IN VITRO ASSESSMENT OF ACUTE AND CHRONIC TOXICITY OF LOVASTATIN TO CULTURED HEPG2 HEPATOMA CELLS

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By

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A thesis submitted to the School of Graduate Studies in partial fulfilment of the requirements for the degree of Master of Science

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October, 1996

St. John's Newfoundland

ISBN 0-612-17592-8

Abstract

The investigations reported here highlight the cytotoxic effect of 3-Hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) Reductase inhibition by lovastatin on the cultured human hepatoma cell line HepG2. This investigation focused on the toxic effects of lovastatin at exposure levels at and above those currently used clinically. The laboratory approaches used included electron microscopy, cell cycle analysis by flow cytometry, investigation of peripheral cell damage by enzyme leakage and other protein studies, and analysis of intracellular and extracellular lipids for various exposure concentrations and times. The experiments were also extended to consider the effects of adding oleic acid to the cell cultures as a nutritional supplement to enhance lipogenesis. The findings are discussed in light of current understanding on the significance of protein prenylation on the cell cycle and the mechanisms of cell death by necrosis and apoptosis. The lower lovastatin concentrations used in this study (0.1 - 2.5 μM) did not kill cells, even after exposure for 8 days. However, if cell cultures containing otherwise nontoxic lovastatin concentrations were supplemented with oleic acid/BSA, cell viability was significantly reduced at all lovastatin concentrations (α=0.05). This work shows that lovastatin supplemented by fatty acid can significantly affect HepG2 cell morphology and functions. The results of this work suggest also that HepG2 cells could be used as an efficient and practical model system to investigate the process of cell death *in vivo*. Also, it is a convenient model for investigations on mevalonate-dependent cellular mechanisms.

This study provides evidence to support the potential role of lovastatin treatment in cancer management.

Acknowledgements

I wish to extend my sincere appreciation to my supervisors, Dr. K.M. Kutty, who believed in me and has been there with encouragement and support, and Dr. V. Prabhakaran, for the sound advice and help. Special thanks go to Dr. R. Payne, member of my supervisory committee, for his patient guidance in this research project, the critical reading of the thesis draft and support during the entire period of my preparation of this manuscript. I would also like to extend my acknowledgements to Drs. W. Andrews and B. Virgo, for their useful guidance as supervisory committee members for various durations.

I would like to thank Dr. M. Khalifa, Faculty of Medicine, for his constructive comments on the thesis draft, especially the electron microscopy part and for knowing all the right buttons to push to get things done. My sincere gratitude goes to Drs. S. Pushpanathen and A. Liepins, who gave useful and sound advice, and to Dr. Atef Mansour for sharing his expertise in statistical analysis with me.

Special thanks to the Janeway Foundation, Dr. Charles A. Janeway Child Health Centre and the Faculty of Medicine, Memorial University of Newfoundland for their financial support. Lots of thanks to the technologists at the Janeway Chemistry, Cytogenetics and Histology laboratories for their assistance and endless encouragement.

Lastly, I wish to acknowledge the love and support from my family and friends. To A. Saoudy (husband), Melissa (daughter) and Mustafa (son), a heartfelt appreciation and thanks go to them for just being there even when I was not, for putting up with my late nights and early morning, and for having to listen to me talk about everything and anything concerned my thesis. To my parents, sisters and brothers in Egypt, I thank them for there love and belief in me all my life even more than I did. Finally, special thanks go to my friend Dr. Mona Abdelbarry for her kindness and caring given to my children and for offering me a friendship which principally contributed to the finishing of this thesis.

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ALT	Alanine aminotransferase.
AST	Aspartate aminotransferase.
EM	Transmission electron microscopy.
EXCEL	Expanded Clinical Evaluation of Lovastatin
GC/MS	Gas chromatography/mass spectrometry.
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A.
LDH	Lactate dehydrogenase.
OA/ BSA	Oleic acid/bovine serum albumin complex.
SRB	Sulforhodamine B.

CHAPTER 1

INTRODUCTION

The major cause of death and disability in western countries is atherosclerotic heart disease (Gotto et al., 1990). There are several reasons for this high incidence of atherosclerotic heart disease (Turley and Dietschy, 1982) but hyperlipoproteinemia is predominant. In the aqueous environment of the systemic circulation cholesterol, triacylglycerols and phospholipids are solubilized in complex with apolipoprotein to form particles known as lipoproteins. There are many different types of apolipoproteins, the major ones being apo-AI, apo-AII, apo-CI, apo-CII, apo-CIII, apo-B and apo-E. Each of the five different major species of lipoprotein particles in human blood have specific apolipoproteins associated with them. Apo-B, for example, is found in very-low-density-lipoprotein (VLDL) and low-density-lipoprotein (LDL) while apo-AI is characteristic of high-density-lipoprotein (HDL) (Yang et al., 1986; Knott et al., 1986).

Several epidemiological studies have confirmed higher risk for heart disease in the presence of hypercholesterolemia (Langer et al., 1972). This is especially true in the presence of increased LDL-associated cholesterol. Treatment directed at reducing LDL-cholesterol has been shown to lower the risk for ischaemic heart disease (Avogaro et al., 1979; Sniderman et al., 1980; Waters et al., 1995).

1.1 Properties of 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase Inhibitors

In both the liver and the peripheral tissues, cholesterol inhibits its own endogenous synthesis by a classic negative feedback mechanism. The committed step in cholesterol

synthesis and the focus of negative feedback control is the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) (Sirtori, 1993; Oates and Wood, 1988). Important weapons in the pharmacological arsenal against hypercholesterolemia are the mevinic acid derivatives (vastatins) shown in Figure 1.1 (Robisno et al., 1994; Blum, 1994). These compounds act as specific inhibitors of HMG-CoA reductase. The rationale behind this mode of treatment is that if hepatic endogenous synthesis of cholesterol is blocked, the liver will be starved for cholesterol needed for its own metabolic purposes (mainly synthesis of bile salts). The response of the liver is to scavenge cholesterol from the circulation (Sirtori, 1993). This has been shown to dramatically reduce the level of total cholesterol in the blood and, perhaps more important, to reduce the level of LDL particles (Tobert, 1987; Tobert, 1988; Alberts, 1988; Hunninghake, 1988; Shepherd, 1995; Corsini et al., 1995; Hsu et al., 1995). The inhibition of cholesterol synthesis by vastatins has been demonstrated *in vitro* in a number of animals and human cultured cells line and, *in vivo* in several animal species (Chao et al., 1991; Corsini et al., 1995).

The chemical structures of the four available HMG-CoA reductase inhibitors are shown in Figure 1.1. Lovastatin is a natural product of Aspergillus terreus (Alberts et al., 1980; Alberts, 1988; Corsini et al., 1995); and simvastatin and pravastatin are produced by chemical modification of the parent lovastatin molecule (Serajuddin et al., 1991). These three compounds have a hydronaphthalene ring that interacts with the coenzyme

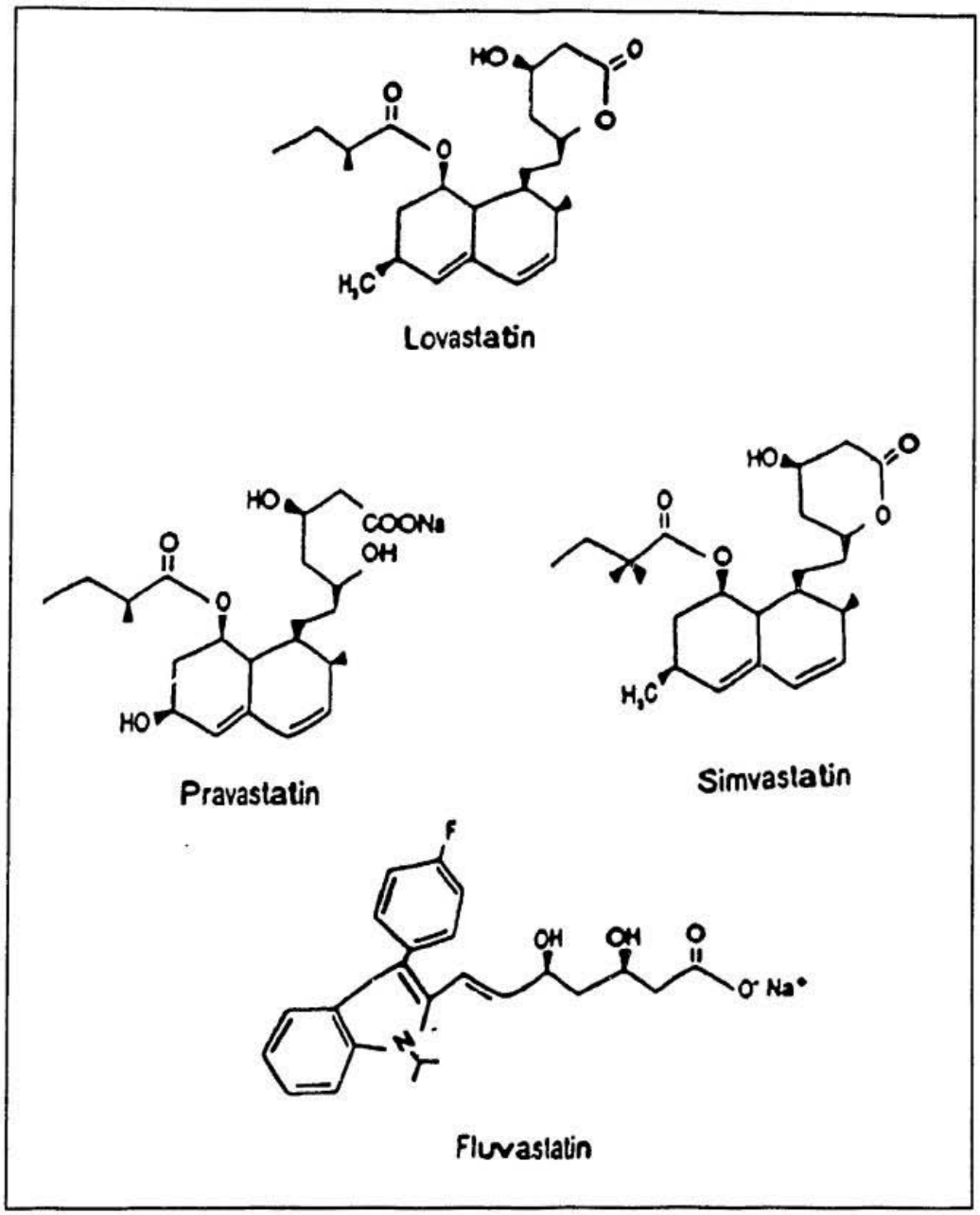

Fig.1.1. Chemical structures of currently available 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors.

A recognition site of HMG-CoA reductase (Alberts, 1988). Additionally, they have a hydroxy-acid side chain that mimics mevalonate. Fluvastatin, the first totally synthetic HMG-CoA reductase inhibitor (Kathawala, 1991; Tse et al., 1990; Tsa et al., 1990; Robisno et al., 1994), has a structure that is distinct from that of the fungal products. Fluvastatin is a mevalonolactone derivative of a fluorophenyl substituted indole ring. The fluorophenyl indole portion of fluvastatin mimics coenzyme A in interacting with HMG-CoA reductase and the side chain mimics mevalonate (Keilson et al., 1990).

Lovastatin and simvastatin are provided as inactive lactones that must be hydrolysed to the corresponding B-hydroxy acids for pharmacologic activity (Smith, 1991; Halpin et al., 1993). Thus they are prodrugs. Pravastatin and fluvastatin are provided in their active hydroxy acid forms.

Fluvastatin, which is chemically synthesized, is racemic, with equal amounts of the highly active (3R, 5S) and weakly active (5R, 3S) enantiomers. The highly active enantiomer of fluvastatin has more than 30 times the potency of the weakly active one (Kathawala, 1991). Because fungal metabolites are biosynthesized, they contain only an active enantiomer.

1.2 Lovastatin Toxicity

Lovastatin toxicity has been studied in non-primate species, particularly rats,

rabbits, and dogs (Chao et al., 1991; Corsini et al., 1995). It appears that high doses of lovastatin, on a long-term basis, can affect the morphological features of rodent liver cells. Foci of cellular alteration appear either as basophilic or eosinophilic areas and are randomly distributed throughout the liver lobule. Hyperplasia of hepatic bile ducts has been also observed. These changes occur reproducibly in rats not only after treatment with lovastatin but also with several other HMG-CoA reductase inhibitors of similar and differing structures and the response is dependent on dosage and exposure time (the no-effect dose for the appearance of these changes in long-term studies is 5 mg/kg/day) (MacDonald et al., 1988). No similar changes were reported in dogs, rabbits or monkeys.

The above findings are not considered to be important for human safety evaluation. Central to this conclusion is the fact that rodents respond to inhibition of HMG-CoA reductase in a manner distinct from dogs and humans (Halpin et al., 1993). In response to inhibition of the enzyme, these species exhibit marked induction of HMG-CoA reductase sufficient to prevent sustained decreases in serum cholesterol concentrations (Bensch et al., 1978; Endo et al., 1979; Fears et al., 1980). This intense enzyme induction occurs predominantly in the periportal regions of the liver lobule (Singer et al., 1984) which is coincident with the atypical cellular areas identified histologically. Studies by Singer (Singer et al., 1984; Singer et al., 1988) have demonstrated that these pathological areas have extensive proliferation of smooth endoplasmic reticulum, the membrane in which HMG-CoA reductase is bound. It is this membrane proliferation that

is recognizable at the light microscopic level as cellular atypia.

A dose of 200 mg/kg/day of lovastatin administered for 6 months to rats produced a significant morphologic changes in the liver but coadministration of mevalonic acid (the product of the inhibited enzyme) had a pronounced protective effect. Livers of rats treated with lovastatin and mevalonic acid simultaneously could not be distinguished from control tissue. These data demonstrated that for rodent liver, while the mechanism of induction of foci of cellular alteration was not known, the pathology is clearly related to inhibition of mevalonate synthesis.

High doses of lovastatin and other HMG-CoA reductase inhibitors were rapidly lethal in rabbits. Preliminary data indicated that high plasma levels of these compounds were observed at comparable dosage levels in dogs (MacDonald et al., 1988) were achieved in these species. The rabbit is uniquely sensitive to lovastatin. The probable cause of death in rabbits receiving high dosage levels of lovastatin was centrilobular hepatocellular necrosis (Kornbrust et al., 1989). This occurs in a dose-dependent fashion. Increasing degrees of hepatic damage are observed as the dose is increased from 100 to 200 mg/kg/day. As for rats, co-administration of mevalonate with a frankly hepatotoxic dose of lovastatin completely prevents hepatic damage. Studies reported by Kornbrust (Kornbrust et al., 1989) indicated that this prevention was not simply due to inhibition of drug uptake in the liver; similar concentrations of drug in the liver were observed with

or without mevalonate co-administration.

In addition, data are available to show that co-administration of mevalonate can reverse hepatic damage in rabbits even after it is allowed to progress for approximately 4 to 5 days (Kornbrust et al., 1989). These findings clearly demonstrate that the observed toxicity in this species is related to marked and sustained inhibition of mevalonate synthesis.

Exposure of cultured cells to lovastatin not only blocks mevalonic acid production, thereby inhibiting cholesterol synthesis, but also prevents DNA replication and cell cycle progression (Habenicht et al., 1980; Fairbank al., 1984; Ortiz et al., 1995). As both of these phenomena are reversible with an exogenous supply of mevalonate (Goldstein and Browen, 1990; Sepp-Lorenzino et al., 1991), it would appear that the mechanism for lovastatin-induced interference with DNA replication and the cell cycle involve depletion of mevalonate or its metabolites (Sumi et al., 1992; Sumi et al., 1994).

Using this general method of exploring the pathogenesis of observed changes (supplementation with the product of the enzyme inhibited), it has been possible to show that many of the effects produced by lovastatin and other HMG-CoA reductase inhibitors were a direct result of inhibition of mevalonate synthesis. In addition to the findings on the prevention of lovastatin-induced liver damage in rats and rodents with mevalonic acid,

this substance is also effective in preventing the slight increases in serum transaminase activity observed in dogs undergoing experimental administration of lovastatin. Mevalonate also prevents the renal and gallbladder changes observed in rabbits receiving high dosage levels of lovastatin (Kornbrust et al., 1989).

Another characteristic feature of HMG-CoA reductase inhibitor toxicity is a hyperplastic change in the squamous epithelium of the nonglandular stomach of the rat (acanthosis). Hyperplasia in this unique anatomic structure (not present in humans) has been shown to be related to inhibition of mevalonate synthesis as it can be induced by a wide range of HMG-CoA reductase inhibitors of varying structure (14 compounds have been investigated), the effect is antagonized by co-administration of mevalonate, and is not produced by pharmacologically-inactive epimeres of potent inhibitors (MacDonald et al., 1988)

1.3 Acute Lovastatin Toxicity in Humans

The general conclusion extrapolated from the non-human studies reported above is probably that the dosage regime required to induce clinically-significant lowering of blood LDL-cholesterol levels in humans is below the toxicity threshold. However, clinical studies discussed below demonstrate that the effect of lovastatin in humans is not entirely benign.

Small increases in serum transaminases, particularly in glutamic pyruvic transaminase (alanine transaminase), sometimes occur, often within 6 weeks of starting therapy (Lovastatin Study Group II, 1986; Havel et al., 1987; Lovastatin Study Group III, 1988 and Illingworth et al., 1988). Such increases are often transient and have not necessitated withdrawal of therapy. The same phenomenon has been reported with most other lipid-lowering drugs (Brown and Goldstein, 1985). In a large clinical trial comparing lovastatin and cholestyramine (Lovastatin Study Group III, 1988), both drugs raised transaminase levels equally (Hunninghake, 1988). Because cholestyramine is not absorbed from the gastrointestinal tract, small increases in transaminases may be an indirect response to changes in lipid metabolism, rather than a direct effect on the liver. A more important finding is that 1.9% of the patients treated with lovastatin in clinical trials have had asymptomatic but marked and persistent transaminase increases, particularly serum glutamic pyruvic transaminase (Tobert, 1987; Mevacor, 1988; Tobert, 1988; Sirtori, 1993). When the drug was discontinued, transaminases returned to pretreatment levels, usually within a few weeks. In contrast to the small increases in transaminases that appear early in therapy, larger increases have usually occurred between 3 and 12 months after starting therapy. This side effect is clearly related to the dose of the drug. In the 48-week EXCEL study (Expanded Clinical Evaluation of Lovastatin), persistent transaminase elevation of > 3 times normal was found in 0.1% of persons treated with 20 mg daily; in 0.9% of persons treated with 40 mg daily and in 1.5% of persons given 80 mg daily (Dujovne et al., 1991). To date, no permanent liver damage

has been documented with lovastatin. Alkaline phosphatase remained essentially normal, indicating that the effect is most probably hepatocellular rather than cholestatic. All the patients who have had increased transaminase levels have been asymptomatic throughout.

