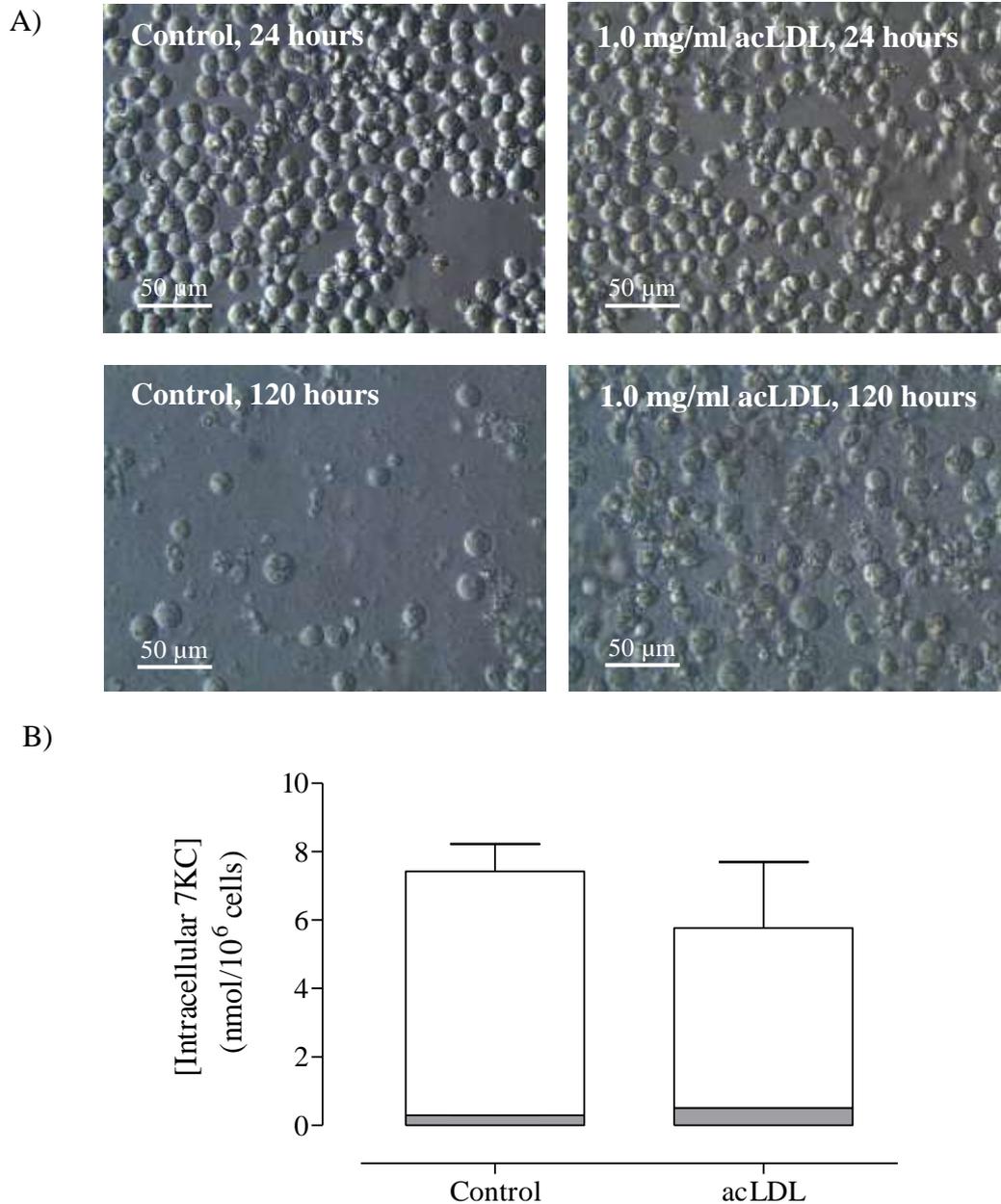


Figure 3.19 Intracellular free and total 7KC in U937 cells, during cell death.

U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 with and without 1.0 mg/ml acLDL for 24 hours. Cells were then washed and re-suspended in RPMI-1640, flushed thoroughly with argon gas and sealed during the following 120 hour incubation. A) Cells were viewed *in situ* in tissue culture wells using an inverted microscope at 400x magnification at 24 hours and 120 hours. Images were taken using a Leica C-Mount camera and processed with Leica Applications Suite software. B) Following incubation, free (grey bars) and total (white bars) intracellular 7KC were measured via HPLC analysis. Data shown are the mean \pm SEM of triplicates.

3.4.3 The effect of native and modified LDL on U937 cell viability

U937 cells were incubated in RPMI cell medium with a final concentration of 0.2 mg/ml of native and modified LDL for 24 hours before cell viability was assessed using the MTT reduction assay (Figure 3.20). The results of this experiment are expressed as a percentage of the viability of a cell-only control. The cell viability of treatments containing native LDL, oxLDL, acLDL and 7KC-acLDL were 98, 5, 121 and 127% relative to the control, respectively. Native LDL, acLDL and 7KC-acLDL (at 0.2 mg/ml LDL) did not show any significant degree of toxicity to U937 cells. In fact, the viability of cells incubated with acLDL and 7KC-acLDL was slightly increased.

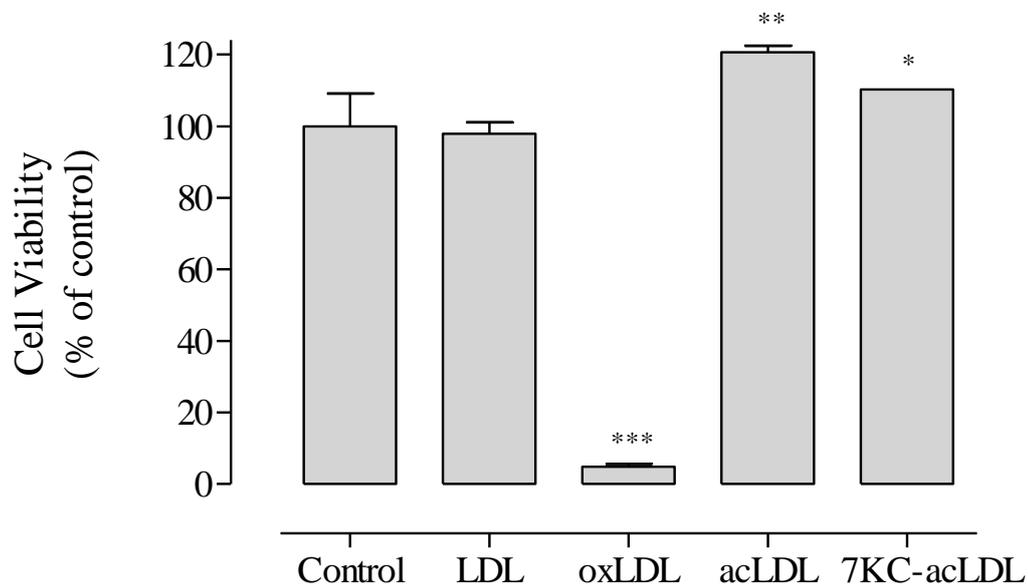


Figure 3.20 U937 cell viability following incubation with native and modified LDL.

U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 without LDL (control) or with 0.2 mg/ml LDL, acLDL, oxLDL or 7KC-acLDL for 24 hours. Results are expressed as a percentage of the control and significance is also indicated from the control. Data shown are the mean \pm SEM of triplicates.

3.5 7-Ketocholesterol-loaded acetylated LDL

3.5.1 7KC-acLDL uptake by U937 cells

U937 cells were incubated with increasing concentrations of 7KC-acLDL for 24 hours before measurement of cell viability using the MTT reduction assay (Figure 3.21). Note that the free 7KC content of every separate 7KC-acLDL preparation was measured by HPLC analysis so that the final concentration of 7KC-acLDL may be presented as mg/ml LDL or as μM 7KC. This result confirms that 7KC-acLDL was internalised by U937 cells and that uptake occurred in a concentration-dependent manner. Incubation with a final 7KC-acLDL concentration of 0.2 mg/ml LDL (equivalent to 18 μM 7KC) resulted in an intracellular free 7KC concentration of 9.4 nmol/ 10^6 cells. The intracellular 7KC content increased as the 7KC-acLDL concentration was increased, reaching a maximum at approximately 20 nmol/ 10^6 cells, induced by 7KC-acLDL concentrations of 1.5 and 2.0 mg/ml LDL (equivalent to 135 and 180 μM 7KC, respectively).

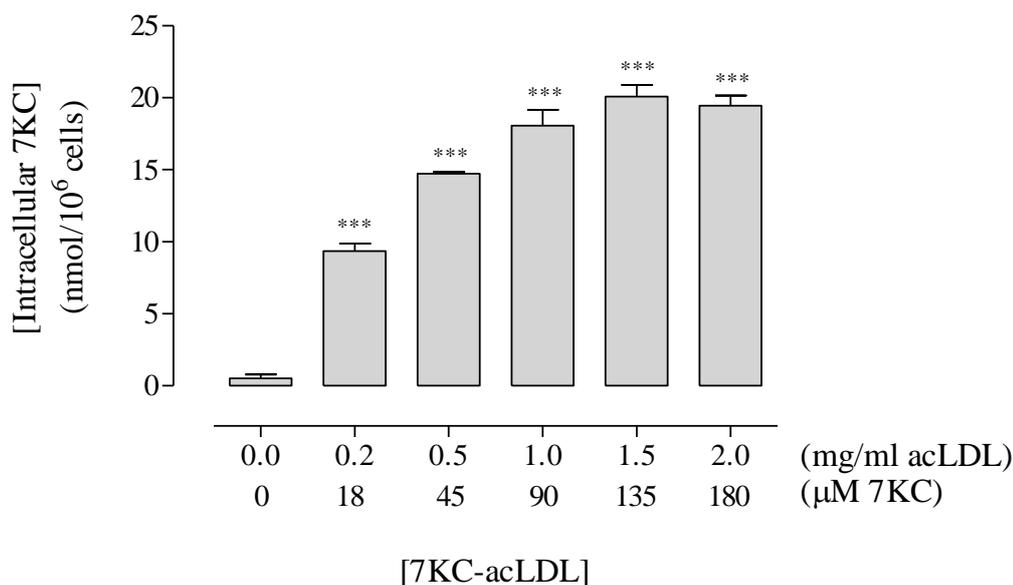


Figure 3.21 The effect of 7KC-acLDL on U937 intracellular 7KC concentration.