1.4 Long-term Lovastatin Toxicity in Patients.

Most of the long-term data on lovastatin has been provided by a collective study of patients who participated in the original four multicenter controlled studies (Lovastatin Study Group II, 1986; Havel et al., 1987; Lovastatin Study Group III, 1988 and Lovastatin Study Group IV, 1988). In study by Tobert (1988) 744 patients had been taking lovastatin for an average of 2.5 years. Along with lovastatin, approximately half of these severely-hypercholesterolemic patients were taking other lipid-lowering agents, usually resins, at some point during their therapy. Therapy for 15 patients (2.0%) was discontinued because of drug-attributable adverse events. Therapy for most of these patients was discontinued because of persistent asymptomatic increases in transaminase levels to 3 times the upper limit of normal. In an EXCEL study, the percentage of individuals affected was dose dependent, increasing from 0.1% at 20 mg lovastatin / day to 1.5% on 80 mg/day (Bradford et al., 1991; Dujovne et al., 1991).

In conclusion, the clinical trials indicate that there is no evidence of adverse events appearing after prolonged use. The drug-attributable discontinuation rate is low and demonstrates the good tolerability and adverse-effect pattern of the drug at the dosage

levels employed for therapeutic lowering of LDL-cholesterol.

1.5 The HEPG2 Cell Line.

The HepG2 cell line was established in. 1979 from minced human hepatoblastoma tissues that were initially overlaid on feeder cultures of irradiated mouse cells. After several months of passage, a feeder-independent proliferating cell line was obtained (Aden et al., 1979; Knowles et al., 1980).

Many investigations on hepatic lipoprotein metabolism have used HepG2 cells (Ellsworth et al., 1986; Dashti and Wolfbauer, 1987; Dashti et al., 1989). This human hepatoblastoma derived cell line expresses many of the functions of normal human hepatocytes (Grant et al., 1988; Silvers et al., 1994). It has been well documented that this cell line can synthesize and secrete lipoprotein fractions within the density ranges of VLDL, LDL and HDL (Ishak and Glunz, 1966; and Ellsworth et al., 1986). They have also been shown to secrete the apolipoproteins B, E, AI, AII, AIV, CII and CIII. The cell line has been used as a model system to study lipoprotein synthesis and metabolism (Moberly et al., 1990; Cianflone et al., 1992; Dixon and Ginsberg, 1993). Apo B-100, the sole apo B species secreted by the human liver, is also the only apo B species synthesized and secreted by the HepG2 cell line (Furukawa et al., 1992; Cianflone et al., 1992 and Dixon and Ginsberg, 1993). However, although HepG2 cells can synthesize and secrete lipoproteins in the VLDL and LDL ranges, it appears that the apo B containing

lipoproteins that are secreted have the buoyancy of LDL rather than VLDL. The particle size and shape of apo B containing particles have been found to be similar in both size and morphology to that of plasma LDL (Javitt, 1990).

HepG2 cells have also been shown to synthesize bile acid (Javitt, 1990; Axelson et al., 1991; Cooper et al., 1994) and many liver-specific proteins (Knowles et al., 1980). HepG2 cells maintained only in Eagle Minimum Essential Medium will continue to synthesize cholesterol (Javitt, 1990). Vastatins effectively inhibit cholesterol synthesis in HepG2 cell (Chao et al., 1991) and experimental evidence suggests that they also interfere with intracellular cholesterol esterification by reducing the activity of ACAT (Cianflone et al., 1990; Corsini et al., 1995).

The lipid composition of HepG2 cells grown to confluence in serum-containing medium has recently been compared to that of human liver (Wang et al., 1988). HepG2 total lipid content (255μg/mg protein) was much higher than that of normal liver tissue (143μg/mg protein). The increased total lipid content of HepG2 cells was the result of an elevated triacylglycerol and phospholipid content, whereas the cholesterol content was comparable to that of human liver (Javitt, 1990). Cholesterol or cholesterol esters are available in HepG2 cells in quantities adequate to support apo B secretion at most levels of triacylglycerol synthesis (Dixon and Ginsberg, 1993). The availability of cholesterol or cholesterol ester may only be rate limiting under certain conditions. The effects of

delivery of lipoprotein cholesterol esters to HepG2 cells may depend on the status of cholesterol metabolism in the cells and on the particular lipoprotein used. Although these comparisons did not take into account the fact that normal human liver contains four major cell types; parenchymal, endothelial, kupffer and stellate cells; they do indicate that HepG2 cells, although a rapidly growing cell line, are not triacylglycerol or phospholipid poor (Dixon and Ginsberg, 1993).

It must always be remembered that HepG2 cells are an immortalized cell line and there could be many differences between these cells and human hepatocytes *in vivo*. Therefore, many of the results obtained with HepG2 cells should be corroborated in primary cultures of human hepatocytes, being careful to ensure that the culture conditions are adequate to maintain the continued transcription of liver-specific genes and to prevent the dedifferentiation of these cells in culture (Jefferson et al., 1985; Clayton et al., 1985).

1.6 Toxicity Studies using the HepG2 Cell Line.

In vitro cellular models have been proven to be extremely useful in some areas of toxicology research because the mechanism of action of potentially toxic chemicals and drugs and their metabolism can be studied under strictly controlled conditions (Acosta et al., 1985; Ball et al., 1995). The organ-specific or basal acute toxic effects of a particular compound can be assessed with the combination of metabolically competent cells from the target organ in parallel with non-differentiated cell lines. The species-specific effect

of a chemical can also be easily evaluated by using *in vitro* cellular systems from different species (Ekwall and Ekwall, 1988). *In vitro* cytotoxicity data provide a relatively quick and inexpensive way of ranking chemicals according to their potential toxicity (Ekwall et al., 1988; Ekwall et al., 1989).

In vitro cytotoxicity assays can also reduce the use of live animals for toxicity testing of chemicals such as newly developed drugs; a procedure which is expensive and ethically controversial. However, the relevance and reliability of *in vitro* systems for predicting toxicity in humans have not been adequately evaluated. The validation of *in vitro* assays is the key step to increase the use of *in vitro* methods to complement or replace *in vivo* testing. Recently, validation strategies have been proposed and discussed as part of several programmes to assessing the relevance and reliability *in vitro* toxicological methods (Ekwall et al., 1991). In, 1989, the Scandinavian Society of Cell Toxicology started a multicentre evaluation of *in vitro* cytotoxicity (the MEIC programme), whose main purpose was to the correlate results from a substantial number of *in vitro* experimental toxicity assay methods with human toxicity data (Bondesson et al., 1989).

The liver is frequently the target organ of toxic chemicals because the hepatic bioactivation of xenobiotics may generate metabolites that are more toxic than the originally administered compound (Okey et al., 1986). Since xenobiotic biotransformation

may differ significantly from one species to another (Hucker. 1970; Quinn et al., 1958; Tee et al., 1987), cultured human hepatocytes, which retain most liver-specific functions, represent an acceptable simplified model for predicting the *in vivo* hepatotoxicity and metabolism of new drugs in humans. The advantage of cultured human hepatocytes has been shown in a study by Jover et al. (1992) who demonstrated that the acute toxicity to humans of the first ten MEIC chemicals was more accurately predicted using human hepatocytes than using rat hepatocytes or mouse non-hepatic 3T3 cells.

Cultured cells of the human hepatoblastoma-derived line HepG2 display morphology and function similar to that of liver parenchymal cells *in vivo* including the production of the major blood serum proteins. HepG2 cells also retain drug-metabolizing capabilities, including the cytochrome P450-dependent mixed function oxidases and glucuronic acid and sulfate-conjugating activities (Silvers et al., 1994). Being a permanent, stable cell line; drug metabolizing activities should not diminish in culture as happens with primary cultures of human hepatocytes (Grant et al., 1988; Forrester et al., 1992; Gugen-Guillouzo et al., 1988). It was shown that the culture medium composition can affect drug metabolising enzyme activities of the HepG2 cell line (Doostdar et al., 1988). Due to these characteristics, HepG2 cells are widely used as an *in vitro* model of human hepatocytes for studies of environmental carcinogen activation (Liu et al., 1993), chemical mutagenicity (Silvers et al., 1994) and drug metabolism and cytotoxicity (Duthie and Grant, 1989; Hall et al., 1991; Hall et al., 1993; Viau et al., 1993; Babich et al.,

1993). HepG2 cells have also been used in cytotoxicity and genotoxicity investigation of environmental pollution and have been suggested as a convenient and sensitive model for screening complex pollutant mixtures (Naji-Ali et al., 1994; Duthie et al., 1994).

HepG2 cell line retains many morphological and biochemical characteristics of normal human hepatocytes (Aden et al., 1979; Bouma et al., 1989). It has been extensively used to study hepatic function, including cholesterol, lipoprotein and bile acid metabolism (Javitt, 1990; Thrift et al., 1992; R.N; Cooper et al., 1994). Other studies with cultured HepG2 cells (Ellsworth et al., 1986; Cianflone et al., 1990; Dashti et al., 1989) have used supplementation with oleic acid to promote lipogenesis and VLDL secretion. In the presence of oleate, lovastatin reduced apoB secretion, diminished cholesterol ester synthesis, but did not affect triglyceride formation (Cianflone et al., 1990). However, these effects of lovastatin on apoB secretion were not seen in the absence of oleate (Pullinger et al., 1989, Cianflone, 1990). These results *in vitro* observation appear to complement the *in vivo* observations (Arad et al., 1990). HepG2 was found by treatment of lovastatin to coordinately regulate the expression of LDL receptor, HMG-COA reductase and HMG-COA synthase (Molowa and Cimis, 1989).

1.7 Detection of Cell Death

Cell death, the irreversible loss of vital cellular structure, can take place by either of two mechanisms; necrosis or apoptosis (Wyllie, 1981; Ledda-Columbano et al., 1991;

Majno and Joris, 1995). While cells undergoing necrosis or apoptosis can readily be distinguished morphologically, the underlying molecular mechanisms are incompletely understood (Uchiyama, 1995).

Necrosis is recognized by cellular metabolic collapse and acute cellular injury; and occurs when a cell no longer maintains ionic homeostasis. As ATP levels become exhausted and transmembrane ion gradients are dissipated, the cell swells and internal organelles become distended (Wyllie, 1981; Uchiyama, 1995). At a critical point the cell membrane ruptures and spills out lysosomal enzymes which mediate non-specific inflammation in the surrounding tissue. Necrosis is typically induced by extracellular trauma such as hypoxia, membrane-active toxicants, and respiratory poisons such as cyanide. When viewed by light microscopy, cells undergoing necrosis demonstrate uniform eosinophilic staining of the cytoplasm and substantially normal staining of the nucleus (Uchiyama, 1995). Some nuclei show hyperchromatinism without shrinkage or complete dissolution (Wyllie, 1981).

The term apoptosis describes a phenomenon traditionally interpreted as programmed cell death (Kerr et al., 1972) which is characterized by controlled autodigestion of the cell. *In vivo*, apoptotic cells lose contact with their neighbours as they decrease in size; they exhibit chromatin condensation; and plasma membranes undergo a characteristic pathological extrusion-generation known as "blebbing" (Amenta

et al., 1993; Majno and Joris, 1995). Apoptosis also involves characteristic changes within the nucleus. The nucleus undergoes condensation as endonucleases are activated and begin to degrade nuclear DNA. In many cell types, DNA is degraded into fragments the size of oligonucleosomes, whereas in others larger fragments are produced. Apoptosis is also characterized by a loss of mitochondrial function. This has led to speculation that mitochondria may have an important function in regulating apoptosis (Vayssiere et al., 1994; Reipert et al., 1995). While the dying cells maintain their gross plasma membrane integrity, subtle plasma membrane changes signal neighbouring phagocytic cells to engulf them and thus complete the degradation process (Ellis et al., 1991; Uchiyama, 1995). Cells undergoing apoptosis that are not immediately phagocytosed disintegrate into smaller membrane-bound fragments called apoptotic bodies. An important feature of apoptosis is that it results in the elimination of the dying cell without induction of an inflammatory response. In contrast, necrotic cell death is associated with an early loss of cell membrane integrity which results in leakage of cytoplasmic contents and the induction of an inflammatory response (Majno and Joris, 1995).

It has been shown (Reedquist, et al., 1995) that high concentrations of lovastatin can inhibit cell proliferation and differentiation in lipopolysaccharide-activated murine B lymphocytes, perhaps through induction of programmed cell death (apoptosis). Also, treatment of human malignant glioma cells with a lovastatin concentration as low as 10 mM over a period of 72 hours led to DNA degradation into nucleosome-sized fragments

characteristic of apoptosis (Jones et al., 1994). The cell cycle of a human bladder carcinoma T24 cell line was arrested in G1 phase (an indication of apoptosis) at 10 μM lovastatin (Jakobisisak et al., 1991). Morphological changes typical for apoptosis was confirmed after 72 hours of exposing a human prostate cancer cell line, PC-3, to 10 μM lovastatin (Borner et al., 1995).

Several methods have been employed to assess lovastatin-induced HepG2 cell death and cytotoxicity by lovastatin. Some involve detection of increased membrane permeability; such as trypan blue uptake, a characteristic feature of dead cells; plasma membrane lactate dehydrogenase (LDH) leakage through the plasma membrane; or sulforhodamine B (SRB) binding to unleached intracellular proteins, so providing an indicator of relative protein leaching. Other cytotoxicity assays measure functional aspects of living but not dead cells (Rubinstein et al., 1990). Thus, the tetrazolium dye MTT is converted into its insoluble formazan derivative by active mitochondrial dehydrogenase; and uptake of neutral red into lysosomes is evidence of cell viability; both exclusive properties of living cells (Keepers et al., 1991). Whilst it is relatively easy to determine by any of these methods whether necrosis has taken place, it is unclear whether they are reliable determinants of apoptosis.

Sulphorhodamine (SRB) is a sulfonated aminoxanthene dye with histochemical properties similar to that of related dyes such as Coomassie brilliant blue, bromophenol

blue and naphthol yellow S, which are used widely as protein stains. Under mildly acidic conditions, SRB binds to basic amino acid residues of proteins in TCA-fixed cells to provide a sensitive index of cellular protein content that is linear over a cell density range of at least 2 orders of magnitude (Skehan et al., 1990). The protein-bound stain can be solubilized under alkaline conditions and the optical density of the solution used to determine relative cell growth or viability in cultured cells (Keepers et al., 1991). Colour development in the SRB assay is rapid, stable and visible.

Electron microscopy (Wyllie, 1980; Beaver and Waring, 1994; Uchiyama, 1995) permits clear identification of apoptotic cells on the basis of the characteristic morphology outlined above (Sonc and Wick, 1994).

Flow cytometry, using propidium iodide as a fluorescence DNA stain, permits cell subpopulations to be identified. Apoptotic cells can be recognized on the basis of a 'low subdiploid peak'; that is the presence of cells having DNA contents less than typical of normal cells in G1 transition (low subdiploid peak) (Gorczyca et al., 1993; Zaleskis et al., 1994; Aten et al., 1995). Flow cytometric analysis is a very rapid and objective way of quantifying apoptotic cells in a mixed population. Flow cytometry with propidium iodide is the quickest and most efficient method for measuring apoptosis and is particularly suitable for large-scale *in vitro* studies (Sonc and Wick, 1994).

1.8 Aim of the Work

This work aims at studying the cytotoxicity and metabolism of lovastatin at various concentrations on human cultured hepatic cells. HepG2 cells has been chosen for the proven similarity of their metabolic pathways to those of human hepatocytes. Since the primary target organ for inhibition of cholesterol synthesis and metabolism is the liver it is important to study lovastatin cytotoxicity with a human liver cell line. Both acute and chronic effects of lovastatin are reported.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

HepG2 cells were obtained from the American Tissue Culture Collection (Rockville, MD). Tissue culture medium, fetal calf serum, delipidated fetal calf serum, trypsin, trypane blue and other tissue culture supplies were obtained from Gibco (Gaithersburg, MD). Tissue culture flasks and multiplate-wells were purchased from Falcon (Cockeysville, MD). [^{14}C]-Oleic acid was purchased from DuPont-New England Nuclear (Mississauga, Ontario). Oleic acid (sodium salt), fatty acid-free bovine serum albumin Fraction V (BSA), o-phthalaldehyde, propidium iodide, sulforhodamine B and all other reagents were obtained from Sigma Chemical Co., (St. Louis, USA). LDH, AST and ALT slides were supplied by Kodak Ektachem Clinical Chemistry Co. (Rochester, NY). N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) for derivatization was from Pierce (Rockville, IL). The Apo A and Apo B kits were from Orion Diganostica (Espoo, Finland). Lovastatin was generously provided by Merck, Sharp and Dohme Research Pharmaceuticals (Rahway, NJ).

2.2 Methods

2.2.1 Cell Cultures

HepG2 cells were grown in Falcon tissue culture T-25 flasks in 5.0 mL of Eagle Minimal Essential Medium supplemented with 10% fetal calf serum and 0.1 mM non-essential amino acid mixture, 1mM sodium pyruvate, 10,000 units/mL penicillin/ streptomycin (Furukawa et al., 1992). The cells were grown at 37°C under a humidified

atmosphere of 95% air, 5% CO_2. The cell medium was replaced on the third day. By day 5-6 each flask reached almost 80% confluency and the cells were sub-cultured as follows:

The growth medium was removed and the adherent cells were washed once with Ca^{2+} free Mg^{2+} free phosphate buffer (CMF-PBS). The cells were detached by treatment with 3 mL of CMF-PBS containing 0.25% trypsin solution for 5-10 min at room temperature. Detached cells were suspended by gentle pipetting and 2 mL complete medium was added as substrate for residual tryptic activity to counter over-trypsinization of the cells. The cells were transferred from the culture flasks to capped conical centrifuge tubes which were centrifuged at 1000 to 1200 rpm for 5 min. The cell suspension was mixed vigorously with complete medium. Cell viability and number was measured by exclusion of trypan blue (0.04% solution). The cell viability was 90-95% on each day of culture. The desired volume of fresh complete medium was added and cells were resuspended gently by pipetting up and down. The cultures were diluted 3-fold every 5-6 days.

2.2.2 Acute and Chronic Treatment with Lovastatin

1 X 10^6 HepG2 cells/well in complete medium were added to flat bottom 24- well plates and left overnight to ensure adherence. For all following treatments cells were cultured in complete medium except that 5% delipidated calf serum was used in place of

10% fetal calf serum. At Day 0, lovastatin, oleic acid/BSA complex, lovastatin and oleic acid/BSA, or medium alone was added to the cells. In all cultures where oleic acid/BSA was added, either alone or with lovastatin, the final concentration of oleic acid was 0.8 mM and the treatment time was 24 hours. In case of lovastatin alone, the concentration ranged from 0.1 to 100 μM, and the treatment time 1-8 days, with medium change every 2 days. HepG2 cell morphology was assessed every two days by phase microscopy and electron microscopy.

2.2.3 Lovastatin preparation and administration.

Lovastatin was generously provided by Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey. A 5 mM stock solution was prepared in isopropanol and stored frozen. An appropriate volume of stock solution was added to culture medium containing HepG2 cells to produce the desired lovastatin concentration in a final concentration of isopropanol not exceeding 0.01%. Control cultures were grown with isopropanol alone (Cianflone et al., 1990).

2.2.4 Preparation of oleic acid/bovine serum albumin complex.

Sodium oleate (40 mg) was dissolved with gentle heating in 5.0 mL of 0.9% NaCl. Delipidated bovine serum albumin (BSA) was used. The purity of the fatty acid and BSA was verified by thin layer chromatography (TLC). The oleic acid/BSA complex was prepared as described by Van Harken et al. (1969): 1.0 g of defatted BSA was dissolved

in 5.0 mL of 0.9% NaCl, the pH of the solution was adjusted to 7.4 and the solution was placed on ice. The pH adjustment was critical for the formation of stable, optically clear fatty acid/BSA complexes. The warm fatty acid solution was added to the cold albumin solution, and the mixture was stirred well overnight at room temperature. The complex was sterilized by passage through a 0.45 μm filter and stored at room temperature. The ratio of oleic acid to albumin after sterilization by filtration, was 9.0 mol/mol ± 0.01 (mean of 16 preparations). For the control experiments, a 1.0 g sample of albumin was carried through the procedure in the absence of oleic acid.

2.2.5 [^{14}C]-Oleic acid preparation and administration.

Radiolabelled tracer [^{14}C]-oleic acid was added at 0.5 μCi/mL medium. Subsequently, triacylgylcerol and cholesteryl ester were separated by silica gel TLC and the appropriate regions of silica were scraped into vials containing scintillation cocktail (ScintiVerse 1 from Fisher). Activity was measured in a scintillation counter (1214 Rackbeta LSC from LKB WALLAC).

2.2.6 Lipid extraction and thin layer chromatography.

After incubation, cells were washed twice with ice-cold PBS and the cell pellet was treated for 60 min with 2 mL 2:1 (v/v) chloroform/methanol twice and the extracts were pooled. The residual insoluble material was dissolved in 1 mL 0.1 M NaOH for protein measurement by the Lowry's method (Lowry et al., 1951). The lipid extracts

were evaporated under a stream of nitrogen and redissolved in 100 µL chloroform/methanol. 50 µL aliquots of extract were applied to silica gel TLC plates and the lipids were separated by development with 75:25:1 hexane/diethyl ether/acetic acid. Aliquots of the medium were also extracted with 3 volumes of chloroform/methanol 2:1 (v/v) for 60 min twice. The organic phase was washed twice with 1 ml chloroform/methanol 2:1 (v/v) and 1 ml 0.05% KOH to remove the remaining radioactive free oleate. The sample was then processed in same way as the cell extracts to measure medium triglyceride, cholesterol, and cholesterol ester.

2.2.7 Measurement of cholesterol and cholesteryl ester by gas chromatography/mass spectrometry

Measurement of free and esterified cholesterol was accomplished by gas chromatography/mass spectrometry (GC/MS) using a modification of the method of Linnet (1994). In outline, the method involves extracting the cholesterol and cholesterol ester spots from silica gel TLC plates with 2:1 chloroform/methanol, adding 0.1 mL N-methyl-N-trimethylsilylfluoroacetamide and incubating for 30 min at 50°C to produce volatile derivatives. For the measurement of cholesterol ester, the ester fraction eluted from the TLC was hydrolyzed with ethanol and KOH for 3 hours at 37°C (Linnet, 1994) prior to derivitization. The derivatized extracts were injected into a Hewlett-Packard GC 5890 Gas Chromatography coupled to a Hewlett-Packard MS 5970 Mass Selective Detector at a port temperature of 240 °C. The resulting mass spectra were compared

automatically with the cholesterol spectrum from the machine's stored library of mass spectra. Tropic acid was used as an internal standard.

2.2.8 Determination of Cholesterol and Cholesteryl Ester with o-Phthalaldehyde.

After silica gel TLC, lipids were visualized temporarily with iodine vapour and the fractions corresponding to cholesterol and cholesterol ester were scraped into glass-stoppered tubes and extracted with 5ml chloroform. The tubes were then centrifuged for 5 min at 1000 g. Aliquots (1 mL) of the chloroform layer were pipetted in duplicate into colorimeter tubes and the solvent was evaporated under nitrogen. Then 2 mL o-phthalaldehyde reagent (prepared as in Rudel and Morris, 1973) was added to each tube with thorough mixing to ensure complete solution of the lipids. The solution was stood for approximately 10 min later. Then 1 mL sulphuric acid was added with care and the absorbance read at 550 nm within 10 to 90 min.

2.2.9 Sulforhodamine B (SRB) assay

The SRB assay provides a sensitive method for estimating cell viability in cytotoxicity studies. It is claimed (Rubinstein et al., 1990) to offer several advantages over the tetrazolium bromide (MTT) assay (Kubota et al., 1993) for very large scale drug screening. The SRB assay provides a better linearity with cell number, a higher sensitivity, and its staining properties are not cell-line dependent (Keeper et al., 1991;

Kubota et al., 1993). The optical densities of SRB can be measured over a broad range of visible wavelengths in either spectrophotometers or well plate readers.

This procedure is essentially that of Skehan et al.(1990) with minor modifications. The cells were washed with CMF-PBS twice and then fixed in situ by addition of 50 µl of cold 50% (wt/vol) trichloroacetic acid (final concentration, 10%) . After incubating for 60 minutes at 4°C, the supernatant was discarded and the plates washed five times with tap water and dried. One hundred microliters of SRB solution (0.4% wt/vol in 1% acetic acid) were added to each well and the culture was kept for 30 minutes at room temperature (Hall et al., 1993). The unbound SRB was removed by washing five times with 1% acetic acid. Then the plates were air dried. The bound stain was solubilized with unbuffered 10 mM Tris and the optical density read on an automated spectrophotometric plate reader at 540 nm.

2.2.10 Enzymatic Investigations on Cell Membrane Permeability

The basis of this approach is that damaged cell plasma membranes leak cytosolic enzymes into their surrounding extracellular environment. The presence of these enzymes can be used as a qualitative and quantitative index of cell damage.

After cells were incubated for various periods of time with the experimental reagents, the culture medium was analyzed for lactate dehydrogenase (LDH), alanine

aminotransferase (ALT) and aspartate aminotransferase (AST). Levels of these three enzymes are routinely used in clinical chemistry as indices of liver damage. The LDH leakage to the extracellular medium was used as a cytotoxicity index for HepG2 cells in culture by Neuman et al. (1993).

All measurements were made using Kodak Ektachem Clinical Chemistry slides on a Kodak Ektachem 700 Analyzer. The Kodak Ektachem Clinical Chemistry Slide system consists of a dry, multilayered, self-contained analytical element coated on a clear polyester support. An 11 µL drop of sample is deposited on the slide and evenly distributed through the spreading layer which also contains the appropriate substrates for the enzyme being assayed. Enzymes are assayed by following the change in absorbance at 340 nm and 37°C as NADH is oxidised to NAD^+.

2.2.10.1 Lactate dehydrogenase

The assay for lactate dehydrogenase (LDH) uses pyruvate and NADH as substrates to generate lactate and NAD^+.

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD+}$$

2.2.10.2 Alanine aminotransferase

Alanine aminotransferase catalyzes the transfer of the amino group of L-alanine to α-ketoglutarate to produce pyruvate and glutamate. Pyridoxal-5-phosphate (PyP) is a cofactor. Lactate dehydrogenase (LDH) then catalyzes the conversion of pyruvate and NADH to lactate and NAD^+.

ALT

Alanine + α-Ketoglutarate ----------> Pyruvate + Glutamate

PyP

LDH

Pyruvate + NADH + H^+ ----------> Lactate + NAD^+

2.2.10.3 Aspartate Aminotransferase

Aspartate aminotransferase catalyzes the transfer of the amino group of L-aspartate to α-ketoglutarate, using pyridoxal-5-phosphate (PyP) as a cofactor, to produce glutamate and oxaloacetate. Oxaloacetate and NADH are converted to malate and NAD^+ by malate dehydrogenase.

AST

Aspartate + α-Ketoglutarate ----------> Oxaloacetate + Glutamate

PyP

MDH

Oxaloacetate + NADH + H^+ ----------> Malate + NAD^+

2.2.11 Quantitation of cell cycle composition by flow cytometry

Flow cytometry was conducted only on HepG2 cell cultures grown for 24 and 48 hours with oleic acid/BSA complex alone (0.8 mM), lovastatin alone (10, 50 and 100 μM), or oleic acid\BSA and lovastatin. The procedure was performed as described previously with slight modification (McClosky et al., 1994). The basis of the method is that DNA is complexed with ethidium cations and the DNA concentration of each individual cell is measured fluorometrically as it passes across a detector: relative DNA concentration permits determination of the position of each cell in the cell cycle.

To prepare cells for flow cytometric analysis, 2 X 10^6 cells were harvested and fixed in 95% ethanol for 20 minutes at 4°C. The fixed cells were washed with PBS and resuspended in 250 μL of RNase solution (100 μg/mL) and incubated at 37° C for 30 minutes. After washing the cells with buffer to remove RNase, 250 μL of propidium iodide solution (50μg/mL PI in 0.6% Triton (TX-100 in PBS)) was added, mixed gently, and held at 4°C overnight in the dark. Next day, the volume was increased to 1 mL with PBS. Propidium fluorescence of each cell in the specimen was measured on a Becton Dickinson Immunocytometry System (FACStar Plus). Data on relative membership at different stages in the cell cycle and the presence of a sub-G0 (hypodiploid or M1) peak

were collected, stored and analyzed with Becton Dickinson LYSYS II software.

2.2.12 Morphological Analysis using Electron Microscopy

Cells were washed with PBS and fixed overnight at 0°C in mixture of 4% paraformaldehyde, 5% glutaraldehyde and 0.1M sodium cacodylate buffer (pH 7.4). After fixation, cells were scraped out and washed three times for 5 min each in a 0.2 M sodium cacodylate buffer, the cells were post-fixed in 1% osmium tetroxide in cacodylate buffer, dehydrated through a graded series of alcohols and embedded in TAAB epoxy resin. Ultrathin sections were cut on a REICHERY OMU3 ultratome and examined with a JOEL, 1200 EX, electron microscope. This procedure is based essentially on published methods (Bouma et al., 1989; Pullinger et al., 1989).

2.2.13 Apolipoprotein Quantitation

Apoproteins A and B levels in culture media were measured using specific clinical immunochemical assay reagents from Orion Diagnostica. The principle of the method is that excess specific antibody is permitted to combine with the antigen under conditions where precipitation does not take place. The turbidity of the specimen is proportional to the amount of antigen present and was measured on a Monoarch Autoanalyser at 340 nm.

2.2.14 Statistical Analysis

Each cytotoxic determination was done in 6 wells per concentration in 12

experiments and the mean value was expressed as a percentage ± SD of the respective controls. Metabolic assays were carried out with at least 6 values as will be mentioned under Table 3.5. The dose-response curve represents percentage value ± SD of the respective controls. Statistical significance was calculated by the Student's t-test, Analysis of Variance and Multiple Comparison by Scheffe's test using Statistical Analysis System (SAS) Software Version 6.09.

CHAPTER 3

RESULTS

In this investigation, the cytotoxicity effects of acute and chronic exposure of lovastatin on HepG2 cells were studied. A comparison was also made between the effect of treatment with lovastatin alone or supplemented with 0.8 mM oleic acid in complex with bovine serum albumin. The rationale for the addition of oleic acid/BSA was to determine if supplementation with an external source of fatty acid to promote intracellular synthesis of triacylglycerols and cholesteryl ester would have an effect on the response to lovastatin.

Lovastatin toxicity was investigated by the following independent methods:

- Direct Sulforhodamine B staining for cell viability.
- Detection of the intracellular leakage of enzymes lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) into the culture medium as an indicator of plasma membrane disruption.
- Visualization of morphological changes by transmission electron microscopy.
- Cell cycle analysis and detection of apoptosis by flow cytometry.
- Detection of the effects on lipid metabolism

3.1 Results of Sulphorhodamine B (SRB) Analyses of Cell Viability.

For 24-hour acute exposure, the effects of lovastatin concentration on HepG2

cytoviability are shown in (Figures 3.1 and 3.2). At lovastatin concentrations of 10 μM and below no significant reduction in cytoviability was observed. At 50 μM and 100 μM, cell viability was reduced to 79.3% and 61.9%, respectively (α= 0.05).

In the case of chronic exposure (2, 3, 4, 6 and 8 days) the effects of low lovastatin concentrations (2.5 μM and below) were essentially the same as for 24-hour chronic exposure as shown in (Figure 3.1. A-E). Marginally significant change from control with 5 and 10 μM started to show from 2-8 days (α=0.05). However at high concentrations (50 and 100 μM) cell death was much greater. After 8 days of exposure to 50 and 100 μM lovastatin, cell viabilities had been reduced to 15.2% and 0.73%, respectively (α= 0.05, $p < 0.0001$). As shown in Figure 3.3, supplementation of the lovastatin treatments with 0.8 μM oleic acid/BSA had a dramatic effect on cell viability. Although lovastatin concentrations of 10 μM and below had no significant effect on cell viabilities, cell death was evident at these low concentrations when oleic acid was also present in the culture media (α=0.05 , $p < 0.0025$). For comparison, cell viabilities after 24-hour acute exposure to 10, 50 and 100 μM lovastatin and 0.8 μM oleic acid were 81.3%, 55% and 45%, respectively. In the case of lovastatin alone, the comparable viabilities were 93.5%, 79.3% and 61.9%, respectively.

3.2 Results of Enzyme Leakage Studies

Figures 3.4. and 3.5. demonstrate the effect of increasing lovastatin concentration

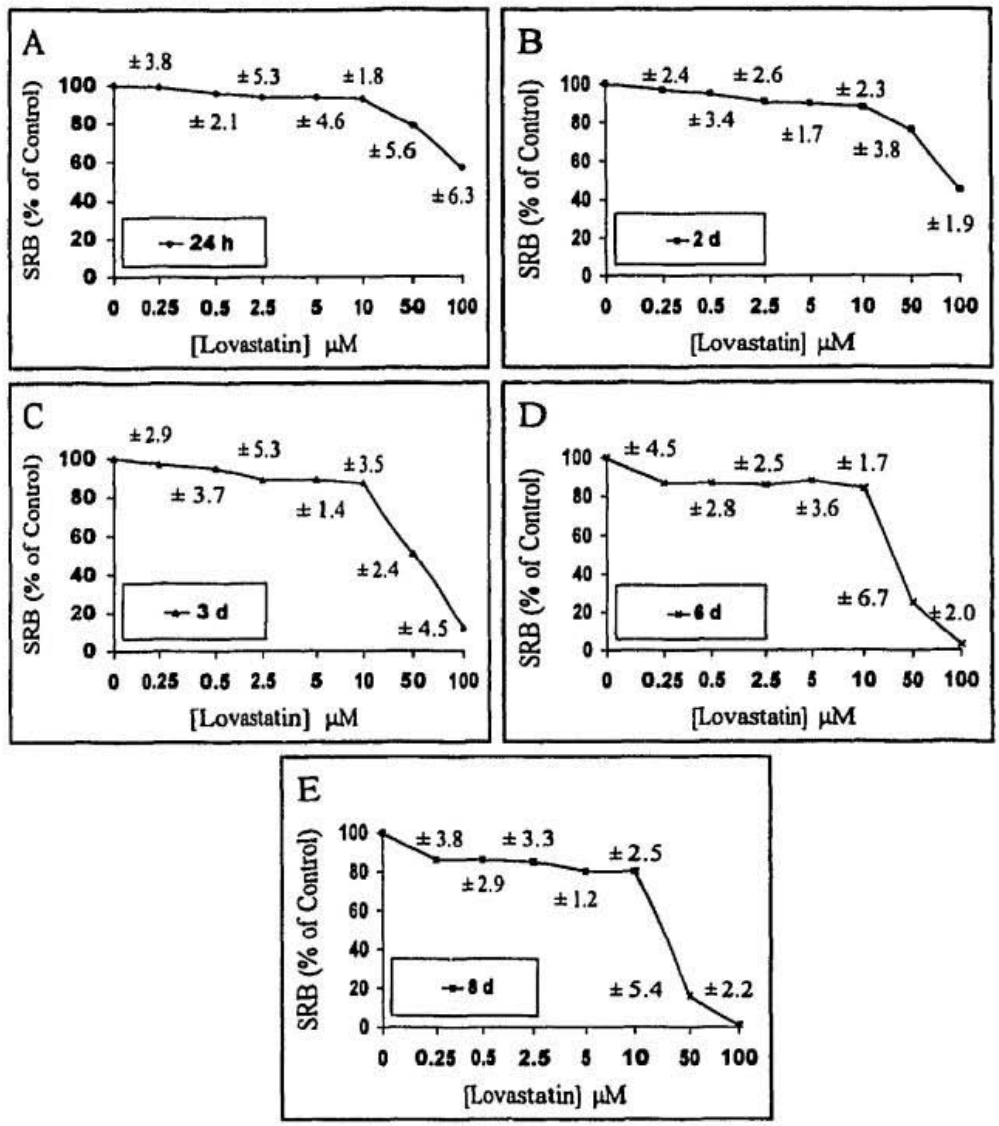

Fig. 3.1. Acute and chronic cytotoxicity effects of lovastatin on HepG2 cells using SRB assay.

HepG2 cells were incubated for 24 hours to 8 days in the presence or absence of lovastatin. Cytotoxicity was assessed by the sulforhodamine B (SRB) assay as described in the method section. Data are expressed as percentages of SRB in control cells. Each point represents the mean (± SD) of 6 replicate wells per point in 12 experiments. ($\alpha = 0.05$). The optical densities in control cultures were 1.636 ± 0.33 for 24 hours, 1.753 ± 0.324 for 2 days, 1.596 ± 0.27 for 3 days, 1.553 ± 0.37 for 6 days and 1.490 ± 0.56 for 8 days.

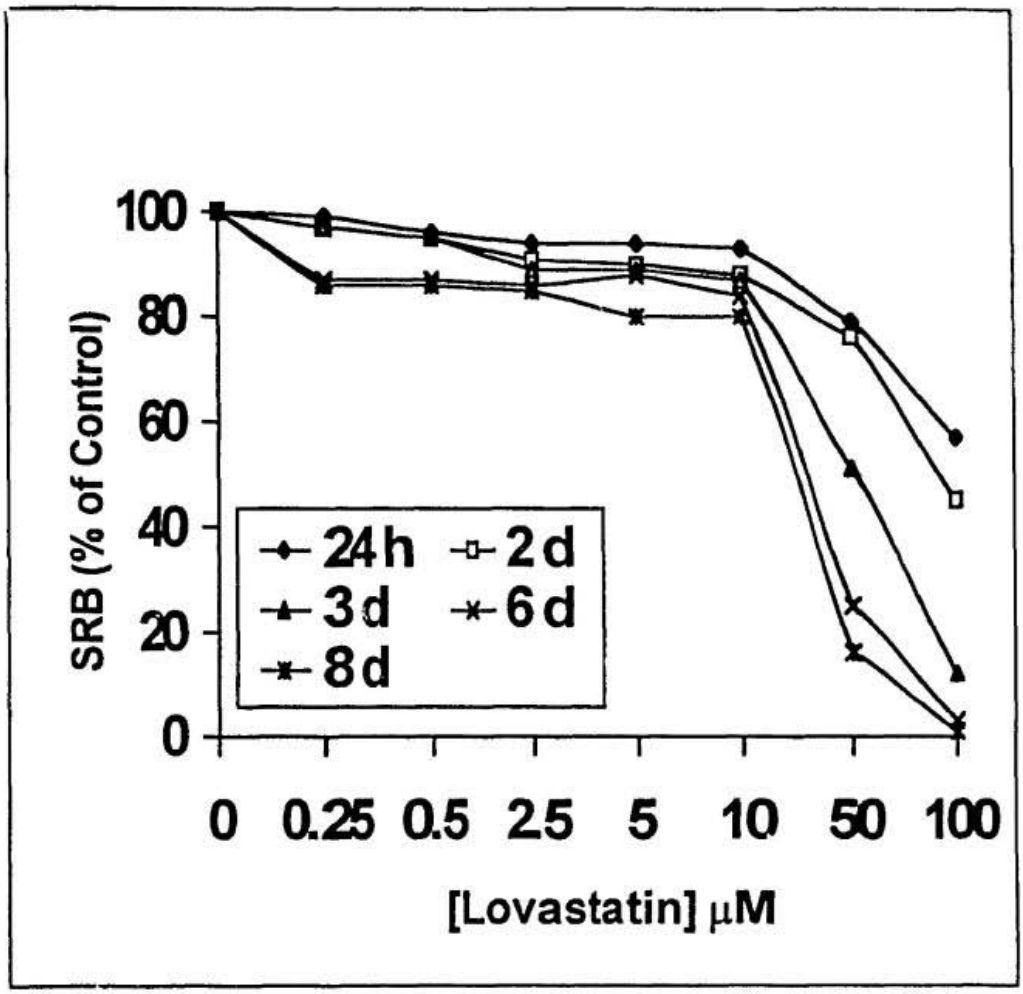

Fig. 3.2. Inclusive representation of Fig. 3.1, Acute and chronic cytotoxicity effects of lovastatin on HepG2 cells using SRB assay.