U937 cells at 5×10^5 cells/ml were incubated with increasing concentrations of 7KC-acLDL in RPMI-1640 for 24 hours. The 7KC-acLDL preparation used contained 225 mol 7KC/mol LDL. The final concentration of 7KC-acLDL incubated with cells is expressed in both mg/ml acLDL and μM free 7KC. Following incubation, intracellular free 7KC concentration was measured via HPLC analysis. Data shown are the mean \pm SEM of triplicates and significance is indicated from the 0 mg/ml 7KC-acLDL control.

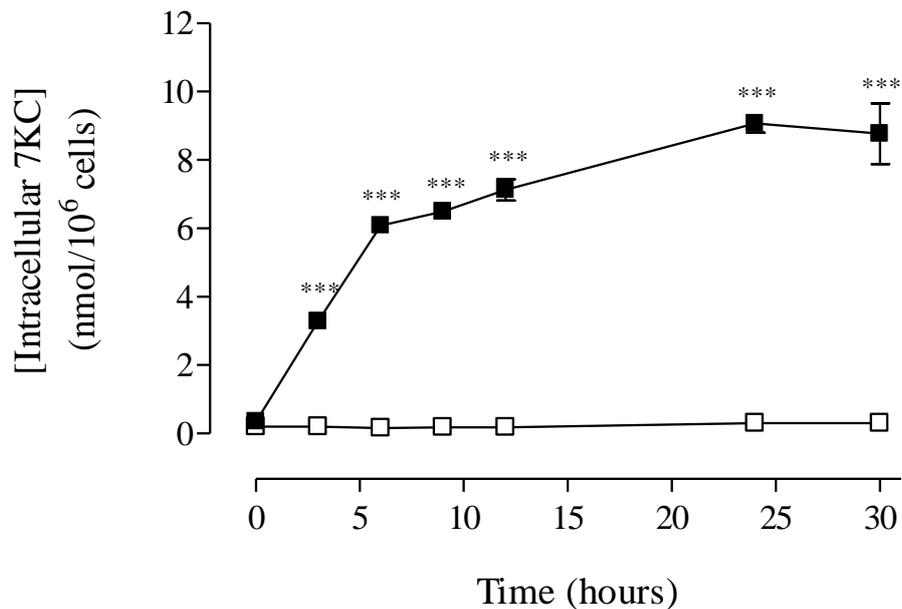


Figure 3.22 Rate of 7KC-acLDL uptake by U937 cells as measured by intracellular 7KC. U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 with (■) and without (□) 0.2 mg/ml 7KC-acLDL. At various times, samples were removed and intracellular free 7KC measured by HPLC analysis. The 7KC-acLDL preparation contained 225 mol 7KC/mol LDL and cells were incubated at a final concentration of 18 μ M 7KC. Data shown are the mean \pm SEM of triplicates. Significance is indicated from the respective control data.

A time course study of the uptake of 7KC-acLDL by U937 cells over a 30 hour time period was performed (Figure 3.22). Intracellular non-esterified 7KC increased rapidly over the initial 6 hours reaching 6 nmol/10⁶ cells and up to 9 nmol 7KC/10⁶ cells after 24 hours, without further increase. All further measurements of 7KC-acLDL uptake have been performed following 24 hour incubation. The progression of 7KC-acLDL uptake (as assessed by intracellular 7KC content) is similar to that observed with oxLDL, with a high proportion of the maximum 7KC content achieved within just 6 hours.

3.5.2 7KC-acLDL and U937 cell viability

Using the same 7KC-acLDL preparation as above (Figures 3.21, 3.22), the corresponding cell viability was measured at the 7KC-acLDL concentrations used previously. Figure 3.23a demonstrates that incubation with 0.2 mg/ml 7KC-acLDL (equivalent to 18 μ M 7KC) did not cause any significant loss of cell viability. Earlier results showed that direct incubation of U937 cells with a similar concentration of 7KC (20 μ M 7KC) caused a 54% decrease in viability relative to the control (Figure 3.6).

Higher concentrations of 7KC-acLDL did appear to cause some degree of cell viability loss, although they are far less than that seen with direct incubation of cells with similar concentrations of 7KC (see Figure 3.24 for a comparison). For example, incubation with 0.5 mg/ml 7KC-acLDL (equivalent to 45 μ M 7KC) caused an 18% decrease in cell viability relative to the control while direct incubation with 40 μ M 7KC caused a 96% decrease in viability. The highest 7KC-acLDL concentration tested (2.0 mg/ml 7KC-acLDL) which is equivalent to 180 μ M 7KC caused only a 47% decrease in viability relative to control cells.

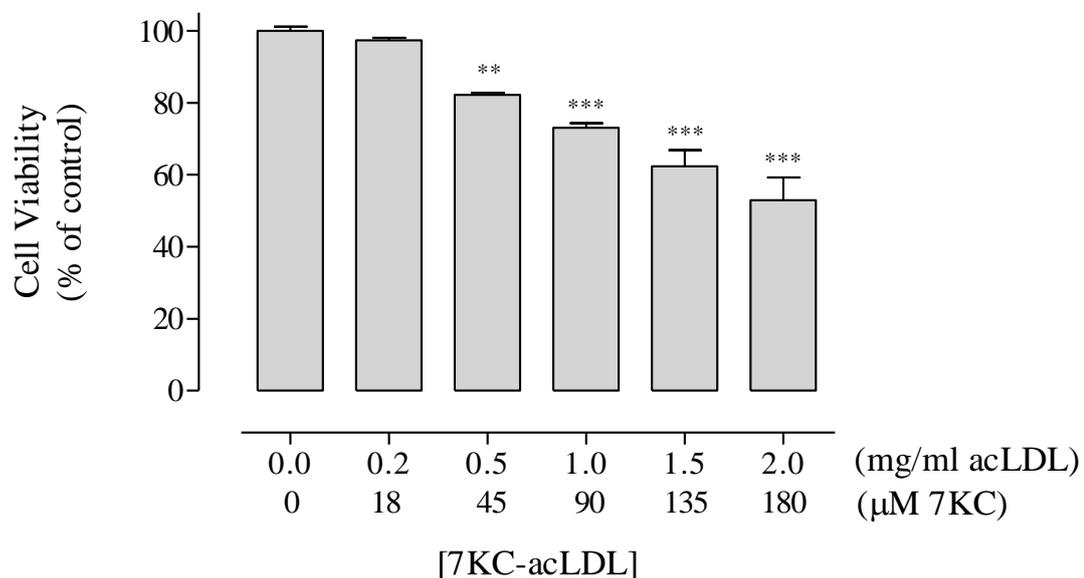


Figure 3.23a The effect of 7KC-acLDL on U937 cell viability.

U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 with increasing concentrations of 7KC-acLDL for 24 hours. Cell viability was measured using the MTT reduction assay. The 7KC-acLDL preparation contained 225 mol 7KC/mol LDL. The final concentration of 7KC-acLDL incubated with cells is expressed in both mg/ml acLDL and μ M free 7KC. Data are expressed as a percentage of the 0 mg/ml 7KC-acLDL control and significance is also indicated from this control. Data shown are the mean \pm SEM of triplicates.

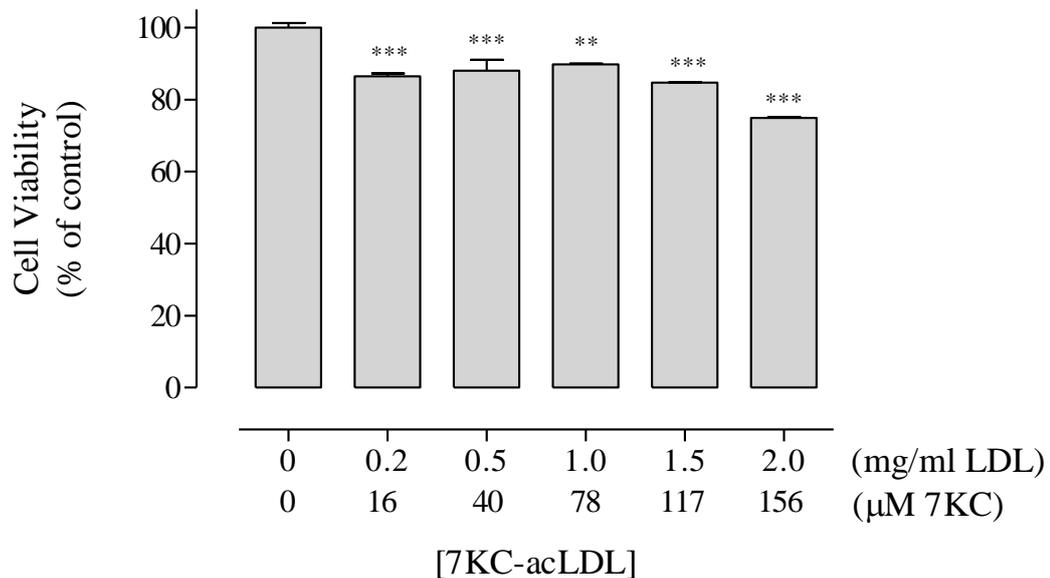


Figure 3.23b The effect of 7KC-acLDL on U937 cell viability.

U937 cells at 5×10^5 cells/ml were incubated with increasing concentrations of 7KC-acLDL in RPMI-1640 for 24 hours. Cell viability was measured using the MTT reduction assay. The 7KC-acLDL preparation contained 195 mol 7KC/mol LDL. The final concentration of 7KC-acLDL incubated with cells is expressed in both mg/ml acLDL and μ M free 7KC. Data are expressed as a percentage of the 0 mg/ml 7KC-acLDL control and significance is also indicated from this control. Data shown are the mean \pm SEM of triplicates.

Figure 3.23b shows the cell viability in response to incubation with a different 7KC-acLDL preparation, which contained a lower free 7KC content. At 0.2 mg/ml 7KC-acLDL, this preparation displayed slightly higher toxicity than that of the previous preparation. However, the overall toxicity induced by incubation at higher concentrations with this 7KC-acLDL preparation was even less than the previous preparation. Incubation with 1.5 mg/ml 7KC-acLDL (equivalent to 117 μ M 7KC) caused just a 15% decrease in viability relative to the control. An equivalent decrease in viability occurred following direct incubation with less than 10 μ M 7KC (Figure 3.6).

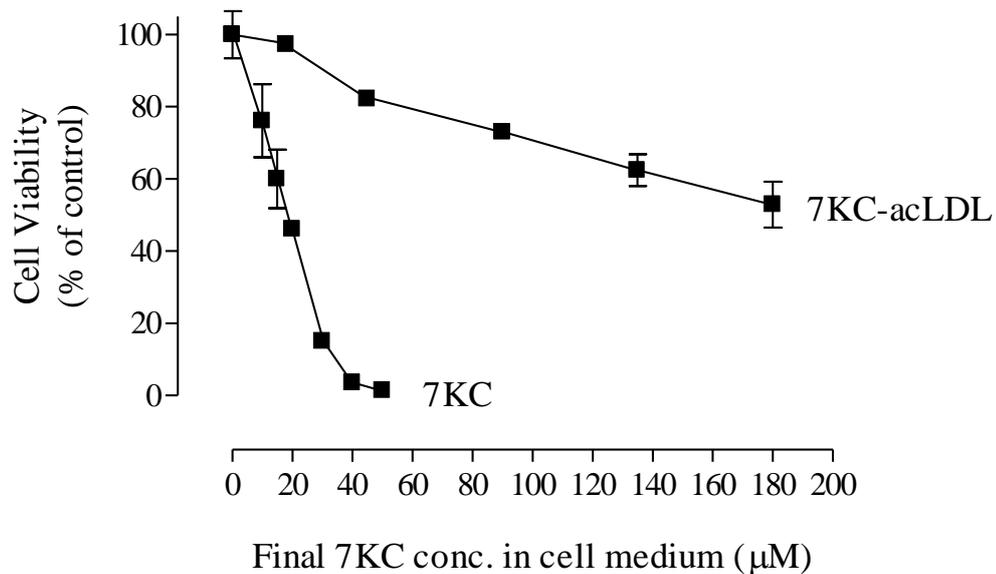


Figure 3.24 A comparison of U937 cell viability with 7KC and 7KC-acLDL.

Data from Figures 3.6 and 3.23a are represented together on this graph. U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 containing increasing concentrations of either 7KC as an ethanol solution or 7KC-acLDL for 24 hours before measurement of cell viability by MTT reduction assay. Data are expressed as a percentage of their respective without-treatment controls. Data shown are the mean \pm SEM of triplicates.

3.5.3 Metabolism of 7KC-acLDL by U937 cells

A previous study using 7KC-acLDL has shown that a high proportion of 7KC becomes esterified following uptake by mouse macrophages (Gelissen *et al.*, 1996). In exploring the metabolism of 7KC-acLDL by U937 cells, intracellular levels of both free and esterified 7KC were measured in response to a 24 hour incubation with 7KC-acLDL (Figure 3.25). In accordance with previous work, these results demonstrate that up to 80% of the total intracellular 7KC detected was esterified. Note that total 7KC represents combined esterified and non-esterified 7KC.

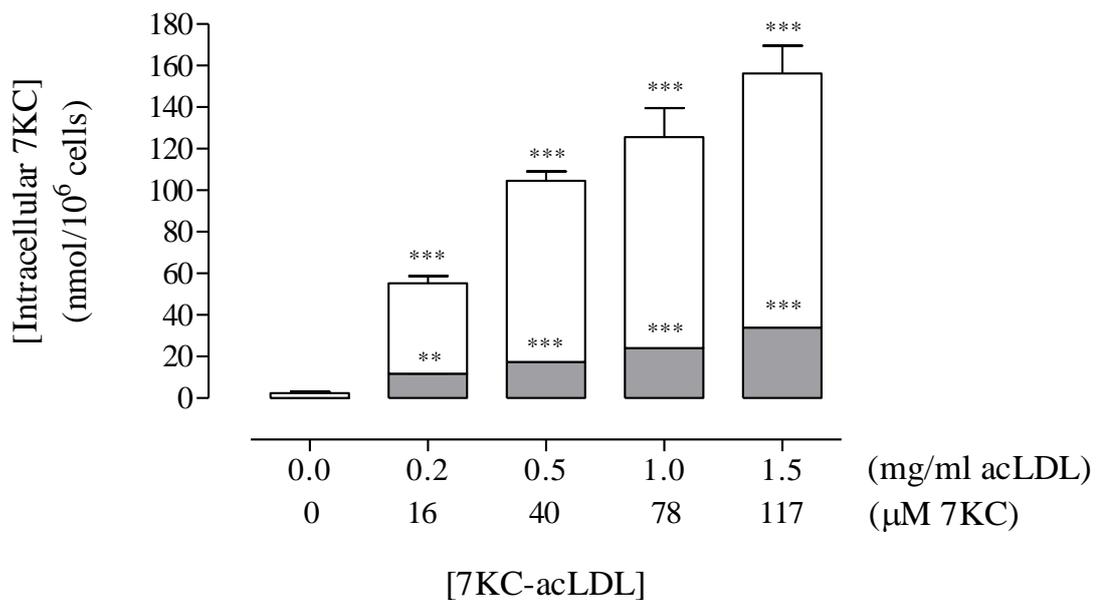


Figure 3.25 The effect of 7KC-acLDL on free and total 7KC in U937 cells.

U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 containing increasing concentrations of 7KC-acLDL for 24 hours. The 7KC-acLDL preparation contained 195 mol 7KC/mol LDL. Following incubation, free (grey bars) and total (white bars) 7KC was measured via HPLC analysis. The final concentration of 7KC-acLDL incubated with cells is expressed in both mg/ml LDL and μM free 7KC. Significance is indicated from the 0 mg/ml 7KC control. Data shown are the mean \pm SEM of triplicates.

A time course study of intracellular free and esterified 7KC was performed during the incubation of U937 cells with 0.2 mg/ml 7KC-acLDL over 30 hours (Figure 3.26). The intracellular free 7KC content increased gradually over 24 hours, before reaching a maximum at approximately 9 nmol/10⁶ cells while the total 7KC (combined free and esterified 7KC) content increased more rapidly to reach an intracellular concentration of 38 nmol/10⁶ cells after 24 hours, before declining to 28 nmol/10⁶ cells after 30 hours.

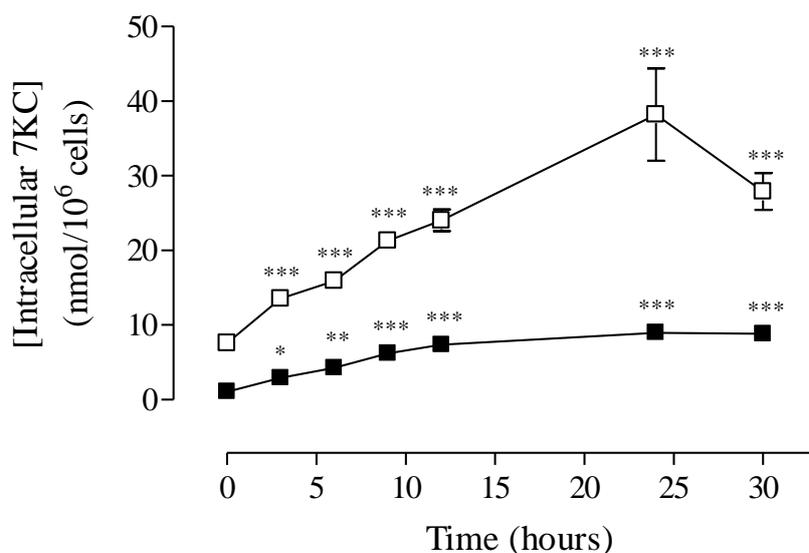


Figure 3.26 Rate of intracellular 7KC esterification during incubation with 7KC-acLDL. U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 containing 0.2 mg/ml 7KC-acLDL (equivalent to 16 μ M free 7KC in cell media) for 30 hours. The 7KC-acLDL preparation contained 195 mol 7KC/mol LDL. At various times, samples were removed and free (■) and total (□) 7KC measured via HPLC analysis. Significance is indicated from the respective 0 hour data. Data shown are the mean \pm SEM of triplicates.

In addition to considering the esterification of 7KC by U937 cells, this work touches upon the question of whether 7KC remains within the cell, is metabolised or effluxed from the cell. U937 cells were incubated in RPMI cell medium containing 0.2 mg/ml 7KC-acLDL (equivalent to 14 μ M 7KC) for 96 hours and the free 7KC content of cells and media was each measured at 24 hour intervals (Figure 3.27). The intracellular 7KC content increased over 48 hours to 4.5 nmol/culture before reaching a plateau. The 7KC content of the cell medium decreased from 22 nmol/culture upon 7KC-acLDL addition, before arriving at a stable level of approximately 15 nmol/culture after 96 hours.

A parallel experiment was conducted with the direct addition of 25 μ M 7KC to the cell culture (Figure 3.28) and showed significant differences compared to incubation with 7KC-acLDL. Intracellular 7KC content increased more gradually, requiring 48 hours to reach the near maximum of approximately 5 nmol/culture. The 7KC content of the culture medium showed a larger and more rapid decrease than that seen with 7KC-acLDL incubation. Media 7KC content decreased from 25.6 to 14.2 nmol/culture over the first 24 hours and then continued a more gradual decrease to reach 10 nmol 7KC/culture after 96 hours.