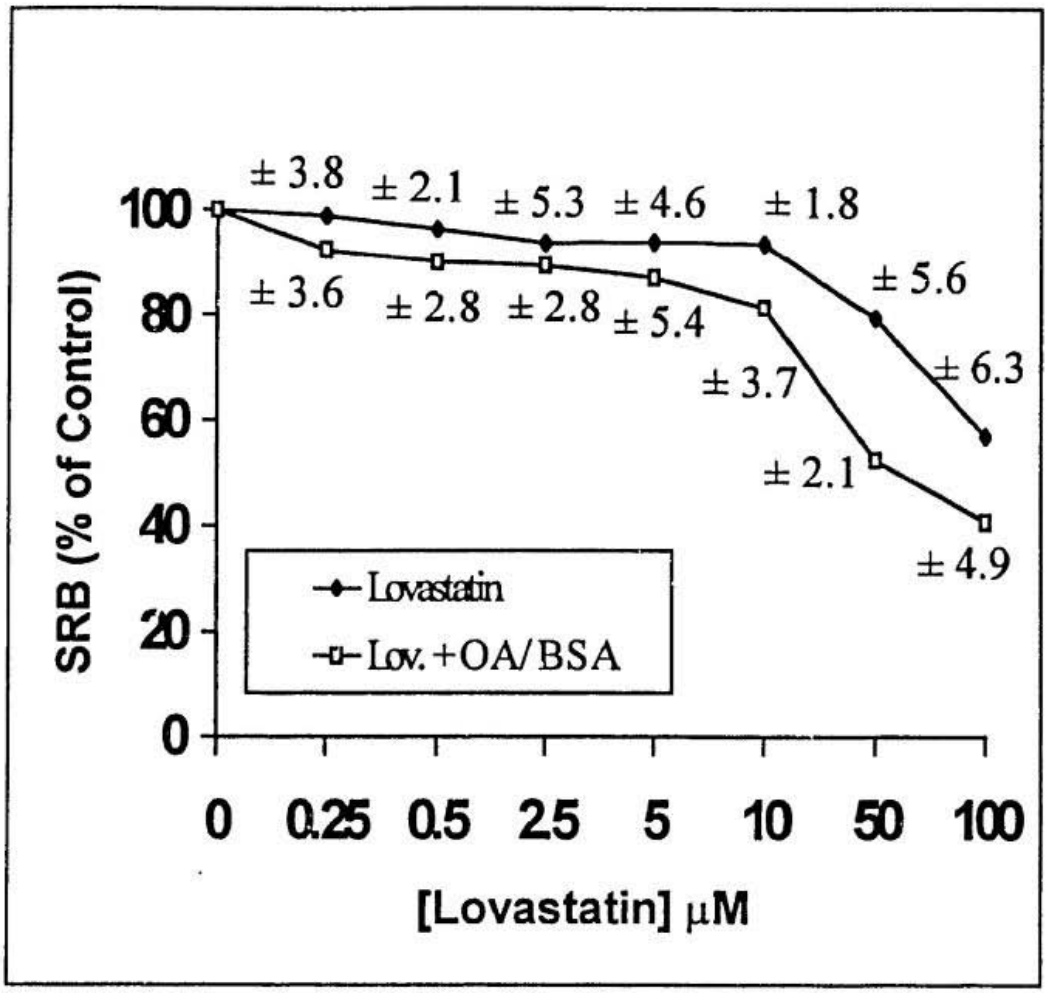

Fig. 3.3. Acute cytotoxicity effects of lovastatin with and without 0.8 mM oleic acid /BSA for 24 hours using SRB assay.

HepG2 cells were cultured for 24 hours in the presence or absence of 0.8 mM oleic acid/BSA with or without lovastatin. Cytotoxicity was assessed by the sulforhodamine B (SRB) assay. Data are expressed as percentage of SRB control cells. The graph shows the means (± SD) of 6 replicate wells per point in 12 experiments. The optical densities in control cultures were 1.636 ± 0.33 for 24 hours without any treatment and 1.538 ± 0.28 for 24 hours with 0.8 mM oleic acid/ BSA. ($\alpha = 0.05$).

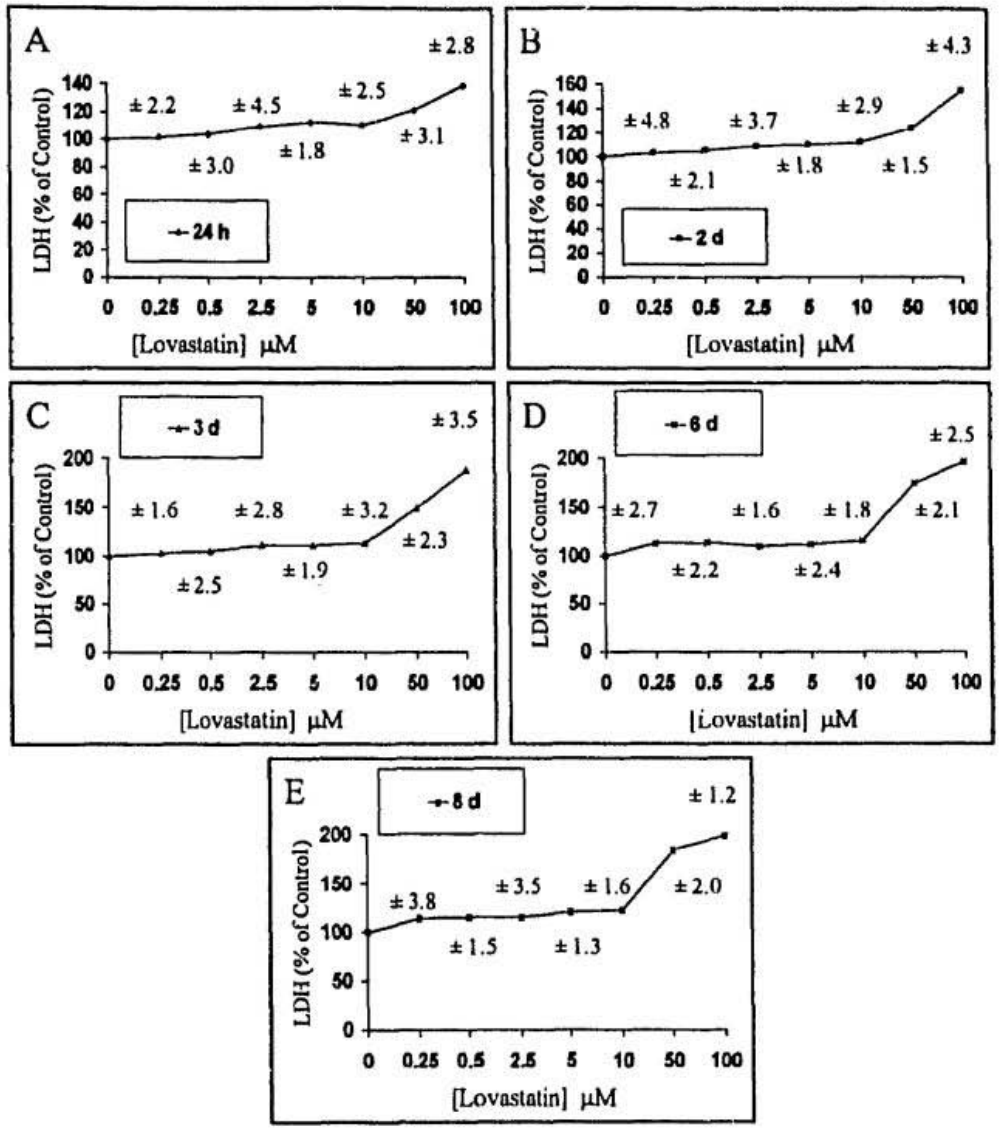

Fig. 3.4. Acute and chronic cytotoxicity effects of lovastatin on HepG2 cells using LDH leakage into the extracellular medium.

HepG2 cells were incubated for 24 hours to 8 days in the presence or absence of lovastatin. Cytotoxicity was assessed by lactate dehydrogenase (LDH) leakage into the extracellular medium. Data are expressed as percentages of LDH activity in control cells. Each point represents the means (± SD) of 6 replicate wells per point in 12 experiments. ($\alpha = 0.05$). The basal rates (U/L) in control cultures were 182.5 ± 6.2 for 24 hours, 181.6 ± 10.7 for 2 days, 178.5 ± 10.56 for 3 days, 185.5 ± 9.35 for 6 days and 190.9 ± 9.87 for 8 days.

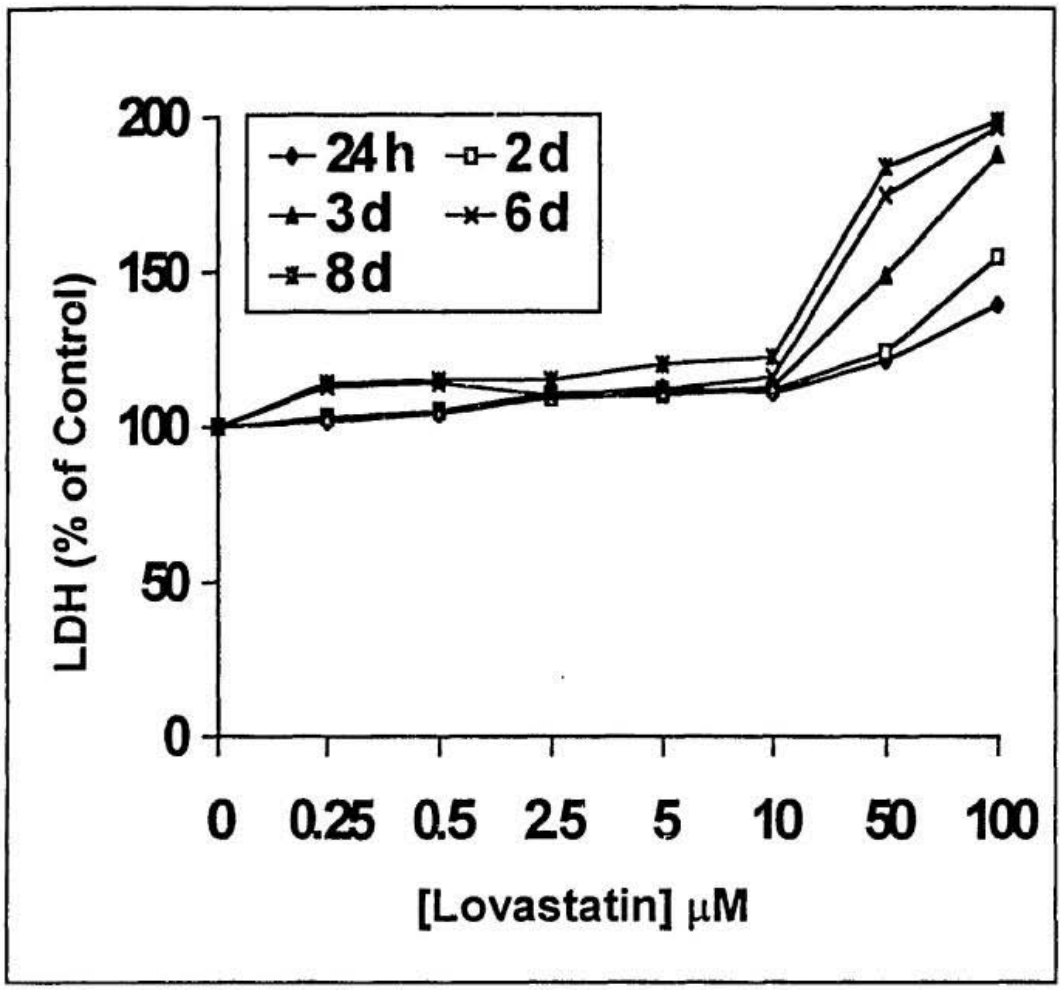

Fig. 3.5. Inclusive representation of Fig. 3.4, Acute and chronic cytotoxicity effects of lovastatin on HepG2 cells using LDH leakage into the extracellular medium.

on the activity of LDH in the culture medium. These results demonstrate that LDH leakage and SRB staining are comparable. The correlation coefficients between LDH and SRB for 24 hours, 3 days and 6 days of exposure are 0.981, 0.986 and 0.992, respectively. LDH was more sensitive for detecting damage at 5 μM after 24 hours (α=0.05 , p <0.001). Lovastatin with 0.8 mM oleic acid/BSA added, LDH leakage analysis was also able to demonstrate more pronounced damage and a lower action threshold (Figure 3.6).

When cell damage was assessed by analysis of AST and ALT leakage (Figures 3.7 - 3.10), it was found that a 10 μM lovastatin concentration was sufficient to elicit enzyme leakage above control values after a 24-hour exposure (Figures 3.8 & 3.10). With cells exposed to 10 μM lovastatin for 3 days, significant AST and ALT leakage values of 19.8 ± 1.7 (p <0.0025) and 6 ± 1.4 (p <0.0001) U/L were noted in comparison to control values of 14.6±0.5 and 1.0 U/L, respectively. An increase in ALT with 5 μM was detected after 3 days (16.9±0.8 U/L) (α=0.05 , p <0.005). By 8 days even the control cultures were leaking significant amounts of AST and ALT (Figure 3.7.E and 3.9.E). In essence, the AST and ALT measurements gave results similar to those of LDH and SRB, except that the AST and ALT were more sensitive in detecting damage after longer incubation time specially ALT after 8 days it recorded leakage for even control and 5 μM after 3 days (0.0025). But LDH is more sensitive at lower lovastatin concentrations,(5 μM after 24 hours), especially during the first three days (α=0.05 , p<0.001).

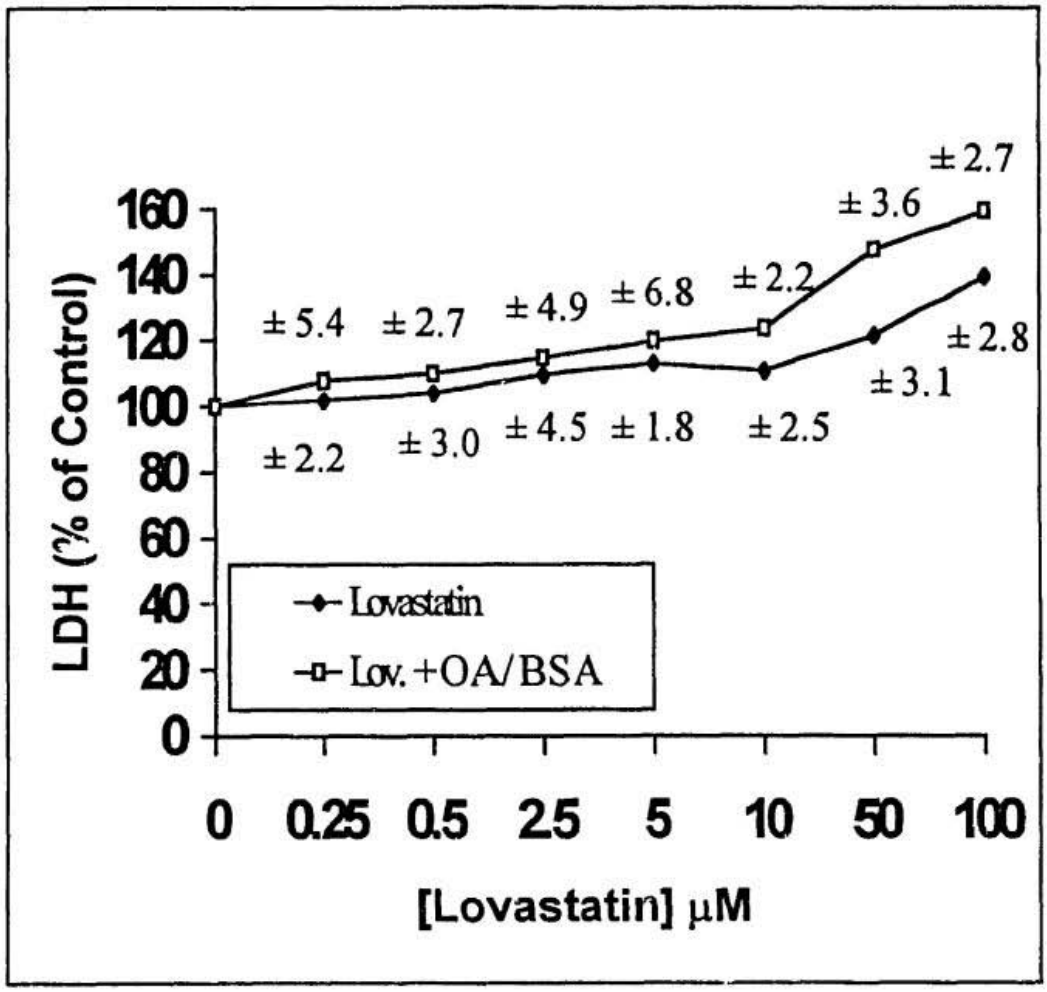

Fig. 3.6. Acute cytotoxicity effects of lovostatin with and without 0.8 mM oleic acid /BSA for 24 hours using LDH assay.

HepG2 cells were cultured for 24 hours in the presence or absence of 0.8 mM oleic acid/BSA with or without lovastatin. Cytotoxicity was assessed by the lactate dehydrogenase (LDH) leakage to the extracellular medium. Data are expressed as percentage of LDH activity in untreated lovastatin cells. The graph shows the means (± SD) of 6 replicate wells per point in 12 experiments. The basal rate in control cultures were 182.5 ± 6.2 for 24 hours without any treatment and 181.8 ± 14.74 U/L for 24 hours with 0.8 mM oleic acid/BSA. ($\alpha = 0.05$).