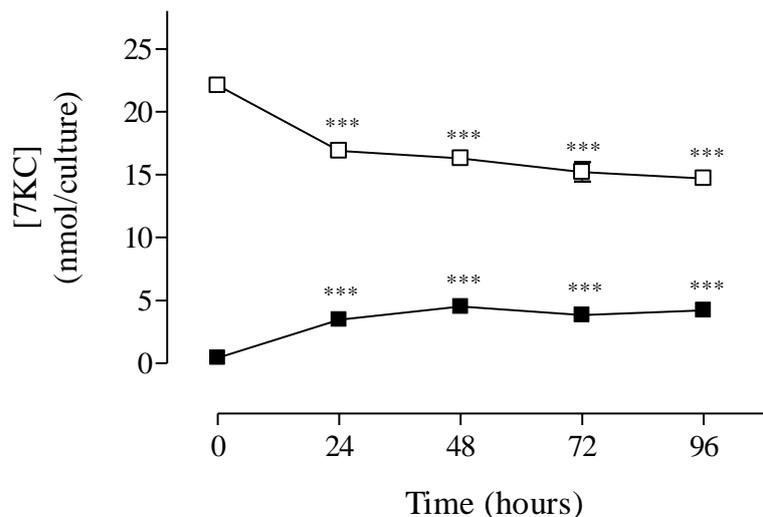


Figure 3.27 Rate of 7KC-acLDL metabolism, as measured by intracellular and media 7KC content. U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 containing 0.2 mg/ml 7KC-acLDL (equivalent to 14 μ M 7KC) for 96 hours. The 7KC-acLDL preparation contained 195 mol 7KC/mol LDL. At various times, samples were removed and free 7KC measured via HPLC analysis. 7KC was measured in cells (■) and in the 1 mL of cell media (□). Significance is indicated from the respective 0-hour data. Data shown are the mean \pm SEM of triplicates.

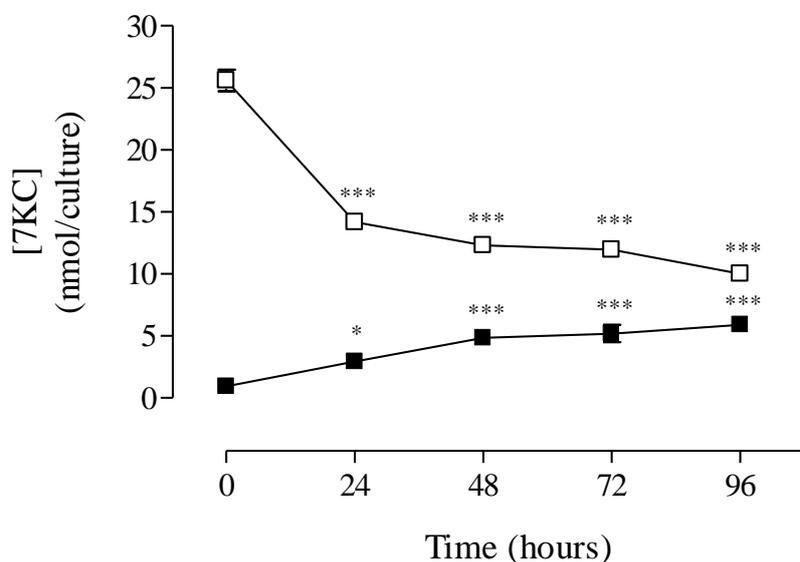


Figure 3.28 Rate of 7KC metabolism, as measured by intracellular and media 7KC content. U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 containing 25 μ M 7KC for 96 hours. At various times, samples were removed and free 7KC was measured via HPLC analysis. 7KC was measured in cells (■) and in the 1mL of cell media (□). Significance is indicated from the respective 0-hour data. Data shown are the mean \pm SEM of triplicates.

3.6 7-Ketocholesterol-loaded oxidised LDL

3.6.1 7KC-oxLDL characterisation

Considering the earlier findings, the higher measured toxicity of oxLDL compared to 7KC-acLDL could possibly result from different processing of the lipoprotein particle by cells. Hence, to eliminate this potentially confounding factor, the toxicity of 7KC-loaded-oxLDL was examined. If 7KC is the toxic agent within oxLDL, we would expect that the selective enrichment of oxLDL with 7KC should increase its toxicity. Human plasma was incubated with 7KC before isolation of 7KC-loaded LDL and subsequent oxidation to create 7KC-oxLDL, by the procedure described in section 2.2. REM and non-esterified 7KC content were examined to confirm that oxidation had occurred and that 7KC had been successfully incorporated into the lipoprotein. The REM of native LDL, oxLDL and 7KC-oxLDL was compared using lipoprotein gel electrophoresis on a non-denaturing gel (Figure 3.29). The relative mobility of native LDL, oxLDL and 7KC-oxLDL was 8, 43 and 19 mm, respectively. Although the oxLDL and 7KC-oxLDL used in this lipoprotein gel were oxidised at the same time and under identical conditions, there is a clear difference in their REM.

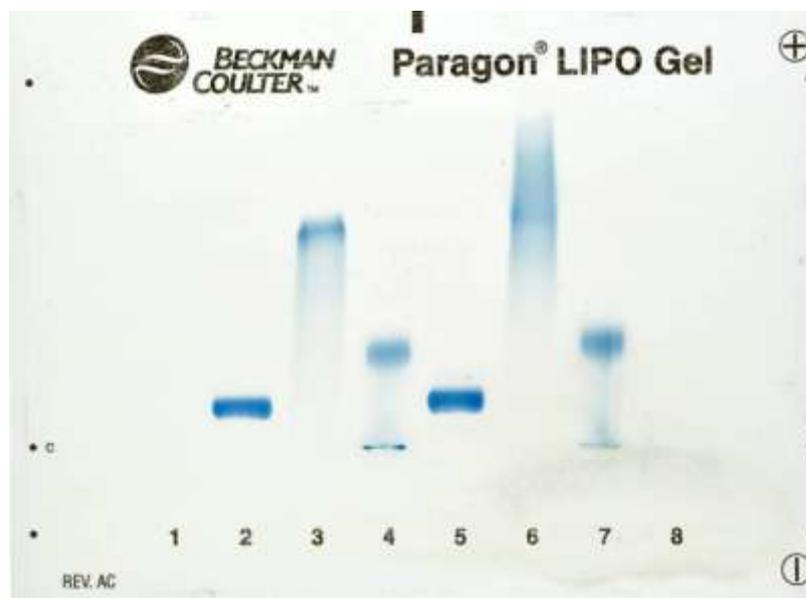


Figure 3.29 Lipoprotein gel of native and modified LDL.

Samples of native and modified LDL at 2.0 mg/ml LDL protein were loaded onto a non-denaturing Paragon lipoprotein gel. Lanes 2 and 5 are native LDL, lanes 3 and 6 are oxLDL and lanes 4 and 7 and 7KC-oxLDL.

The native LDL, oxLDL and 7KC-oxLDL were analysed for non-esterified 7KC content by HPLC analysis (Figure 3.30). No 7KC was detected in native LDL, 87 mol 7KC/mol LDL was measured in oxLDL and 501 mol 7KC/mol LDL measured in 7KC-oxLDL, which represents more than a 5-fold increase in 7KC content as a result of the 7KC incorporation.

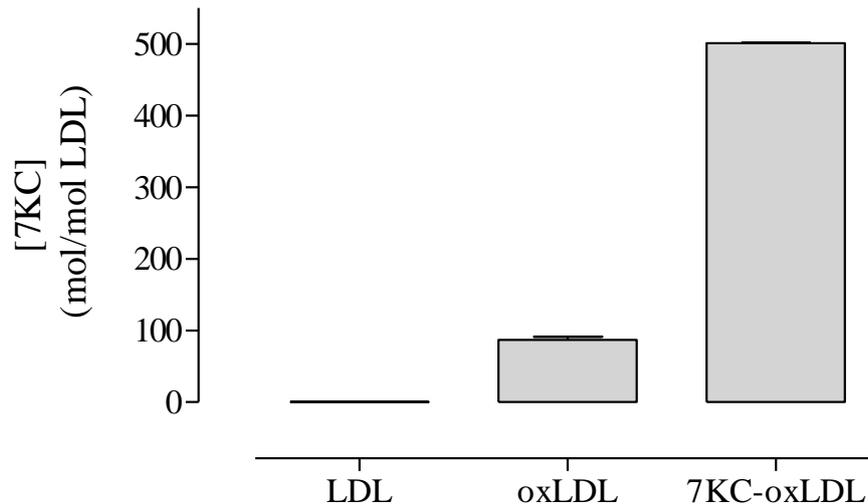


Figure 3.30 7KC content of native and modified LDL.

The free 7KC content of native LDL, oxLDL and 7KC-oxLDL (at 2.0 mg/ml LDL protein) was measured via HPLC analysis. Data shown are the mean \pm SEM of triplicates.

3.6.2 7KC-oxLDL uptake by U937 cells

U937 cells were incubated in RPMI cell medium containing increasing concentrations of 7KC-oxLDL for 24 hours before intracellular non-esterified 7KC was measured by HPLC analysis (Figure 3.31). Cells showed a gradual increase in intracellular 7KC content corresponding to increasing 7KC-oxLDL concentration added to the cell medium. The addition of 0.2 mg/ml 7KC-oxLDL (equivalent to 40 μ M 7KC) resulted in an intracellular 7KC content of 9.5 nmol/ 10^6 cells, which showed little increase until the addition of substantially more 7KC-oxLDL. The addition of 1.5 mg/ml 7KC-oxLDL caused an intracellular 7KC content of 18.4 nmol/ 10^6 cells.