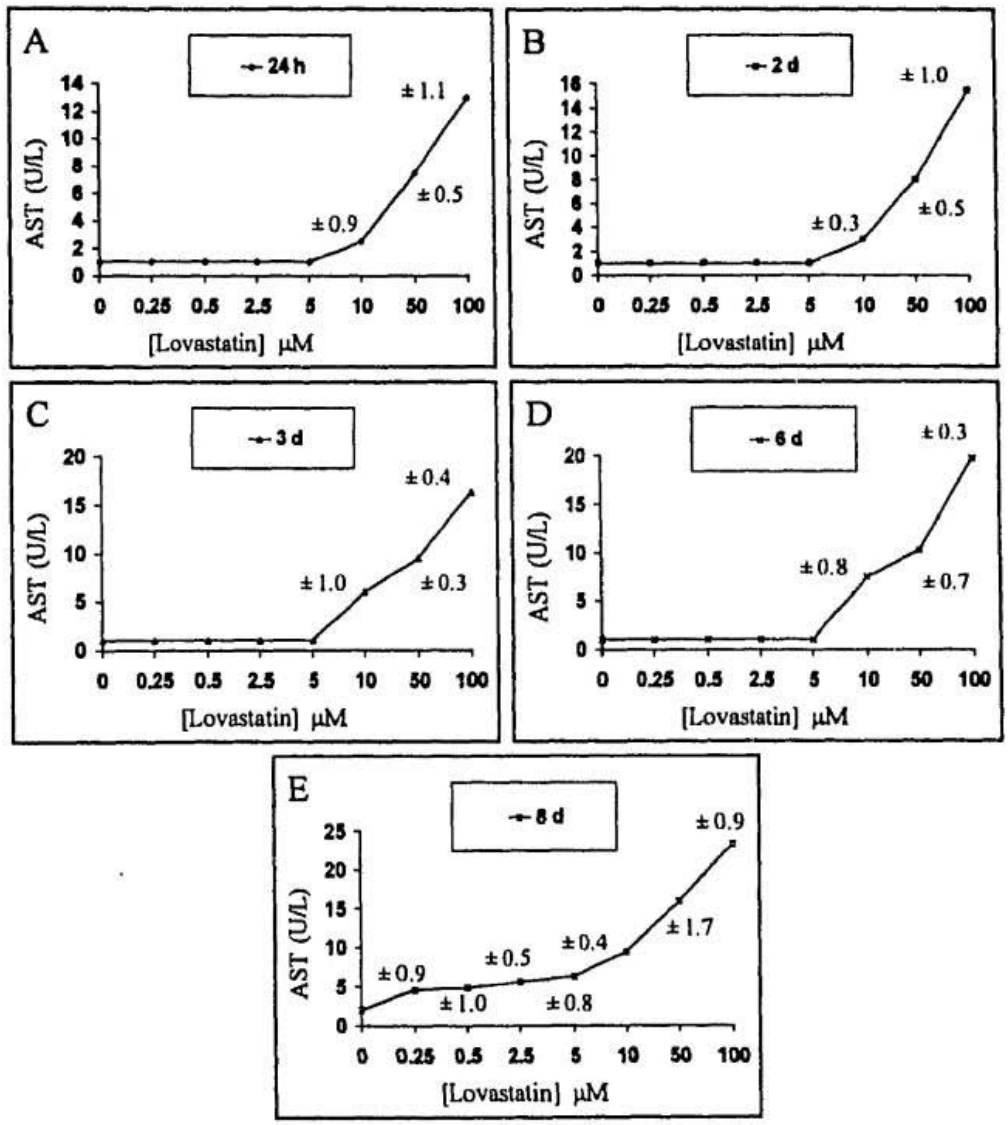

Fig. 3.7. Acute and chronic cytotoxicity effects of lovastatin on HepG2 cells using AST leakage into the extracellular medium.

HepG2 cells were incubated for 24 hours to 8 days in the presence or absence of lovastatin. Cytotoxicity was assessed by AST leakage into the extracellular medium. Data are expressed as U/L. Each point represents the means (± SD) of 6 replicate wells per point in 12 experiments. (α = 0.05).

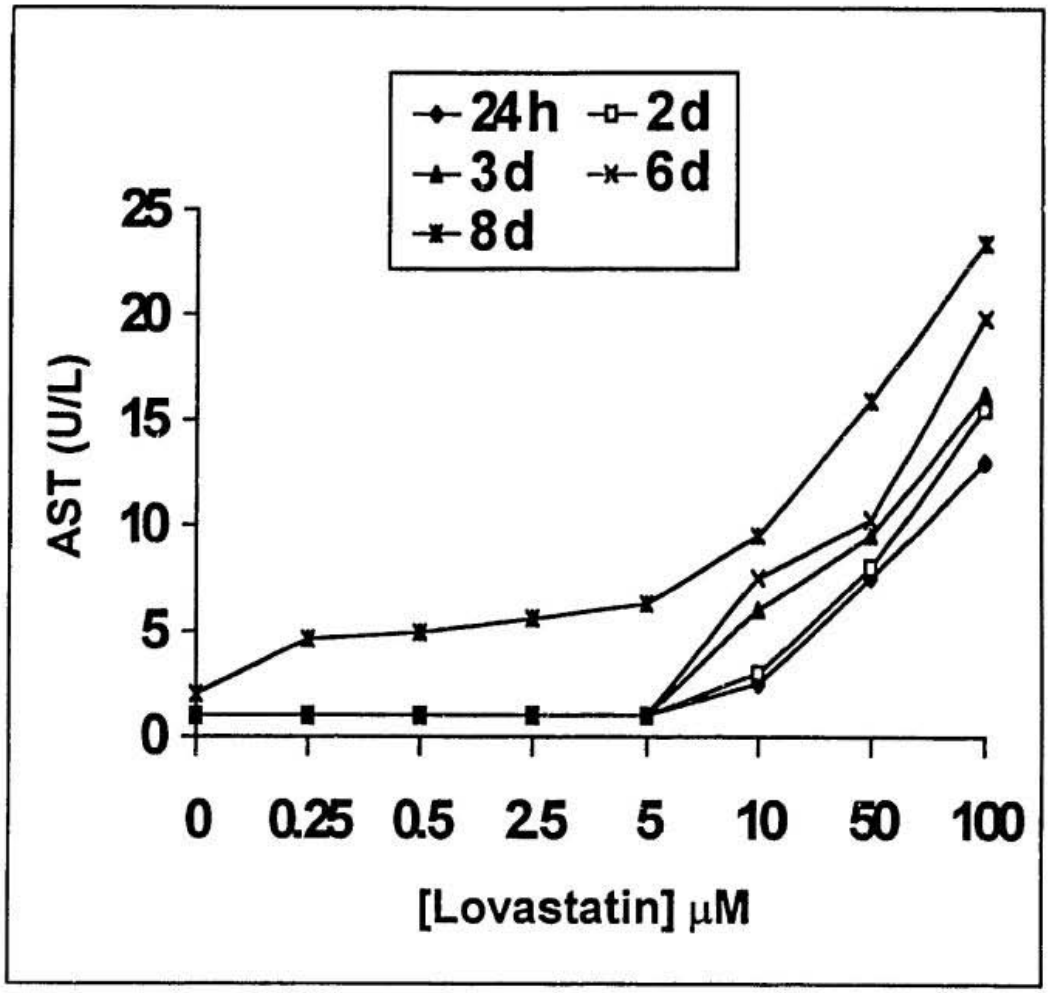

Fig. 3.8. Inclusive representation of Fig. 3.7, Acute and chronic cytotoxicity effects of lovastatin on HepG2 cells using AST leakage into the extracellular medium.

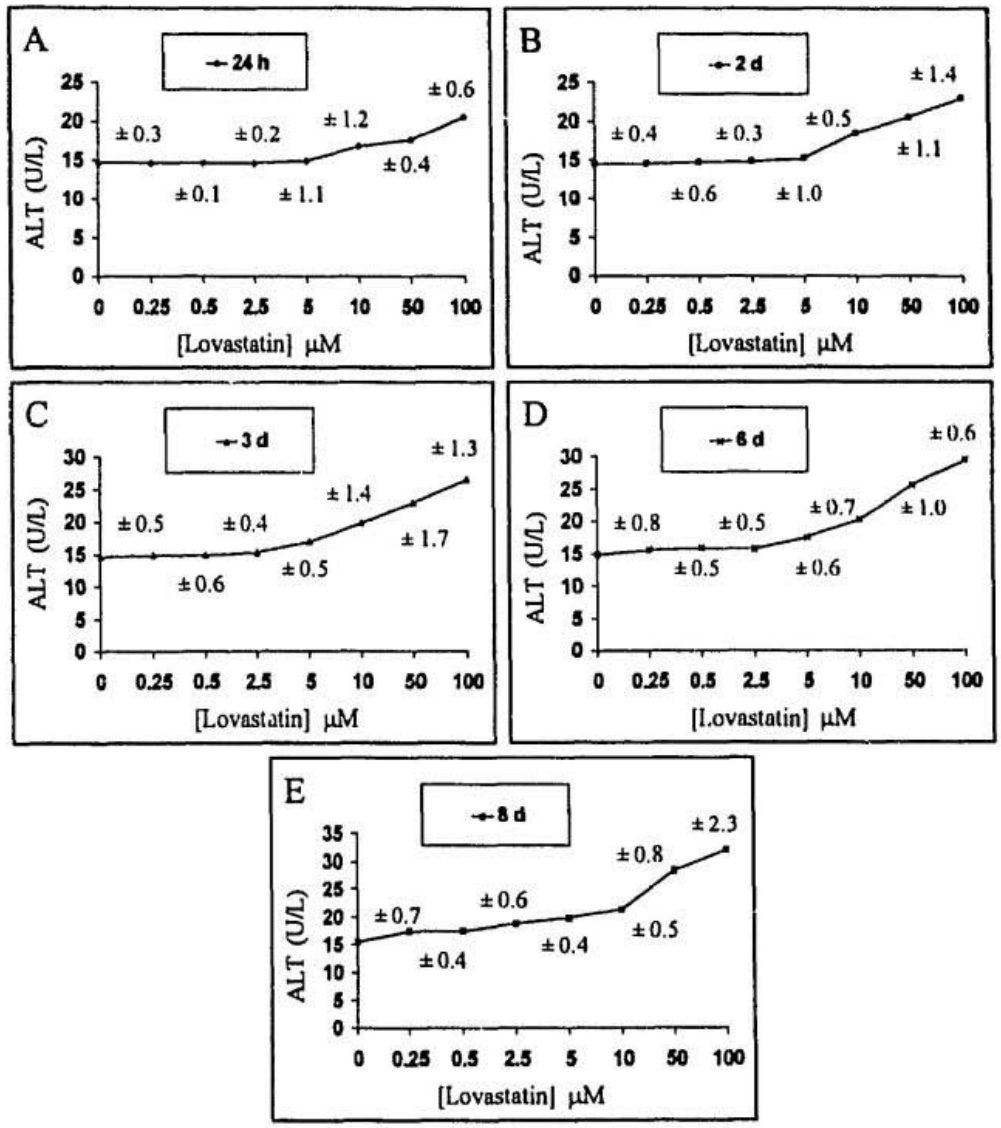

Fig. 3.9. Acute and chronic cytotoxicity effects of lovastatin on HepG2 cells using ALT leakage into the extracellular medium.

HepG2 cells were incubated for 24 hours to 8 days in the presence or absence of lovastatin. Cytotoxicity was assessed by ALT leakage into the extracellular medium. Data are expressed as U/L. Each point represents the means (± SD) of 6 replicate wells per point in 12 experiments. (α = 0.05).

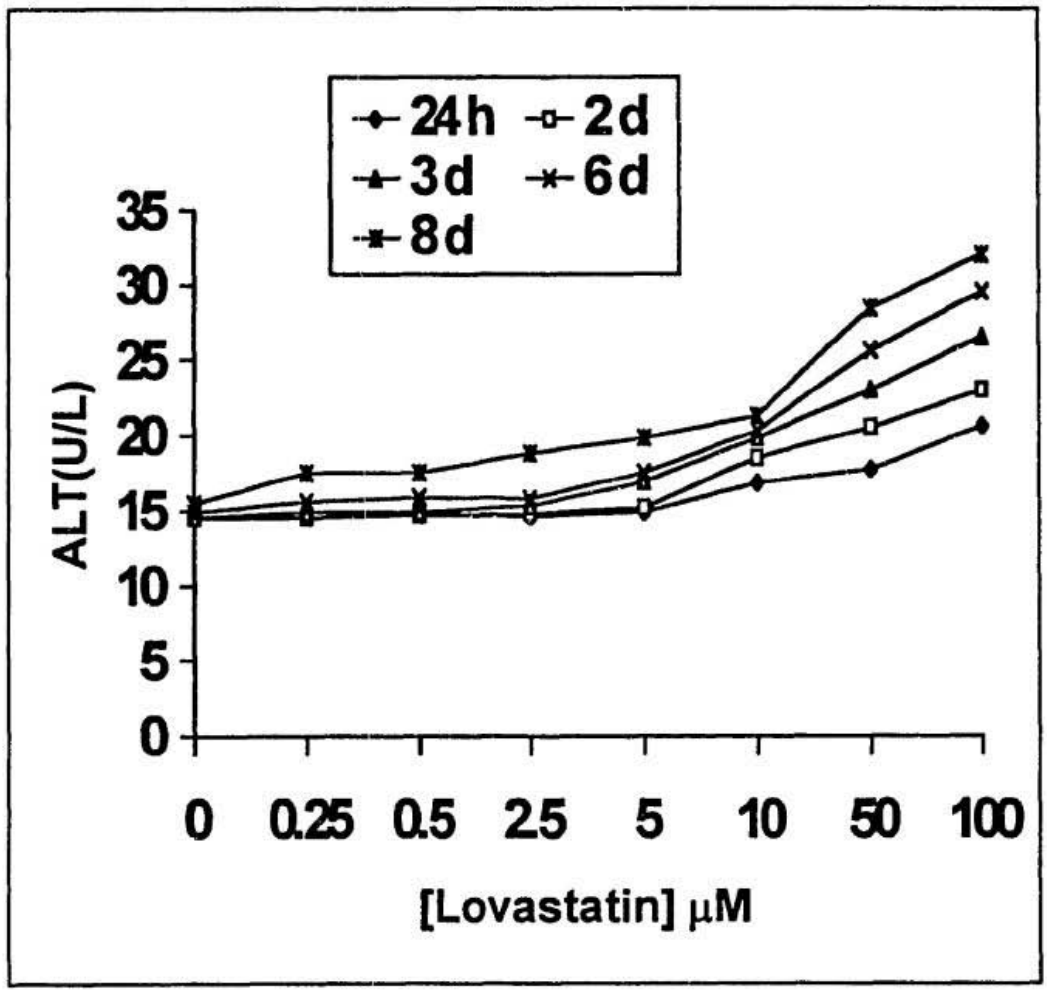

Fig. 3.10. Inclusive representation of Fig. 3.9, Acute and chronic cytotoxicity effects of lovastatin on HepG2 cells using ALT leakage into the extracellular medium.

When 0.8 μM oleic acid/BSA was added for 24 hours, significant AST and ALT leakage was noted; even in the control cultures (Figures 3.11 and 3.12). Clearly, the damage induced by lovastatin at low lovastatin concentrations in the presence of oleic acid was due to the nutritional supplement and its interaction with lovastatin (α=0.05). OA/BSA enhanced the lovastatin toxic effect.

The cytotoxicity of oleic acid is apparent and it is difficult to consider this compound as simply a non-toxic nutritional supplement specially in long-term lovastatin studies. There is a clear synergistic effect and lovastatin and oleic acid produced greater AST and ALT leakage than lovastatin alone.

3.3 Results of Transmission Electron Microscopy

The morphological effects of lovastatin exposure on HepG2 cells were assessed by transmission electron microscopy. Representative ultrastructural features are shown in Figures 3.13-25.

Exposure of HepG2 cells to various concentrations of lovastatin produced detectable morphological changes. Several features were identified and were combined to generate a 3-point scale of damage, mild(+), moderate (++) and severe (+++) (Table 3.1). It was also noted whether the stated features were seen in the majority (A) or minority (B) of cells examined (Tables 3.2-a & b). Cells were considered as

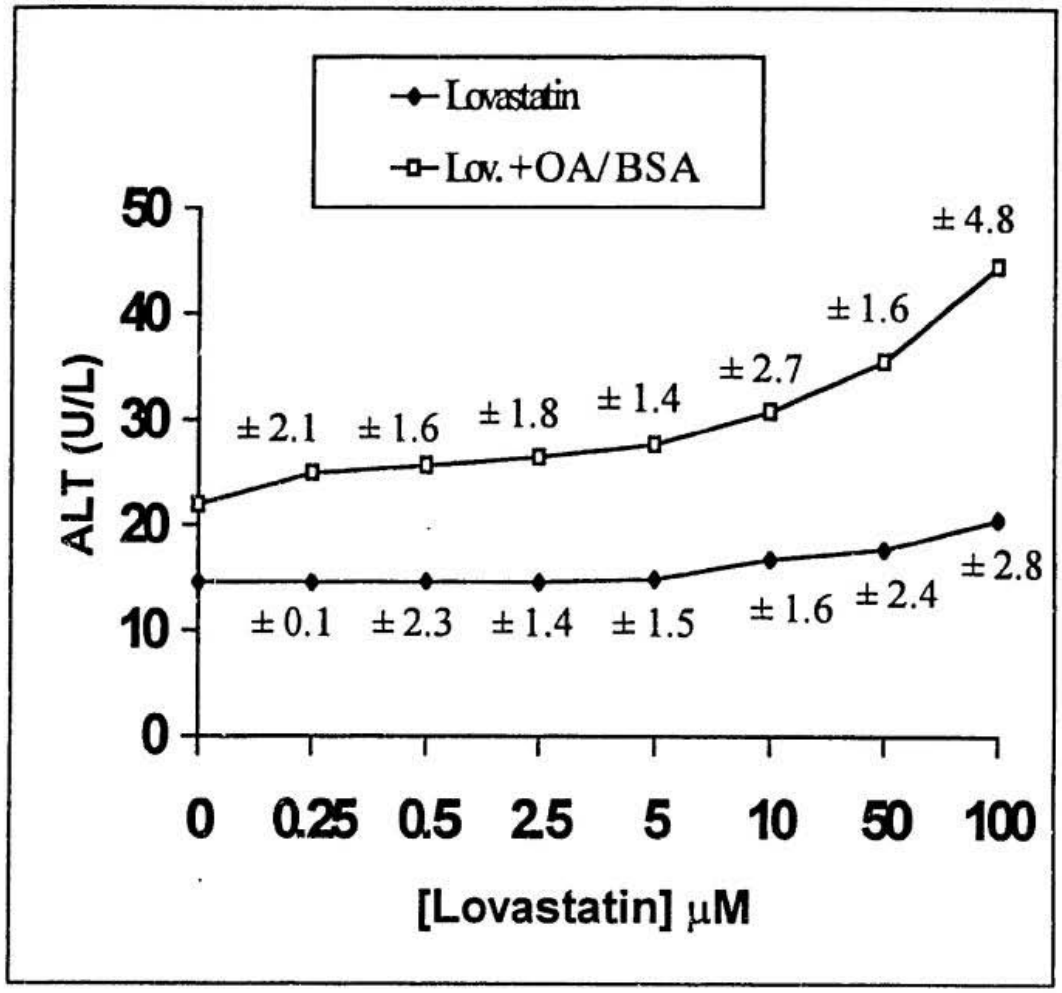

Fig. 3.11. Acute cytotoxicity effects of lovastatin with and without 0.8 mM oleic acid /BSA for 24 hours using ALT leakage into extracellular medium.

Data are expressed as U/L. The graph shows the means (± SD) of 6 replicate wells per point in 12 experiments. ($\alpha = 0.05$).

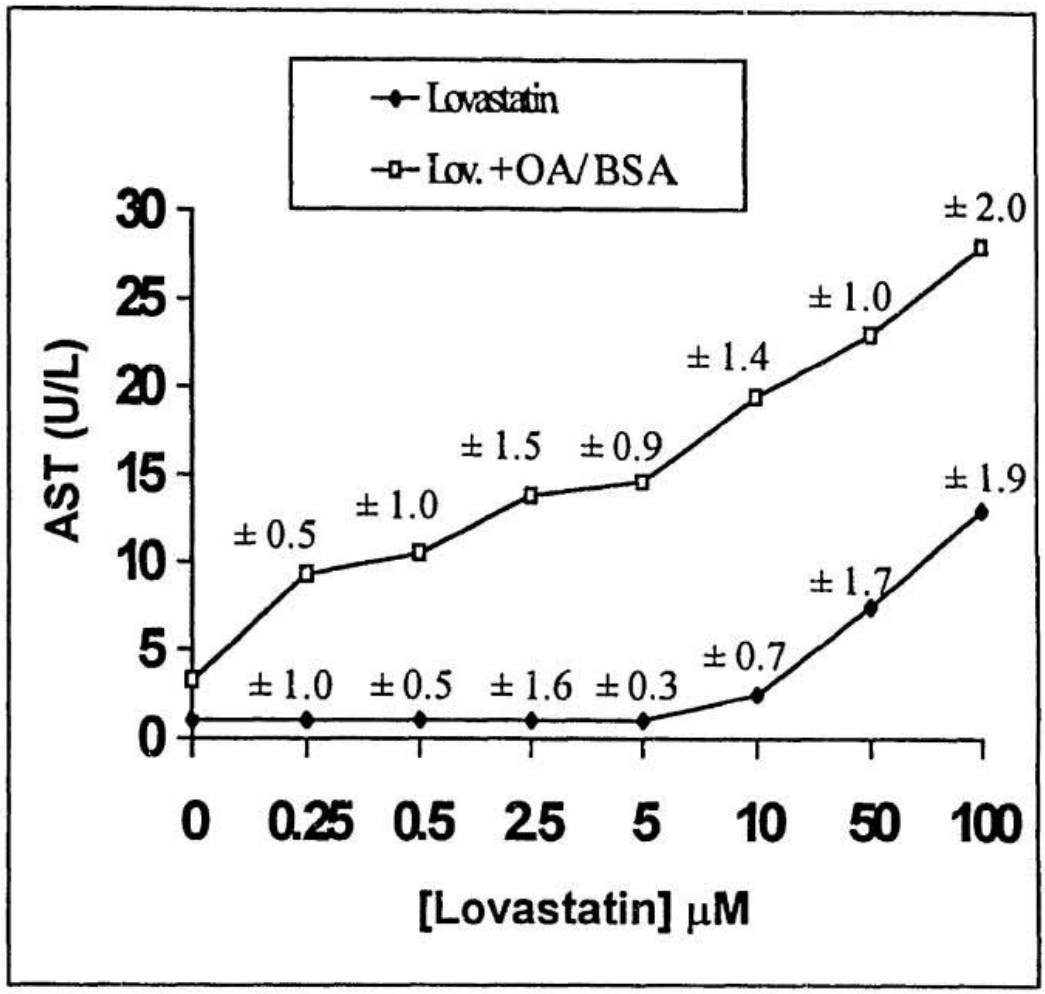

Fig. 3.12. Acute cytotoxicity effects of lovastatin with and without 0.8 mM oleic acid /BSA for 24 hours using AST leakage into extracellular medium.

Data are expressed as U/L. The graph shows the means (± SD) of 6 replicate wells per point in 12 experiments. ($\alpha = 0.05$).

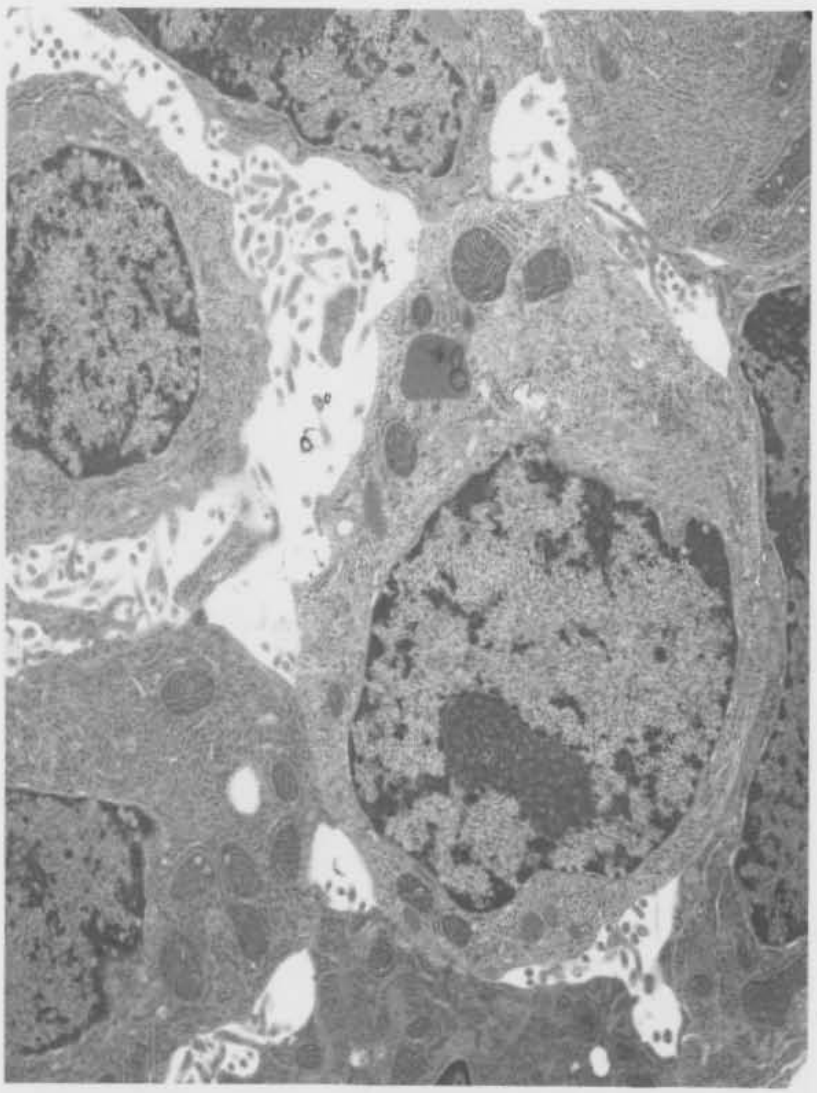

Fig.3.13. Electron micrograph showing an essentially normal cell (control cells after 24 hours). (Original magnification × 10,400).

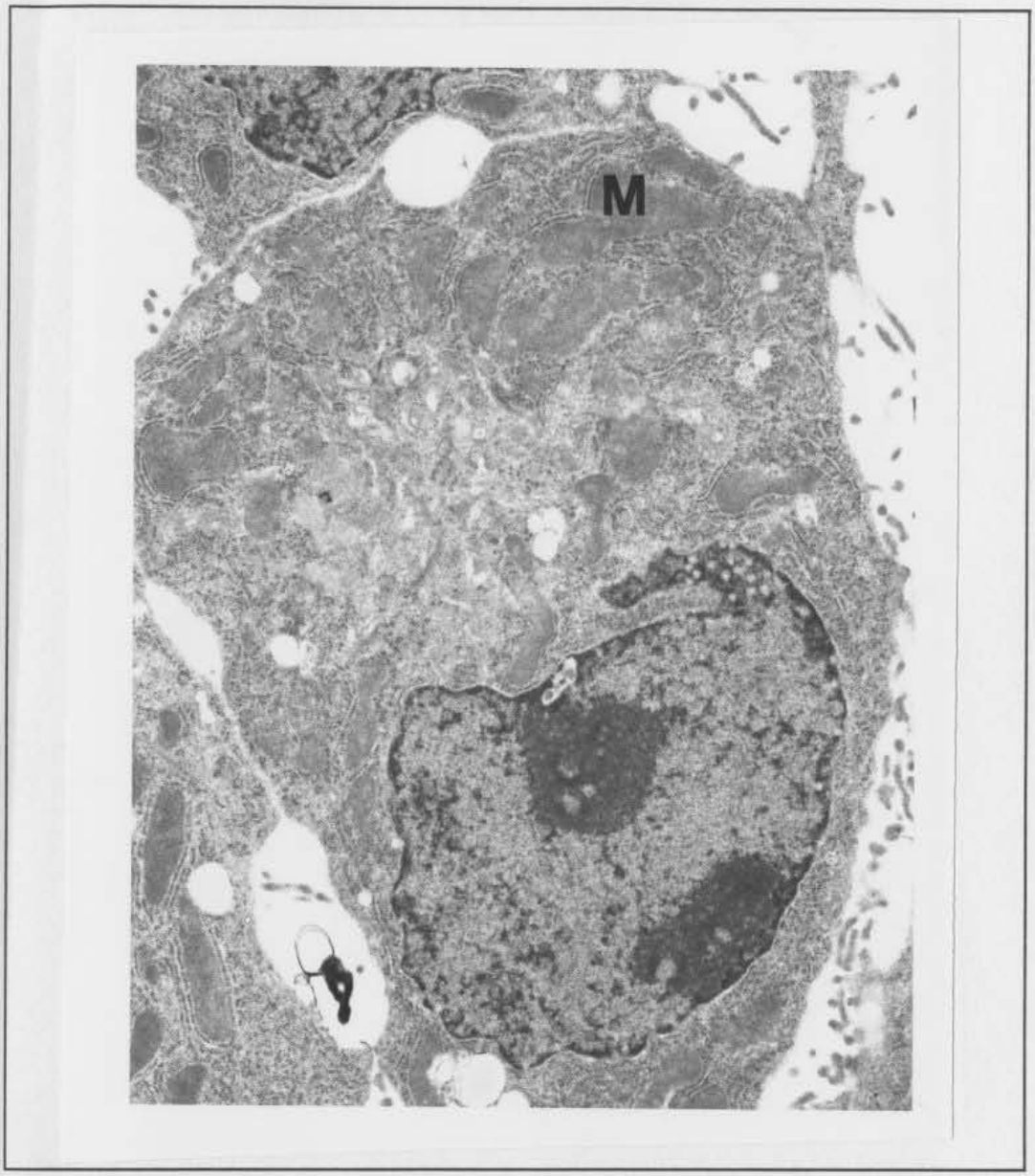

Fig.3.14. Electron micrograph showing generalized cellular swelling and loss of microvilli. Mitochondrial abnormalities are also noted (M), including swelling and cristae reduction (2.5 μM lovastatin after 3 days). (Original magnification × 10,400).

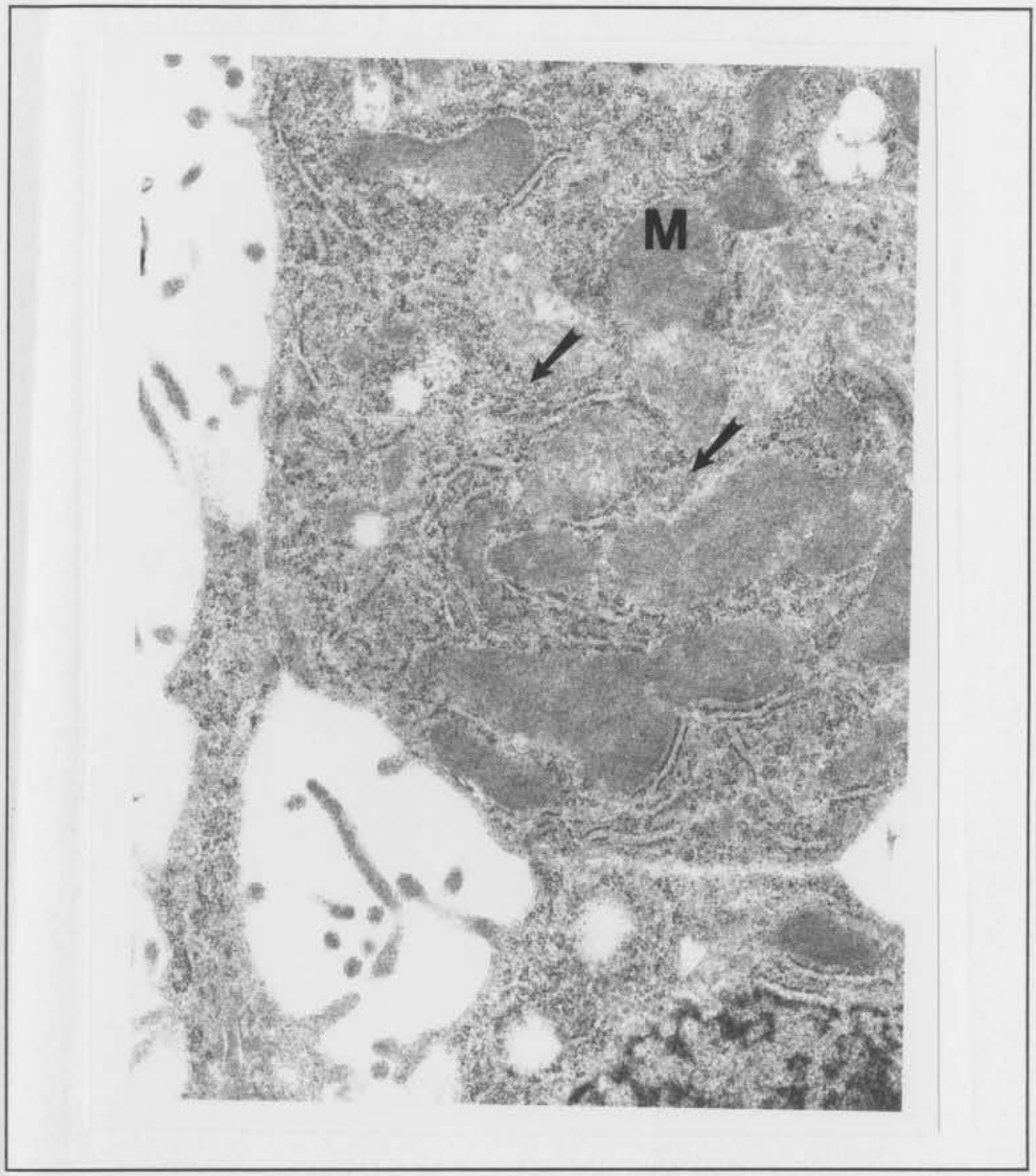

Fig.3.15. Electron micrograph showing early ribosomal separation and dispersion (arrows). Mitochondrial swelling and cristae reduction are also evident (M) (5 μM lovastatin and 0.8 mM OA/BSA after 24 hours). (Original magnification × 10,400).

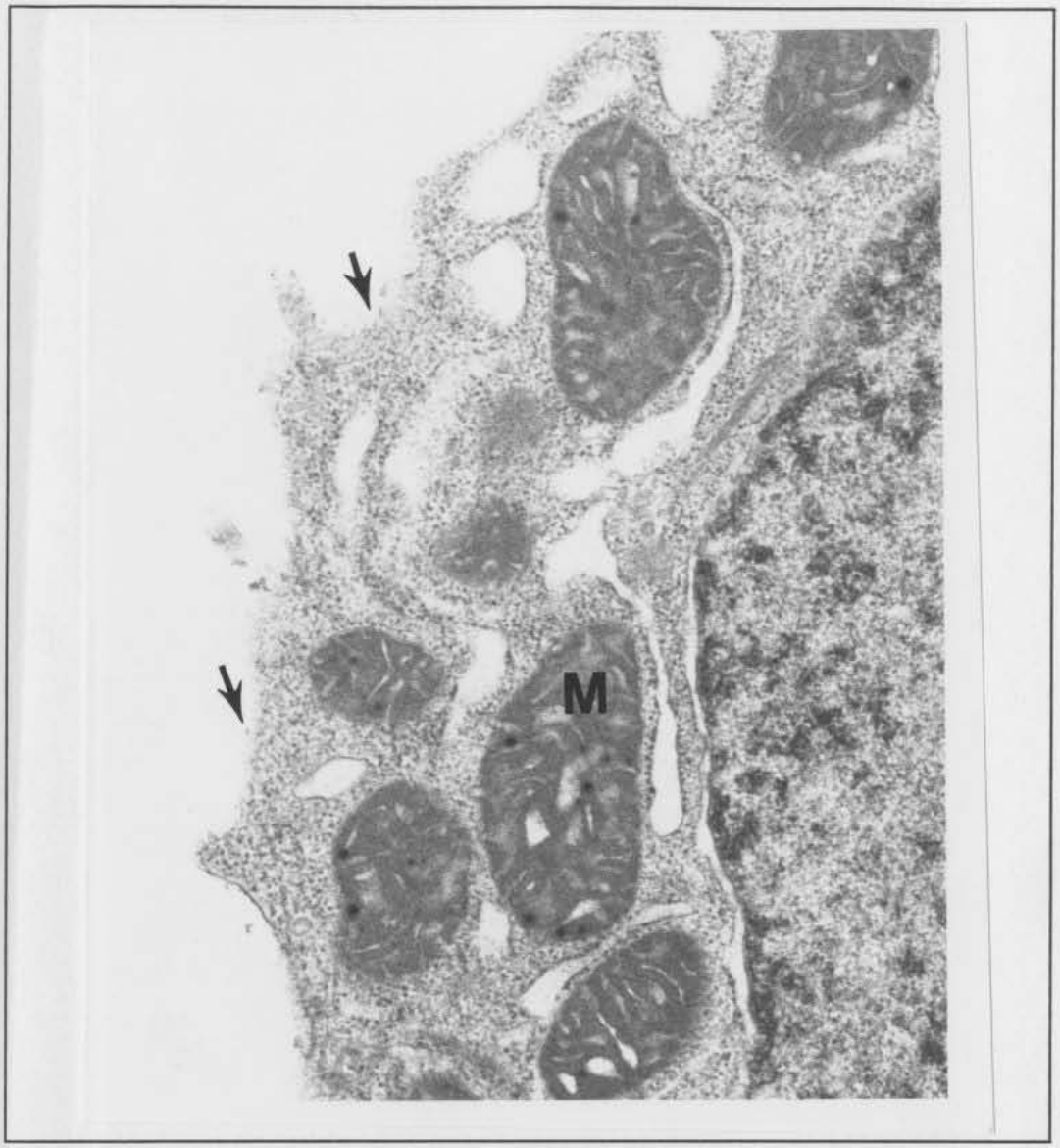

Fig.3.16. Electron micrograph showing mitochondrial abnormalities (M) in the form of swelling, vacuoles and large granules. The cell membrane is also disrupted (arrows) (10 μM lovastatin and 0.8 mM OA/BSA after 24 hours). (Original magnification × 15,600).