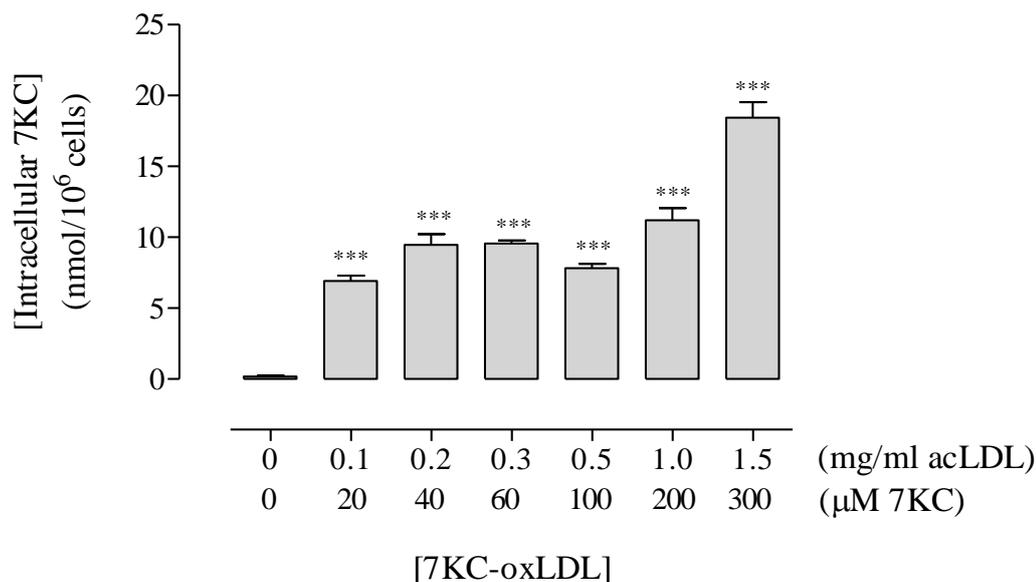


Figure 3.31 The effect of 7KC-oxLDL on intracellular 7KC content of U937 cells.

U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 containing increasing concentrations of 7KC-oxLDL for 24 hours. The 7KC-oxLDL preparation contained 501 mol 7KC/mol LDL. The final concentration of 7KC-oxLDL incubated with cells is expressed in both mg/ml LDL and μ M free 7KC. Following incubation, intracellular free 7KC was measured via HPLC analysis. Significance is indicated from the 0 mg/ml 7KC-oxLDL control. Data shown are the mean \pm SEM of triplicates.

3.6.3 7KC-oxLDL and U937 cell viability

If 7KC is the toxic agent within oxLDL, the selective enrichment of oxLDL with 7KC should make it more cytotoxic. Therefore the effect of incubation with increasing concentrations of 7KC-oxLDL on the viability of U937 cells was analysed (Figure 3.32A). Incubation with 0.1 mg/ml 7KC-oxLDL (equivalent to 20 μ M 7KC) resulted in a small increase in viability. Treatment with 0.2, 0.3 and 0.5 mg/ml 7KC-oxLDL (equivalent to 40, 60, 100 μ M 7KC) caused 11, 12 and 64% decreases in viability relative to the control, respectively. Treatment with 1.0 and 1.5 mg/ml 7KC-oxLDL resulted in near total loss of cell viability.

An experiment was conducted in parallel with the experiment above, so that cells were incubated with increasing concentrations of oxLDL (that was prepared under otherwise identical conditions as the 7KC-oxLDL used above) before cell viability was measured (Figure 3.32B). The results show small differences between the two treatments; such as cell viability differences at the higher end of concentrations tested. Incubation with 1.0 and 1.5 mg/ml oxLDL caused an 83% decrease in viability, while incubation with 1.0 and 1.5

mg/ml 7KC-oxLDL caused near complete loss of viability. Otherwise the trend of cell viability loss is very similar between treatment with oxLDL and treatment with 7KC-oxLDL, which clearly demonstrates that the literature-predicted correlation between increased 7KC oxLDL content and increased cytotoxicity was not observed.

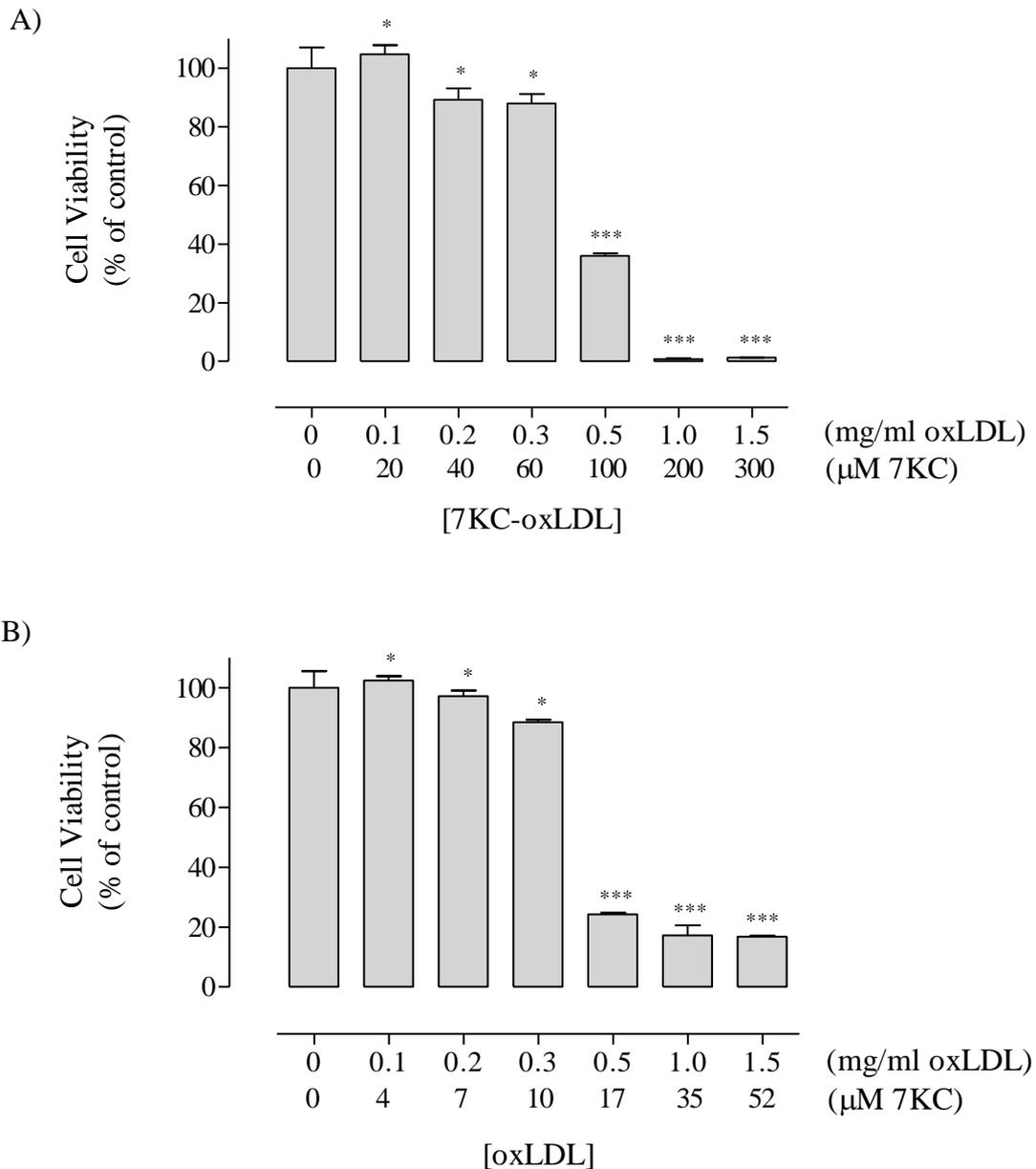


Figure 3.32 The effect of oxLDL and 7KC-oxLDL on U937 cell viability.

U937 cells at 5×10^5 cells/ml were incubated in RPMI-1640 containing increasing concentrations of A) 7KC-oxLDL or B) oxLDL for 24 hours. Cell viability was measured using the MTT reduction assay. The 7KC-oxLDL preparation contained 501 mol 7KC/mol LDL and the oxLDL preparation contained 87 mol 7KC/mol LDL. The final concentration of oxLDL or 7KC-oxLDL incubated with cells is expressed in both mg/ml oxLDL and μM free 7KC. Data are expressed as a percentage of the 0 mg/ml 7KC-oxLDL control and significance is also indicated from the control. Data shown are the mean \pm SEM of triplicates.

4. DISCUSSION

The majority of investigations into the influence of oxysterols on cell cultures so far have involved the direct addition of 7-ketocholesterol and other oxysterols to cell culture media (Christ *et al.*, 1993, Lizard *et al.*, 1998, Nishio *et al.*, 1996). The principal limitation of these studies is that their relevance to the likely physiological means of oxysterol delivery to cells has not been fully considered. Oxysterols are normally associated with lipoproteins *in vivo*, and more specifically, they are thought to be located within the core of the lipoprotein particle (Addis *et al.*, 1989, Javitt *et al.*, 1981, Lin and Morel, 1996). This research instead used 7-ketocholesterol-enriched high-uptake lipoprotein to test the toxicity of 7-ketocholesterol to U937 cells. Across all concentrations, the toxicity of 7-ketocholesterol was greatly reduced when incorporated into acLDL. The enrichment of oxLDL with 7-ketocholesterol also failed to significantly increase the toxicity to U937 cells.

4.1 7KC-enriched LDL and U937 cells

Oxysterol-enriched, high-uptake LDL has been used to explore the effect of 7-ketocholesterol on sterol efflux in mouse J774A.1 macrophages. It was found that 7-ketocholesterol-enriched acetylated LDL (7KC-acLDL) impaired the normal efflux of intracellular sterols to the extracellular acceptor apoA-1, leading to the accumulation of intracellular cholesterol and cholesteryl esters (Gelissen *et al.*, 1996). Although these findings implicate 7-ketocholesterol in the formation of lipid-laden foam cells and hence the fatty streak, they do not link 7-ketocholesterol to the mechanism of oxLDL-induced cytotoxicity and corresponding necrotic core development, which is the focus of this study.