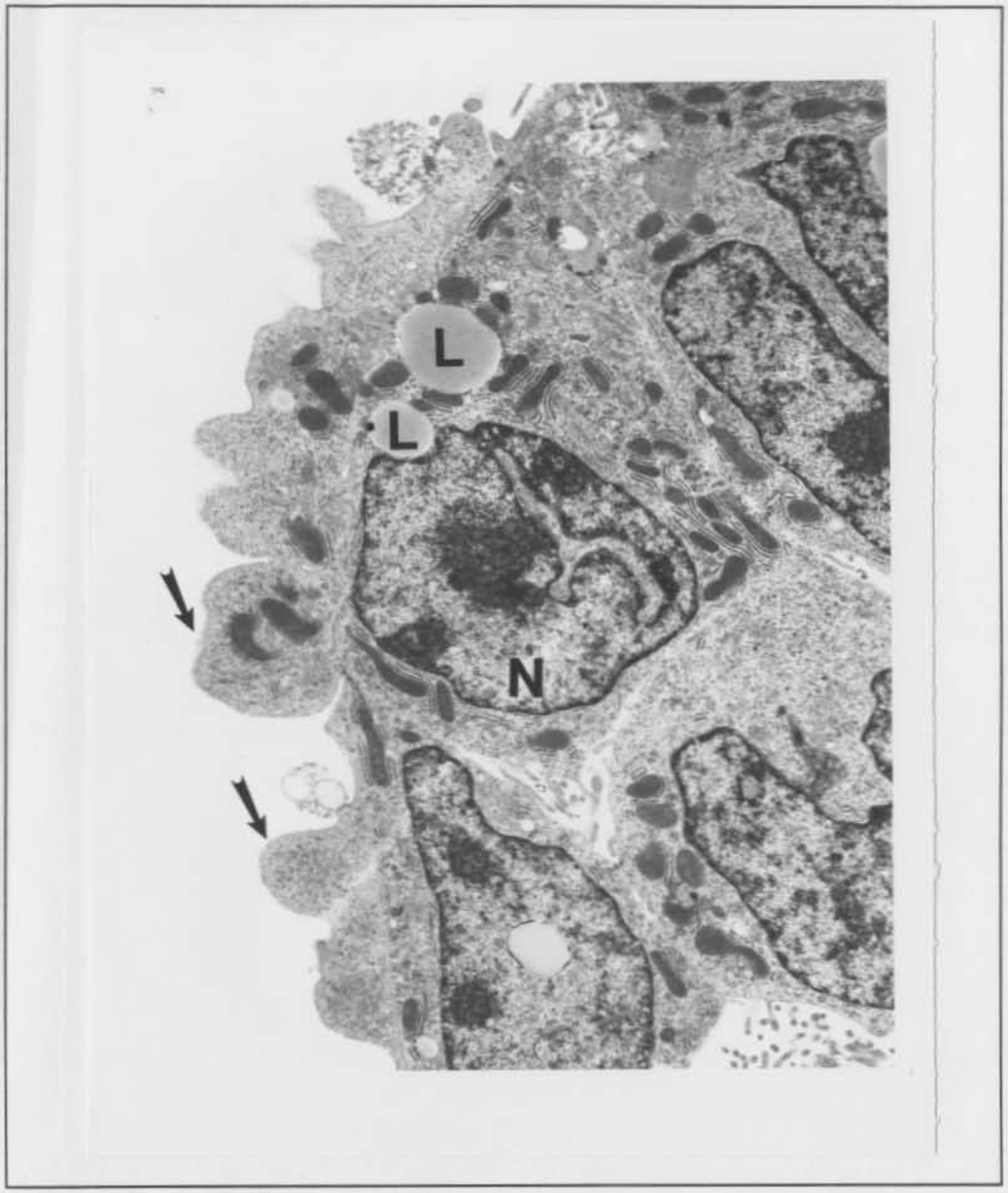

Fig.3.17. Electron micrograph showing nuclear indentation (N), cytoplasmic lipid (L) droplets and blebbing (arrows) (50 μM lovastatin after 3 days. (Original magnification × 7,800).

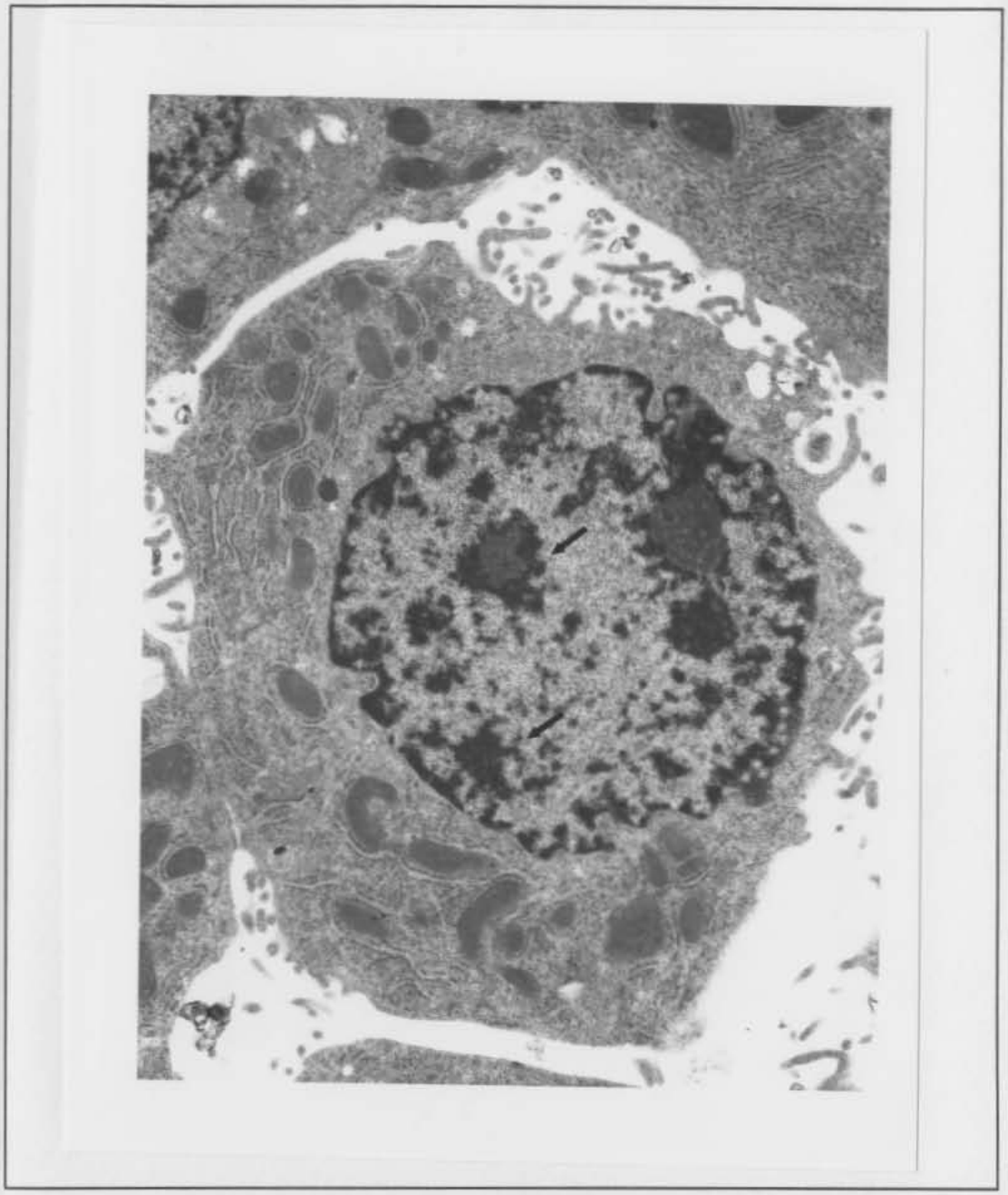

Fig.3.18. Electron micrograph showing nuclear densities and early chromatin condensation (arrow). Abnormal mitochondria are also seen (10 μM lovastatin and 0.8 mM OA/BSA after 24 hours. (Original magnification × 11,500).

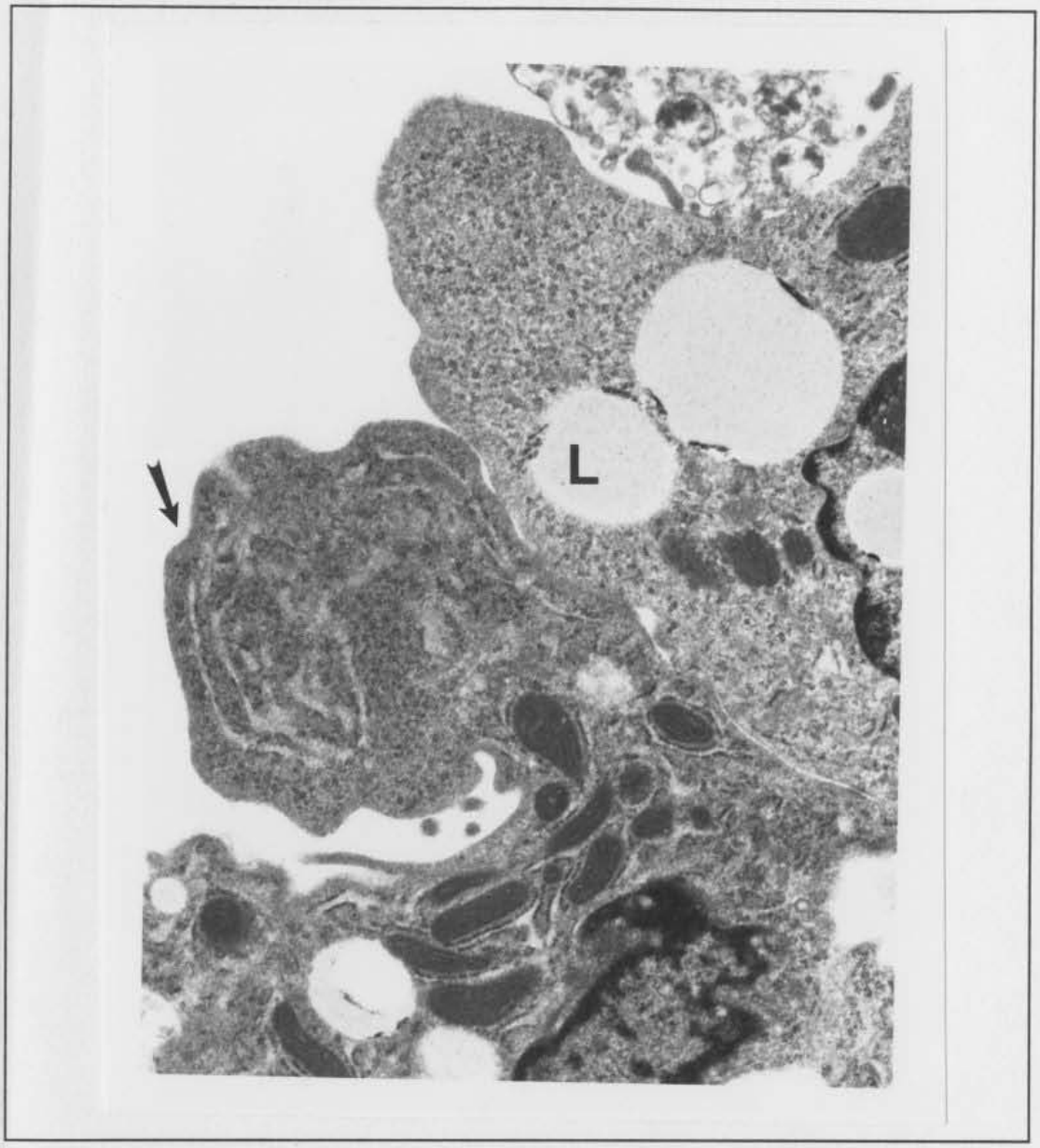

Fig.3.19. Electron micrograph showing ribosomal separation and dispersion along with early endoplasmic reticulum swelling. A myelin figure (arrow) and lipid droplets (L) cytoplasmic lipid droplets are also noted (100 μM lovastatin after 3 days). (Original magnification × 16,800).

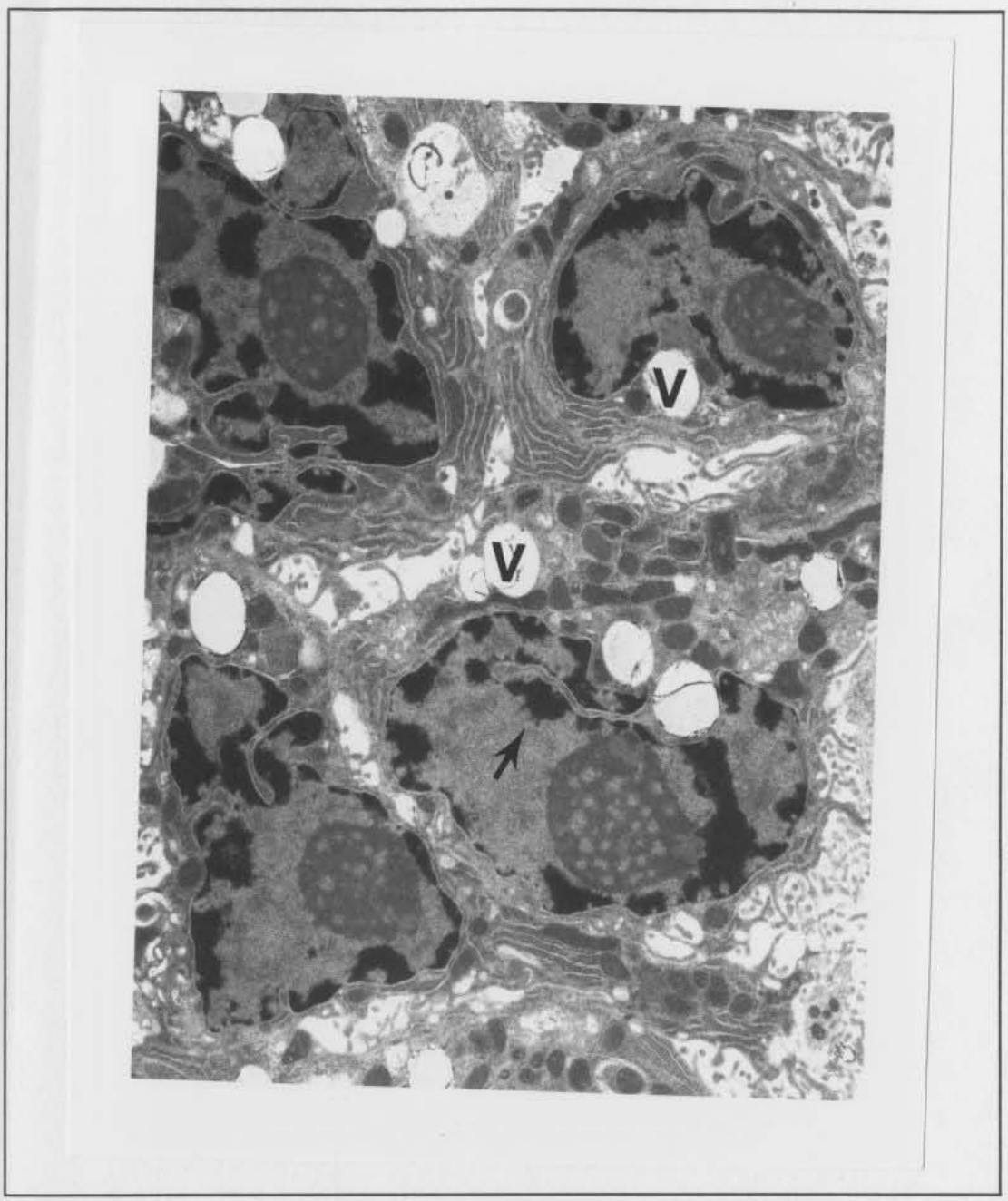

Fig.3.20. Electron micrograph showing cells with nuclear densities (arrow) and chromatin condensation. Cytoplasmic vacuoles are also evident (V) (50 μM after 6 days. (Original magnification × 8,400).

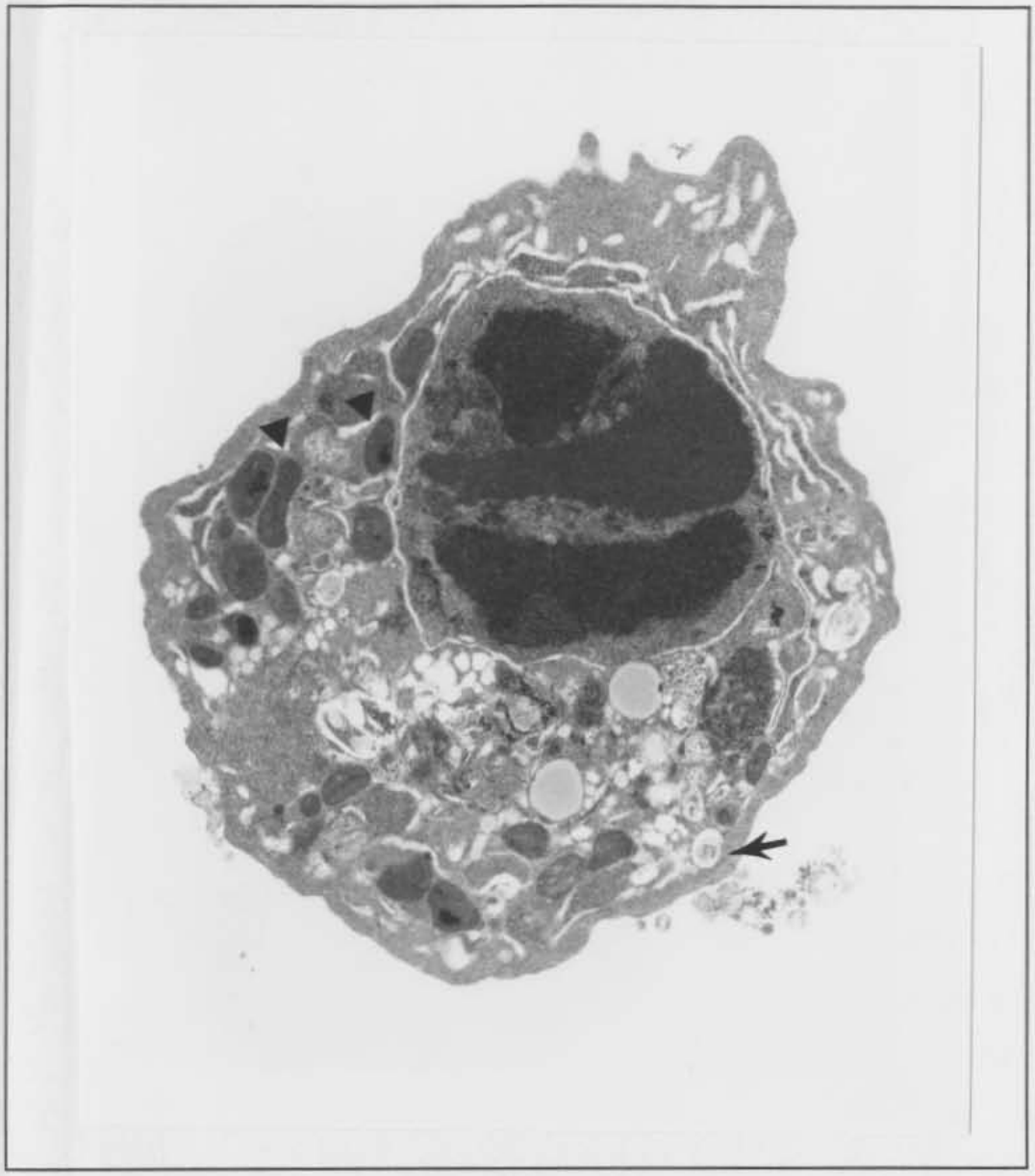

Fig.3.21. Electron micrograph showing early loss of structural details. Preserved organelles exhibit mitochondrial disruption (arrow heads) and a small myelin figure (arrow). The nucleus features chromatin condensation (100 μM after 2 days. (Original magnification × 12,600).