A detailed investigation into the influence of 7KC-acLDL on U937 cell viability revealed that the effect of 7-ketocholesterol on U937 cells, when delivered within lipoprotein, is not comparable to that measured following the direct incubation of 7-ketocholesterol with the cell culture medium. Incubation with 7KC-acLDL induced dramatically lower toxicity to U937 cells compared to 7-ketocholesterol (Figure 3.23), even though similar levels of intracellular 7-ketocholesterol were achieved via each method of delivery (Figure 3.21). To

understand the extent to which the 7-ketocholesterol delivery method affected cell viability, it is necessary to compare the data directly. Incubation of U937 cells with 7-ketocholesterol, as an ethanol solution, at final concentrations of 20 and 40 μM resulted in an intracellular 7-ketocholesterol content of 16 and 17 $\text{nmol}/10^6$ cells and corresponding loss of cell viability of 54 and 98% relative to the control. In contrast, incubation of U937 cells with 7KC-acLDL at similar final 7-ketocholesterol concentrations of 18 and 45 μM resulted in intracellular 7-ketocholesterol content of 9.4 and 15 $\text{nmol}/10^6$ cells and cell viability loss of 0 and 18%, respectively. Across all concentrations tested, the toxicity of 7-ketocholesterol was greatly reduced when incorporated into high-uptake acLDL. The overall levels of intracellular 7-ketocholesterol achieved with 7KC-acLDL incubation were up to 5 times greater than that achieved with oxLDL, yet oxLDL was far more cytotoxic. The concentrations of 7-ketocholesterol that were tested in this study exceed those measured in *ex vivo* advanced atherosclerotic plaque tissue. Reports of 7-ketocholesterol levels in advanced atherosclerotic plaque are in the range of 20-50 nM 7-ketocholesterol (Iuliano *et al.*, 2003).

The relatively small extent of cell viability loss induced by incubation of U937 cells with high concentrations of 7KC-acLDL may be partly accounted for simply by the addition of a large proportion of non cell culture medium. For example, a cell treatment incubated with 2.0 mg/ml 7KC-acLDL contained 200 μl of 7KC-acLDL (at 10.0 mg/ml) of a total 1000 μl culture and therefore contained significantly less RPMI-1640 cell culture medium to sustain normal maintenance and growth over a 24-hour period. A control treatment in an investigation by Warner *et al.* (1995) that involved incubation of mouse peritoneal macrophages with 0.5 mg/ml acLDL, free cholesterol and bovine serum albumin, caused a decrease in cell viability (measured via adenine release) of more than 10% over a 36-hour incubation. It would be worthwhile conducting parallel cell viability studies using equally high concentrations of acLDL as an additional control.

Preliminary experiments were performed using 7KC-acLDL to characterise its composition (Figures 3.15, 3.16) and confirm its uptake by U937 cells (Figure 3.21). The copper ion-mediated oxidation of LDL is typically characterised by an increase in relative electrophoretic mobility (REM) which is caused by the modification of apoB-100 residues; primarily lysine. The reaction of such positively-charged residues with short chain

aldehydes such as 4-HNE and MDA, results in an increase in net electronegativity, which corresponds to greater migration through the lipoprotein gel. As expected, this mobility increase relative to native LDL was observed with oxLDL, acLDL and 7KC-acLDL. It is important to note that the incorporation of 7-ketocholesterol did not influence REM of acLDL, demonstrating that no major changes to the lipoprotein structure or charge had occurred.

Time course studies of 7KC-acLDL uptake by U937 cells provided additional evidence for the incorporation of 7-ketocholesterol within low density lipoprotein. The results illustrated a rapid increase in intracellular 7-ketocholesterol that occurred primarily within the first 6 hours of 7KC-acLDL incubation, congruent with the kinetic progression of intracellular 7-ketocholesterol increase seen during oxLDL incubation. The intracellular 7-ketocholesterol concentration achieved following a 24-hour incubation with 2.0 mg/ml 7KC-acLDL, was 5-fold higher than that achieved following the equivalent oxLDL treatment. Furthermore, consistent with previous findings, the high levels of 7-ketocholesterol esterification following incubation with 7KC-acLDL indicate that cells must have actively metabolised the non-esterified 7-ketocholesterol, which excludes the possibility that oxysterols were bound only at the cell surface (Gelissen *et al.*, 1996).

Further experiments exploring 7-ketocholesterol esterification were conducted to investigate the metabolism of 7KC-acLDL by U937 cells. The results showed that a very high proportion of intracellular 7-ketocholesterol had become esterified during a 24-hour incubation period with 7KC-acLDL (Figures 3.24, 3.25). Oxysterol esterification is likely to have implications for the physiological activity of the oxysterol. The esterification of oxysterols occurs mainly by the action of intracellular acyl coenzyme A: cholesterol acyltransferase (ACAT), an enzyme whose primary role is the conversion of excess cytosolic cholesterol to cholesterol esters within the cell. 7-Ketocholesterol is actually the preferred substrate for intracellular ACAT ahead of cholesterol in mouse J774A.1 macrophage cells (Gelissen *et al.*, 1996). Consistent with this result is the finding that the majority of several oxysterols were also esterified in atherosclerotic plaque samples, and in particular, 83% of total 7-ketocholesterol was esterified (Brown and Jessup, 1999). Such esterification of oxysterols is thought to alter their distribution and association with plasma proteins, in turn, influencing their delivery to cells and tissues. It has been confirmed, using

25-hydroxycholesterol as a model, that the esterification of oxysterols in serum decreases their solubility and immobilises them within the lipoprotein, limiting the diffusional transfer of serum oxysterols to cell membranes (Lin and Morel, 1996). Considering that the esterification of 25-hydroxycholesterol increases its hydrophobicity, which in turn causes more tight and regular packing of the lipoprotein droplet, this provides support for the stable association of non-polar 7-ketocholesterol with lipoprotein *in vivo*. Since previous studies have indicated that the esterification of oxysterols may provide a protective function against oxysterol cytotoxicity, the majority of this investigation was based on detection and measurement of levels of non-esterified 7-ketocholesterol.

Repeated attempts were made to image the loading of U937 cells with different types of modified LDL using oil red-O, a lysochrome diazo dye typically used for staining neutral triglycerides and lipids. The method was adapted from work done by Davies *et al.* (2005) who had successfully employed the staining technique on human vascular smooth muscle cells (vSMCs) and oil red-O was subsequently used for the successful staining of lipids within human monocyte-derived macrophage cells (Amit, 2008). The above experiments both involved differentiated and adherent cell types. The literature contains reports of successful oil red-O staining only in differentiated, adherent cell types, not suspension monocytes. This may offer an explanation for the lipid-staining results observed in this study. Recent work has highlighted the numerous structural changes that occur during the PMA-induced differentiation of U937 human monocyte cells into adherent macrophage cells (Sintiprungrat *et al.*, 2010). These differences may account for the minimal uptake of oil red-O dye by U937 human monocytes.

The U937 human monocyte cell line was selected for use in this model system since monocytes are involved in the primary and ongoing interaction with modified lipoproteins within the artery wall (Lusis, 2000) and lipid-laden foam cells are known to arise from monocyte-derived macrophage cells (Aqel *et al.*, 1984). Most importantly, this laboratory has previously shown that the oxLDL-induced cell death mechanism in U937 cells is the same as that seen in human monocyte-derived macrophages prepared from human blood (Baird *et al.*, 2004). In both cell types, oxLDL causes large reactive oxygen species-induced oxidative stress, loss of cellular glutathione and oxidative loss of regulatory metabolic enzymes, triggering caspase-independent necrosis (Giesege *et al.*, 2009b). U937

cells are a commonly used atherosclerotic model since they allow for the investigation of relatively homogeneous groups of cells, compared to the more heterogeneous nature of comparatively small quantities of human monocyte-derived macrophages isolated from human blood samples. U937 cells have a doubling time of between 3-4 days depending on the presence of serum in the cell culture medium. The use of a rapidly growing cell line over relatively long incubation periods means that cell growth must be taken into consideration during experimental design. For this reason, experiments were designed such that the effects of cell proliferation were minimised; for example, data were expressed as a percentage of, or relative to cell-only control treatments.

In light of the finding that 7-ketocholesterol cytotoxicity is greatly reduced when incorporated into high-uptake acetylated LDL, the possibility remains that the difference in toxicity may be caused by differences in the handling of the differently modified lipoproteins by U937 cells. The toxicity of 7-ketocholesterol-loaded oxLDL (7KC-oxLDL) was therefore tested. If 7-ketocholesterol is the toxic agent within oxLDL, then the selective enrichment of oxLDL with high concentrations of 7-ketocholesterol should increase its cytotoxicity.

7KC-oxLDL contained more than a 5-fold greater quantity of non-esterified 7-ketocholesterol compared to oxLDL and was taken up by U937 cells in a dose-dependent manner, achieving similar intracellular 7-ketocholesterol levels as those achieved following 7KC-acLDL incubation (Figures 3.21, 3.31). 7KC-oxLDL and oxLDL displayed very similar toxicity to U937 cells at the lower and mid-range concentrations tested (Figure 3.32). 7KC-oxLDL induced slightly lower toxicity than oxLDL to U937 cells at a concentration of 0.5 mg/ml oxLDL. Although 7KC-oxLDL displayed slightly increased toxicity at the highest concentrations tested, compared to the oxLDL from the same preparation, the differences in cell viability loss were relatively minor in both cases and occurred well beyond the oxLDL and 7KC-oxLDL median lethal dose. It was only at extremely toxic concentrations of oxLDL (80% viability loss and above), that the corresponding 7KC-oxLDL induced further loss of viability. It is important, however, to take into consideration the variability of oxLDL-induced toxicity measured between different preparations. For example, if the toxicity induced by incubation with 7KC-oxLDL was compared to that of oxLDL from a different preparation (Figure 3.1), it would

appear to have relatively lower cytotoxicity. The median lethal dose of oxLDL is commonly known to vary from one preparation to the next although the exact cause is unknown (Giesege *et al.*, 2009a). Overall, the toxicity of 7KC-oxLDL measured in U937 cells following a 24-hour incubation period, was similar to that measured following oxLDL incubation, which attributes no toxicity to the extensive 7-ketocholesterol loading of the LDL.

Physical characteristics of 7KC-oxLDL were compared to those of oxLDL (non-loaded) which contained known concentrations of non-esterified 7-ketocholesterol. Characterisation of the REM of 7KC-oxLDL showed an increase of 2.4-fold relative to native LDL, while the REM of non-loaded oxLDL was more than 5-fold that of native LDL. The observed alteration in gel migration of 7KC-oxLDL relative to oxLDL may be due to the presence of massive concentrations of 7-ketocholesterol within the lipoprotein, resulting in altered particle mass. Previous comparison of acLDL and 7KC-acLDL REM showed that the presence of 7-ketocholesterol did not induce any major changes in structure or charge (Figure 3.15). For this reason, it would have been worthwhile to have assessed the relative electrophoretic mobility of 7KC-LDL as a further control. Subsequent experiments confirmed that the 7KC-oxLDL was rapidly taken up by U937 cells, indicating that sufficient modification of apoB-100 had occurred, in spite of the unexpected REM measurement. Approximately 15 - 20% of exposed apoB-100 lysine residues must be blocked for recognition by scavenger receptors to occur (Haberland *et al.*, 1984). Alternatively, research groups have assigned a 3-fold increase in LDL REM (for acetylation or copper ion-mediated oxidation) as an indication of sufficient lipoprotein modification to generate the high-uptake lipoprotein (Gelissen *et al.*, 1996).

In the case that 7-ketocholesterol was the toxic agent within oxLDL, then one would expect that the selective enrichment of oxLDL with high concentrations of 7-ketocholesterol should have increased cytotoxicity. This study found otherwise, which suggests that 7-ketocholesterol is not the cytotoxic agent of oxLDL.

4.2 OxLDL-induced damage in U937 cells

This investigation began by examining the effect of oxLDL on U937 cells, in terms of cell viability and glutathione content. As has been demonstrated by previous studies, oxLDL showed a high degree of toxicity to U937 cells, with the median lethal dose found to be between 0.1-0.2 and up to 0.5 mg/ml oxLDL, depending upon the individual oxLDL preparation. This laboratory has previously obtained results to indicate that the equivalent median lethal dose was between 1.5 and 2.0 mg/ml oxLDL (Baird, 2003). A likely explanation is that the different method of copper ion-mediated LDL oxidation caused the observed difference in cytotoxicity. The recently adapted method for LDL oxidation employed in this study eliminates the possibility of decreased yield during processing, and instead involves contained dialysis against copper ions at 37 °C (Gerry *et al.*, 2008). The concentrations of oxLDL used in the experiments are within the range of LDL concentrations *in vivo*, as serum concentrations of LDL in normolipidemic persons are maintained at approximately 3.0 mg/ml (Esterbauer *et al.*, 1992).

Time course studies indicated that the loss of cell viability caused by oxLDL occurred rapidly, within 6 hours (Figure 3.3). This progression for viability loss was very closely correlated with loss of cellular glutathione content (Figure 3.5), although loss of viability appeared to occur slightly ahead of cellular glutathione loss. Glutathione loss provides a direct measure of the cellular antioxidant status and it has been established previously that cellular glutathione loss results in a further increased susceptibility to oxidative stress (Ballatori *et al.*, 2009). OxLDL clearly induces an oxidative stress in U937 cells, causing reduced glutathione content in parallel with reduced cell viability, which is consistent with previous findings by this laboratory (Baird, 2004).

In exploring the mechanism of action of oxLDL to U937 cells, it is necessary to consider firstly whether oxLDL is actually internalised by cells. Although there has been debate as to whether U937 cells express scavenger receptors necessary for the unregulated uptake of oxLDL, it is now widely accepted that they do. The CD36 receptor (belonging to scavenger receptor class B) has been shown to internalise oxLDL in U937 cells (Pietsch *et al.*, 1996). Expression of class A scavenger receptors has been shown to occur during PMA-induced differentiation of U937 monocytes to macrophages (Banka *et al.*, 1991,

Shimaoka *et al.*, 2000) and differentiation of U937 cells by 0.1 mg/ml oxLDL was shown to induce scavenger receptor expression (Lei *et al.*, 2002) which indicates that oxLDL is actively absorbed by U937 cells. The current hypothesis for oxLDL cytotoxicity is that it causes high levels of intracellular oxidative stress, including the formation of highly reactive oxidised lipid and protein species that overwhelm the cells' antioxidant capacity, inducing imbalances in the intracellular redox environment, changes in various signalling pathways and gene expression, leading to cell death via caspase-independent necrosis (Baird, 2003). The mechanism of cellular damage activated by oxLDL is of central importance to this research and also provides for limitless discussion and so this examination of oxLDL-induced cell viability and glutathione loss must be limited to the confirmation of previous findings.

This set of experiments also confirmed that oxLDL-loaded U937 cells contain levels of non-esterified 7-ketocholesterol that are proportional to the degree of oxLDL-loading (Figure 3.12), demonstrating that intracellular non-esterified 7-ketocholesterol content may be a reliable marker for the uptake of oxLDL by cells (for this method of copper ion-mediated LDL oxidation). In an attempt to explore the origin of 7-ketocholesterol during the lipoprotein loading of cells, U937 cells were loaded with acLDL before cell death was induced by hypoxia over 120 hours (Figure 3.19). It was anticipated that 7-ketocholesterol would be generated in acLDL-loaded cells during the cell death process, but the results showed no significant difference in free and total 7-ketocholesterol levels between the treated and control samples, although significant levels of esterified 7-ketocholesterol were detected in both treatments. The failure of this experiment to detect a significant increase in 7-ketocholesterol in acLDL-loaded cells, may have been because cell death progressed past apoptosis to secondary necrosis, causing the rupture of cell membranes and the release of intracellular 7-ketocholesterol into the cell medium. Previous work by Fu *et al.*, (2001) demonstrated that oxysterols can be generated in cells loaded *in vitro* with cholesterol. Following a 72-hour incubation of HMDM cells with acLDL, they observed a 13-fold increase in cellular cholesterol and detectable levels of various oxysterols including 7-ketocholesterol. However, it was not shown that cell death induced an increase in cellular oxysterol content (Fu *et al.*, 2001).

4.3 7KC-induced damage in U937 cells

The purpose of examining cell viability and cellular glutathione content in response to direct incubation with 7-ketocholesterol, was for the confirmation of previous findings and to establish the precise concentrations (both in media and intracellular) that induce toxicity in U937 cells to serve as a comparison for the parallel treatment with 7KC-acLDL.

The potential cyto-protective effect of heat-inactivated foetal bovine serum (HI-FBS) to oxLDL- and 7-ketocholesterol-treated cells was examined. HI-FBS was shown to exert high levels of protection to U937 cells at the lower end of oxLDL and 7-ketocholesterol concentrations tested; in terms of both cell viability and GSH loss (Figures 3.2, 3.4, 3.7). HI-FBS is a complex supplement (normally contained in the culture medium for cell-lines) that contains substances such as albumin, globulin, transferrin and growth factors and various antioxidants that typically favour cell growth (Dominguez *et al.*, 2002). Serum is also known to contain extracellular cholesterol acceptors such as high density lipoprotein (HDL) that promote cholesterol efflux from cells and consequently interfere with cholesterol processing and the potential for foam cell generation. Accordingly, HI-FBS is normally excluded from cell experiments because of its highly complex nature and potential for involvement/interference in the interactions of interest, and was therefore excluded from the majority of experiments.

Mechanism of 7KC-induced cell death

Time course studies of 7-ketocholesterol-induced cell viability loss showed that the decrease in cell metabolic energy as measured by the MTT assay, did not occur immediately but occurred gradually over 28 hours, demonstrating a clear lag phase. This progression is in contrast to the pattern of cell viability loss observed during incubation with oxLDL, which occurred rapidly, during the first 9 hours of incubation. It is clear that different mechanisms of U937 cell death are activated by 7-ketocholesterol and oxLDL. A point of similarity between the two mechanisms however, is that the rate of cellular glutathione loss reflected very closely the rate of cell viability loss, in both cases. Such a close relationship between cellular glutathione loss and viability loss generally indicates the involvement of radical oxygen species. In support of a role of reactive oxygen species in 7-ketocholesterol-induced U937 cell death, the addition of GSH or its precursor, N-

acetylcysteine, was reported to halve the loss of viability that occurs with 7-ketocholesterol (Lizard *et al.*, 1998). Furthermore, Leonarduzzi *et al.*, (2006) demonstrated proof for a primary role of NADPH-oxidase in the over-production of reactive oxygen species in murine macrophages treated with 7-ketocholesterol, by showing that two different NADPH inhibitors prevented 7-ketocholesterol induced cell death in J774A.1 cells.

In this study, the water-soluble antioxidant, 7,8-dihydroneopterin (7,8-NP), was used as a probe for investigating the mechanism of 7-ketocholesterol cytotoxicity to U937 cells. Previous work by this laboratory has shown that 7,8-NP protects U937 cells and human monocyte-derived macrophages from oxLDL-induced cell death by scavenging intracellular oxidants, independently of other antioxidants such as glutathione or α -tocopherol (Baird *et al.*, 2004, Baird *et al.*, 2005). With the previous findings that suggest a role for reactive oxygen species in 7-ketocholesterol-induced cell death, this experiment aimed to determine whether reactive oxygen species represent a cause or product of 7-ketocholesterol-induced cell death. It was found that 7,8-NP does not protect U937 cells from the cytotoxic effect of 7-ketocholesterol (Figure 3.14), which suggests that intracellular oxidative stress was not a key factor in driving cell death. Considering that 7,8-NP prevents intracellular oxidative stress in oxLDL-treated U937 cells, it is likely that much of the cytotoxic effect of 7-ketocholesterol, when incubated directly with the cell culture medium, may occur on the plasma membrane. This finding is consistent with the body of evidence supporting the concept that 7-ketocholesterol exerts its cytotoxic effect via its interference with lipid raft micro-domains within the plasma membrane (Berthier *et al.*, 2004, Massey and Pownall, 2005). The finding that 7-ketocholesterol exerts its toxicity via interaction with the plasma membrane is enough to suggest that 7-ketocholesterol is unlikely to be the toxic component of oxLDL. The location of 7-ketocholesterol within lipoproteins has been suggested to provide a protective function against the cellular actions of the oxysterol by limiting direct access to the cell (Lin and Morel, 1996). It is thought that the accumulation of 7-ketocholesterol within plasma membrane lipid raft domains causes translocation of the Trpc-1 protein to lipid rafts to form store operated Ca^{2+} entry channels that permit calcium influx into the cytosol, initiating the associated calcium-dependent apoptotic cascade (Berthier *et al.*, 2004). These findings indicate that oxLDL and 7-ketocholesterol exert toxicity via different mechanisms and therefore, that oxLDL toxicity is unlikely to be dependent on 7-ketocholesterol toxicity.

4.4 Mechanism of oxLDL-induced cell death

7KC-acLDL had a much lower toxic effect on U937 cells than did similar concentrations of intracellular 7-ketocholesterol resulting from the direct addition of 7-ketocholesterol to the cell culture medium. Furthermore, the treatment of cells with 7KC-oxLDL, with 5-fold greater 7-ketocholesterol content than oxLDL, induced similar loss of cell metabolic activity compared to oxLDL. Additional toxicity of 7KC-oxLDL relative to oxLDL was only observed following treatment with massive and non-physiological concentrations of the oxysterol. The above findings indicate that 7-ketocholesterol, and possibly oxysterols in general, are not the cytotoxic agent of oxLDL, which raises the question, what is the cytotoxic component of oxLDL?

In a review of oxidative modifications (Stocker and Keaney, 2004), it was highlighted that the correlation between levels of oxidised lipids and lesion development, as predicted by the widely accepted oxidative modification hypothesis, is not observed in either *ex vivo* or *in vitro* studies. Together with the results of this study, it seems appropriate that alternative mechanisms of oxLDL cytotoxicity be considered. Although there is solid evidence to suggest that lipid oxidation and toxicity are linked (Clare *et al.*, 1995), indications that protein oxidation may play an important role in cytotoxicity are numerous (Hazell *et al.*, 1996, Stocker and Keaney, 2004, Upston *et al.*, 2002). This provides an opening for the suggestion that the products of protein oxidation may be at the heart of oxLDL toxicity and its role in the development of the necrotic core.

Protein oxidation

This laboratory has shown previously that protein hydroperoxide formation is tightly coupled to lipid oxidation during both copper- and AAPH-mediated LDL oxidation. Also, that THP-1 monocyte cell-mediated LDL oxidation induces the generation of significant levels of protein hydroperoxides (Gieseg *et al.*, 2003). Considering that protein hydroperoxides are found in atherosclerotic plaque (Fu *et al.*, 1998), are known to consume protective cellular thiols and ascorbate (Gebicki, 1997) and can give rise to further radicals and damage other target molecules (Fu *et al.*, 1995, Gebicki, 1997), evidence for a role of such protein oxidation products in oxLDL toxicity seems plausible.

Proteins react readily with hydroxyl, peroxy and alkoxy free radicals, and with oxidants such as hypochlorite, nitric oxide, peroxy nitrite and singlet oxygen (Dean *et al.*, 1997). The ability of many proteins to react with and stabilise metals allows for the formation of very reactive products, leading to the generation of damaged proteins that have the ability to cause further damage to cell components. This was confirmed by the identification of high levels of protein hydroperoxides in cells that had been attacked by reactive oxygen species (Giese *et al.*, 2000). Detection of high concentrations of protein hydroperoxides on apoB-100 occurred in response to copper-, 2,2'-azobis(amidinopropane) dihydrochloride (AAPH)-generated peroxy radical- and cell-mediated LDL oxidation. Protein hydroperoxides can give rise to further radicals that might react with other biological components, thereby propagating damage. In support of this finding, proteins were subsequently identified as the initial cell targets of hydroxyl radicals. The few studies carried out that investigate the kinetic progression of such damage show that protein oxidation is an early event in ROS-initiated events (Du and Gebicki, 2004). The derivatisation of protein is known to occur at the same time that LDL becomes toxic and also correlates with the conversion of LDL to the high-uptake form (Du and Gebicki, 2004).

Of direct relevance is an investigation that aimed to distinguish and examine processes resulting from the direct oxidation of protein (as compared to the derivatisation of lysine residues on apoB-10). The study examined which oxidative species might contribute to protein-bound oxidation products such as chlorotyrosine, dityrosine, 3,4-dihydroxyphenylalanine (DOPA), o-tyrosine and m-tyrosine, that are detected in advanced atherosclerotic plaque. Their findings indicated that hydroxyl radical-induced damage to protein is a plausible influence in atherosclerotic plaque (Fu *et al.*, 1998). Their findings also raised the issue of the source of hugely increased dityrosine levels that are measured in atherosclerotic plaques. Levels of dityrosine (a well-established hallmark for oxidised protein levels) have been shown to increase significantly from the early to late stages of plaque development (Upston *et al.*, 2002). A role for the tyrosyl radical in protein oxidation during plaque development has been confirmed, with the detection of a 100-fold increase in o,o'-dityrosine in plaque-isolated LDL compared to plasma LDL (Leeuwenburgh *et al.*, 1997). Considering the above findings and that active myeloperoxidase, a known source of tyrosyl radicals, is present in human atherosclerotic

tissue, the myeloperoxidase/hypochlorite-generated tyrosyl radical-oxidation of LDL protein components may contribute to the pathological behaviour of oxidised LDL *in vivo*. Furthermore, various studies have used hypochlorite to selectively attack and modify protein components of LDL, an oxidation method which results in only trace amounts of oxysterols (Ermak *et al.*, 2010). OxLDL generated in such a manner, with a near absence of oxysterols, displays a high level of toxicity to U937 cells, comparable to that of copper-ion oxLDL, inducing apoptosis via the classic mitochondrial caspase-dependent pathway (Ermak *et al.*, 2010). This result, consistent with the findings of the present research, demonstrates clearly that oxysterols are not a necessary component of highly cytotoxic oxLDL.

Protein oxidation and uptake by scavenger receptors

The unregulated uptake of LDL via scavenger receptors, in the formation of lipid-laden foam cells characteristic of the fatty streak, is necessitated by oxidative modification of LDL protein components, namely apoB-100. The oxidative modification of apoB-100 by reaction of its lysine residues with lipid oxide derivatives, such as 4-HNE and MDA, is thought to be crucial to the scavenger receptor-mediated uptake of oxLDL by macrophages.

The uptake of plaque-isolated lipoprotein-proteoglycan complexes by human monocyte/macrophages has been shown to stimulate intracellular cholesterol ester accumulation and their subsequent transformation into foam cells. This demonstrates that foam cell formation *in vitro* does not require the oxidation of LDL and its lipid components (Vijayagopal *et al.*, 1992). Further to this, hypochlorite (HOCl)-modified proteins that have been identified in human atherosclerotic plaque have been shown to transform LDL to a high-uptake form without significant lipid oxidation (Hazell *et al.*, 1996). Hypochlorite-mediated LDL oxidation causes modification of lysine residues and protein cross-linking that, once internalised by macrophages, are more resistant to degradation (Jessup *et al.*, 1992). The accumulation of oxidised modifications may decrease the proteolytic susceptibility of some portions of oxidised protein (Grant *et al.*, 1993). This is particularly significant in the case of long-lived resident macrophage cells, where the accumulation of reactive protein oxidation products may cause further damage to other cellular macromolecules in the vicinity.

There is evidence to indicate that oxidised lipids only accumulate late in the disease development, after the accumulation of non-oxidised cholesterol and cholesterol esters. Analysis of the intimal lipoprotein-containing fraction of human aortic lesions, has revealed that the accumulation of non-oxidised lipid precedes that of oxidised lipid *in vivo* (Upston *et al.*, 2002). It was also demonstrated that the overall extent of lipid oxidation in advanced atherosclerotic lesions is substantially less than that needed for the *in vitro* copper-mediated conversion of native LDL to its high-uptake form (Upston *et al.*, 2002). Such findings suggest that lipoprotein lipid peroxidation may represent a response to, rather than a cause for the development of the necrotic core.

The primary finding of the present research is that the major lipid oxidation product detected in advanced atherosclerotic plaques, 7-ketocholesterol, does not display cytotoxicity when delivered to U937 cells within high-uptake lipoprotein, at physiological concentrations and above. Together with the body of evidence discussed above, that explores the formation and catabolism of free radical damaged protein, it seems plausible that protein oxidation may play an important role in the development of advanced human atherosclerotic plaque.

Although these results indicate that the toxicity of 7-ketocholesterol (and possibly other oxysterols) is massively diminished when incorporated into high-uptake non-oxidised LDL, they do not exclude the possibility that 7-ketocholesterol is a significant toxin within atherosclerotic plaque. It is well-established that oxysterols are transported within lipoproteins in plasma *in vivo* (Addis *et al.*, 1989, Javitt *et al.*, 1981, Lin and Morel, 1996) although it remains possible that oxysterols may become dissociated from their associated lipoprotein within the unique environment of atherosclerotic plaque. This, however, appears unlikely given that cellular 7-ketocholesterol efflux to its primary extracellular acceptor, ApoA-1, is dramatically impaired in oxLDL-loaded human macrophages (Kritharides *et al.*, 1996). Investigation into the status of 7-ketocholesterol and other oxysterols in plaque, to determine whether they are lipoprotein associated, free or otherwise, is needed.

4.5 Summary

Oxysterols have been tested for cytotoxicity in numerous *in vitro* studies by their direct addition, primarily as an ethanol solution, to cell culture medium. However, since oxysterols are predominantly located within lipoproteins *in vivo* (Addis *et al.*, 1989, Lin and Morel, 1996), the findings presented here are the result of a more physiologically relevant model system for testing the toxicity of the oxysterol, 7-ketocholesterol, to U937 human monocyte cells.

The U937 cell line used was found to be very sensitive, in terms of cell viability, to both oxLDL and 7-ketocholesterol. Yet when U937 cells were incubated with 7-ketocholesterol incorporated into high-uptake acetylated LDL, the toxicity was greatly reduced. Despite being readily taken up by U937 cells, 7-ketocholesterol-loaded acetylated LDL did not cause a decrease in U937 cell viability at the same 7-ketocholesterol uptake that was earlier confirmed to be highly toxic. Further evidence against the physiological cytotoxicity of 7-ketocholesterol is that the treatment of U937 cells with 7-ketocholesterol-loaded oxLDL, where the 7-ketocholesterol level was increased 5-fold relative to oxLDL, did not show increased viability loss, except at very high oxLDL concentrations.

These results indicate that 7-ketocholesterol, and possibly oxysterols in general, are not the cytotoxic component of oxLDL. Such findings have implications for the direction of future research into oxLDL-induced cell death and the associated formation of the necrotic core within advanced atherosclerotic plaques.

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