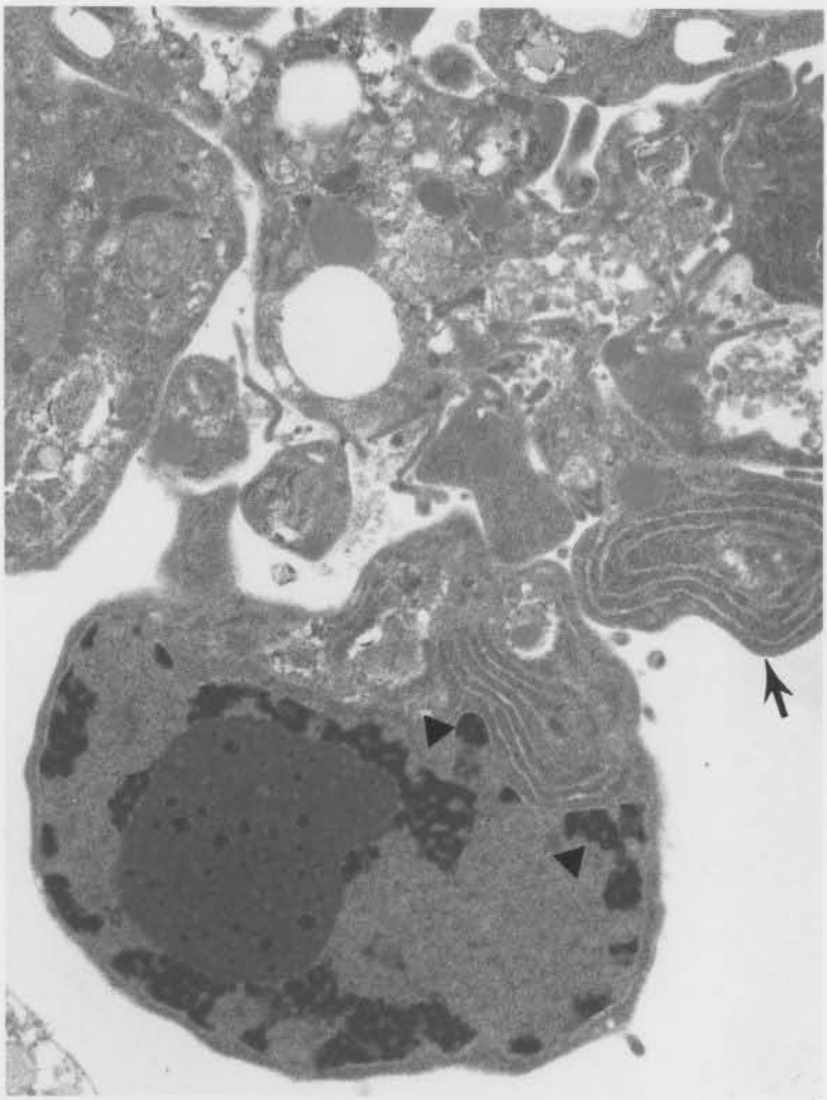

Fig.3.22. Electron micrograph showing myelin figures (arrows) and nuclear densities (arrow heads) (50 μM lovastatin after 6 days). (Original magnification × 15,600).

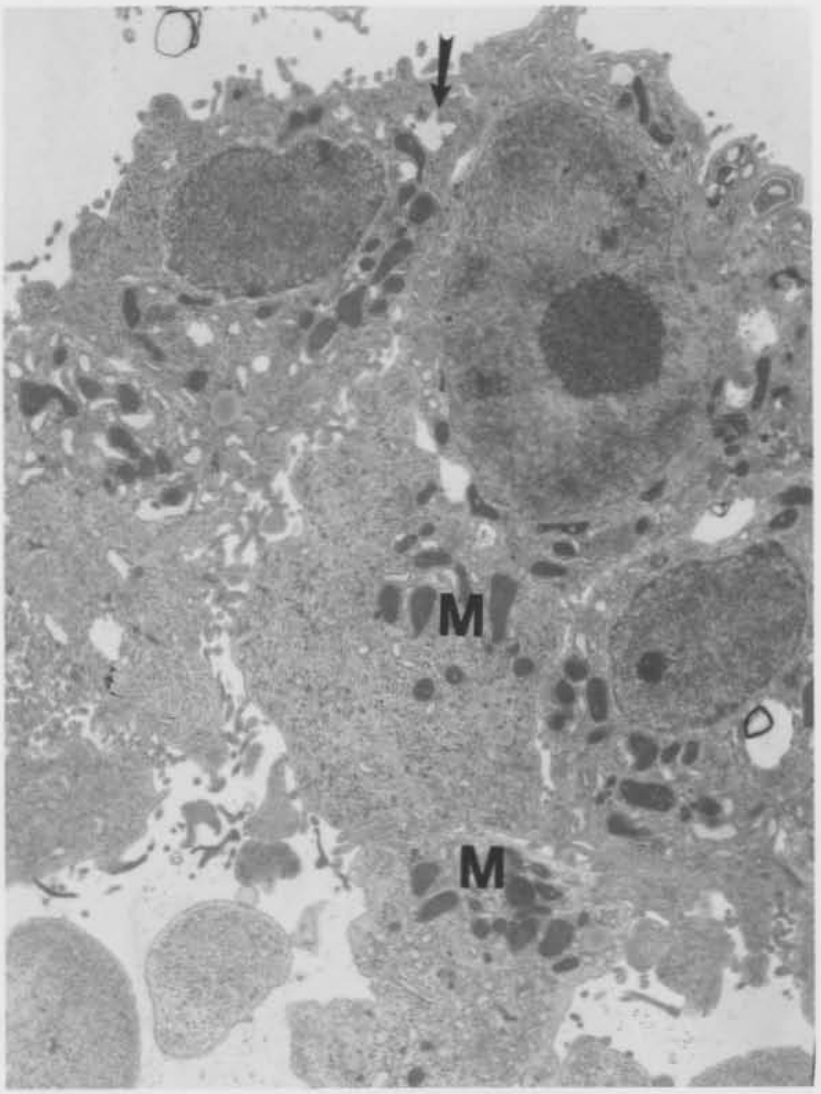

Fig.3.23. Electron micrograph of shrunken cells showing cell membrane disruption and endoplasmic reticulum dilation (arrow). Mitochondrial number is increased (M) (100 μM lovastatin after 24 hours). (Original magnification × 5,700).

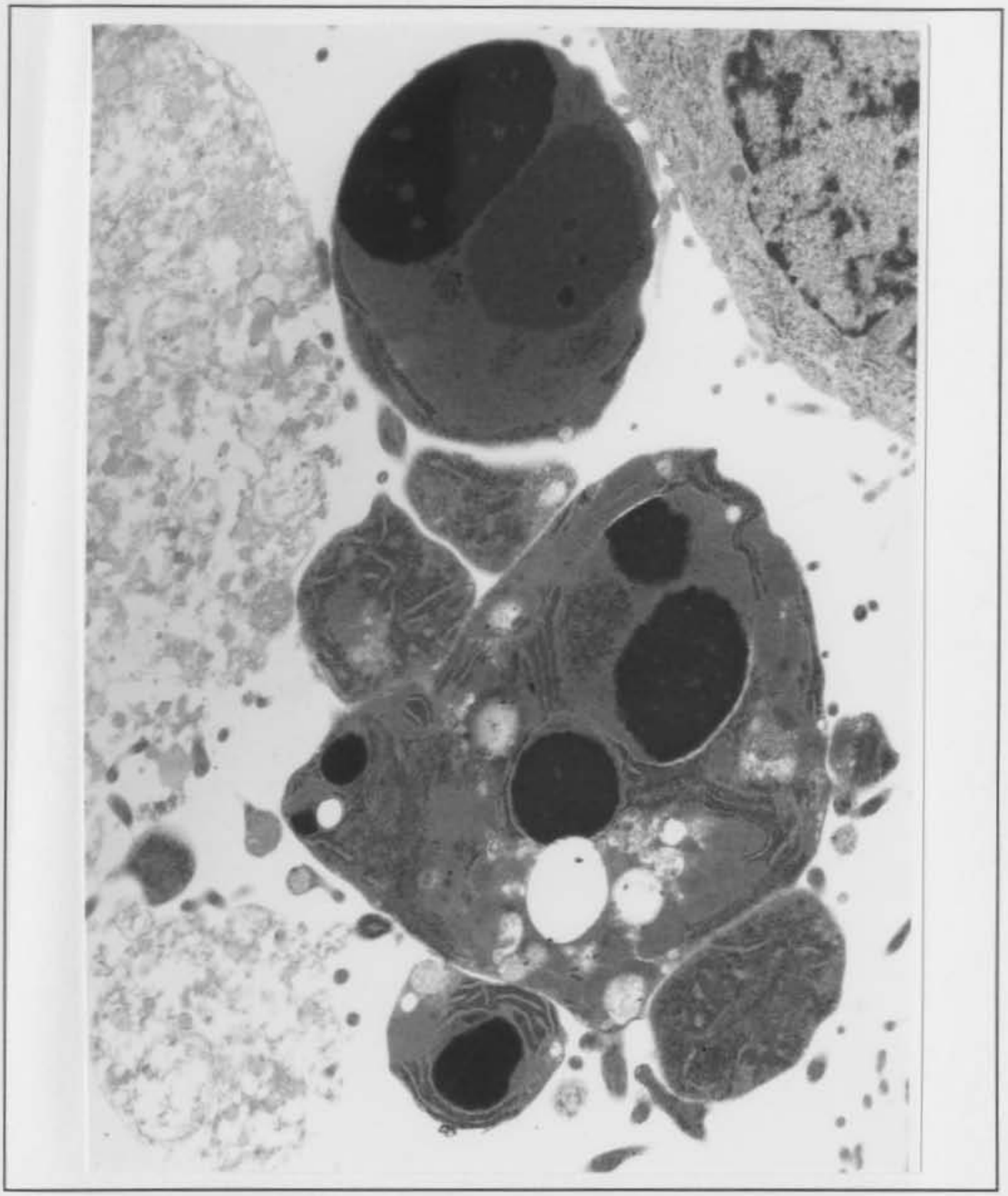

Fig.3.24. Electron micrograph of apoptotic bodies showing condensed nuclear chromatin (50 μM lovastatin and 0.8 mM OA/BSAC after 24 hours). (Original magnification × 14,000).

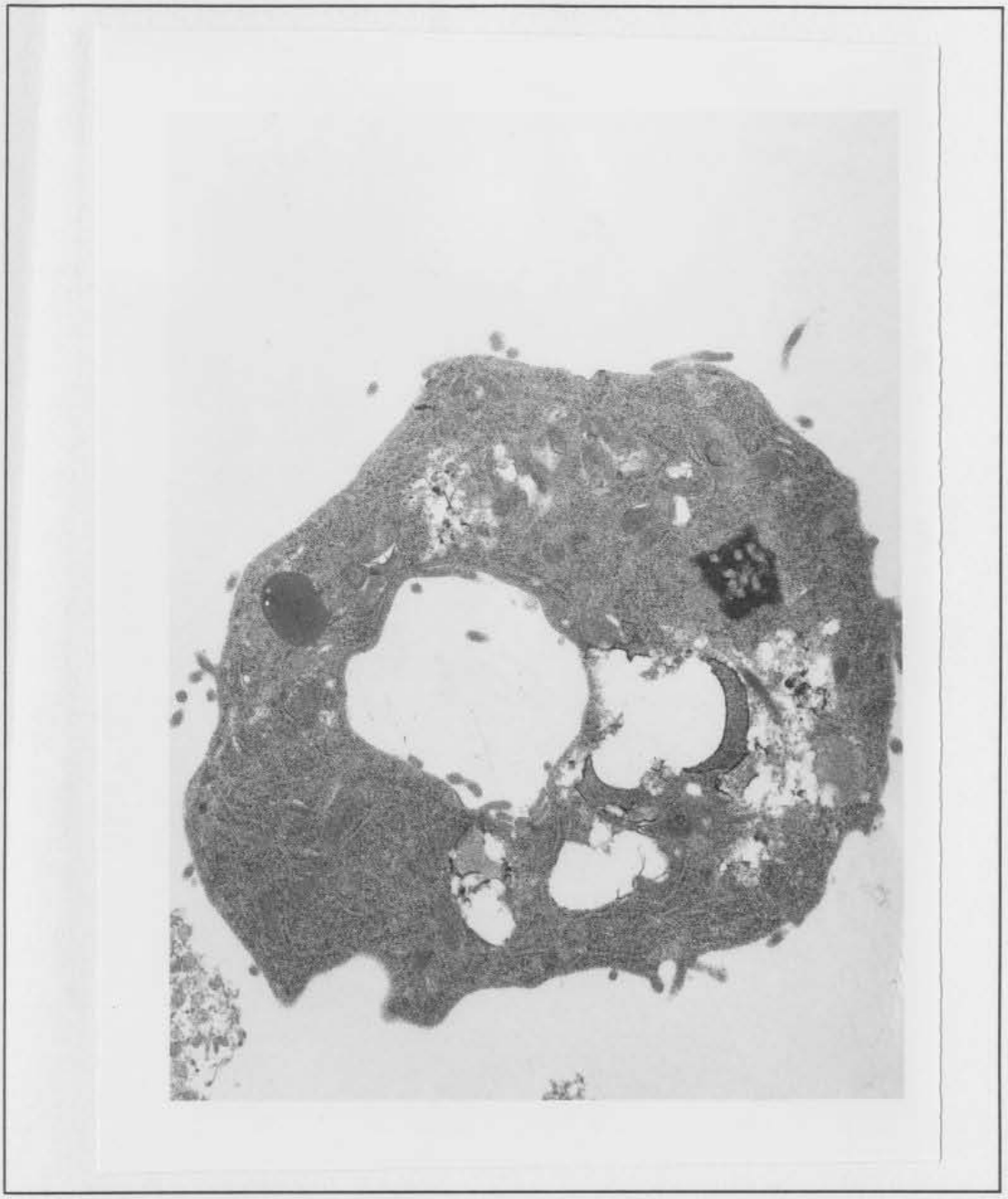

Fig.3.25. Electron micrograph showing a necrotic cell with loss of cytoplasmic and nuclear integrity (100 μM lovastatin after 3 days). (Original magnification × 12,600).

Table 3.1 GRADES OF MORPHOLOGIC CHANGES DETECTED BY ELECTRON MICROSCOPY.

GRADE	FEATURES
Mild(+)	-Cellular swelling (generalized) (Figs. 3.13 & 3.14)
	-Mitochondrial swelling (Fig. 3.14)
	-Mitochondrial vacuoles (granules) (Fig. 3.16)
	-Endoplasmic reticulum swelling (Fig. 3.19)
	-Mitochondrial cristae reduction (Figs. 3.14 & 3.18)
	-Cytoplasmic vacuolization (Fig. 3.20)
Moderate(++)	-Loss of microvilli (Fig. 3.14)
	-Ribosomal dispersion (Fig. 3.15)
	-Blebbing (Figure 3.17)
	-Myelin Figures (Fig. 3.19)
	-Lipid droplets (Figs. 3.17 & 3.19)
	-Condensation of nuclear chromatin (Fig. 3.18)
	-Cell shrinking (Figs. 3.20 & 3.22)
	-Nuclear densities (Figs. 3.18 & 3.20)
Severe(+++)	-Disruption of cell membrane (Figs. 3.22 & 3.24)
	-Mitochondrial disruption (Fig. 3.21)
	-Extreme chromatin clumping (Fig. 3.21)
	-Apoptotic bodies (Fig. 3.24)
	-Loss of structural details (Fig. 3.21)
	-Necrosis (Fig. 3.25)

morphologically damaged if one or more of the following features were noted:

- Cell membrane fragmentation.
- Mitochondria derangement (disappearance of the cristae, increased size).
- Myelin Figures.
- Cellular swelling.
- Cytoplasmic accumulation of globules.
- Presence of blebbing.

Cell death was indicated by the presence of one or more of the following features:

- Chromatin condensation.
- Loss of intracellular structural details.
- Complete disruption of the cell membrane.
- Apoptotic bodies

3.3.1 Effects of lovastatin alone

The morphological changes produced by exposure to lovastatin alone are summarized in Table 3.2.a. These data include lovastatin concentrations from 2.5 to 100 μM and exposure times from 1 to 6 days. Concentrations less than 2.5 μM did not show significant changes as compared to control.

TABLE 3.2-a Grades of morphologic changes in HepG2 cells exposed to lovastatin alone.

Incubation time (days)	Lovastatin concentration (μM)				
	2.5	5	10	50	100
1	-	-	-	++(B)	+++(A)
2	-	-	+(B)	++(B)	+++(A)
3	+(B)	+(B)	++(B)	++(A)	+++(A)
6	+(B)	++(A)	++(A)	+++(A)	+++(A)

TABLE 3.2-b Grades of morphologic changes in HepG2 cells exposed to lovastatin supplemented with 0.8 mM OA/BSA after one day incubation.

	Lovastatin concentration (μM) + 0.8mM OA/BSA				
OA/BSA-1	2.5	5	10	50	100
+(B)	+(B)	++(A)	++(A)	+++(A)	+++(A)

(A) Morphologic changes seen in the majority of cells.

(B) Morphologic changes seen in the minority of cells.

Cells incubated for 48 hours with 2.5 - 5 μM lovastatin exhibited no obvious degenerative changes. Formation of intranuclear dense regions, cellular swelling, and formation of lipid-vacuoles was observed after incubation for 3 days for 2.5 μM. With 5 μM, mitochondrial enlargement and cristae destruction were observed after 3 days. This is classified as 'mild' structural damage. After 6 days, mitochondrial changes and vacuoles (mild chande) appeared in 2.5 μM, whereas reduction in mitochondrial cristae and microvilli (moderate change) was shown with 5 μM lovastatin concentration.

Cells incubated with 10 μM lovastatin developed 'mild' structural damage by day 2. Lipid-filled vacuoles appeared and mitochondrial cristae disappeared by day 3. The cells appeared somewhat shrunken as compared with the controls but the growth rate and general morphology were essentially normal. By day 6, some cells began to demonstrate shrinkage, chromatin condensation around the nuclear membrane, cytoplasmic vacuolation, mitochondrial swelling with cristae reduction, cytoplasmic enlargement, and almost complete disappearance of microvilli. However, some cells preserved normal nuclei.

When cells were incubated for 24 hours with lovastatin in the absence of oleic acid, morphological changes were only observed at high lovastatin concentrations (50 and 100 μM). With 24-hour exposure of 50 μM lovastatin changes were classified as 'moderate'. There was an increase in the number of mitochondria but the mitochondria exhibited swelling, cristae reduction, and large lipid vacuoles. There was also ribosomal

separation, mild chromatin condensation with appearance of dense regions within the nuclei. There were early indications of apoptosis. The nuclear densities were almost twice the size of those observed in control cells. After 48 hours apoptotic cells were evident. By day 6 structural damage was 'severe': myelin figures, blebbing, and nuclear indentation were evident in the majority of cells. Apoptotic bodies appeared. Many cells were dead but a few apparently normal cells remained. The general impression is that a 6-day incubation with 50 μM lovastatin was comparable in morphological changes to 24-48 hours exposure to 100 μM lovastatin.

Severe changes were noted with 100 μM lovastatin for 24-hour exposure. Resulting structural damage was regarded as 'severe' Mitochondria had increased in number but their cristae almost disappeared and vacuoles had appeared. An increased number of mitochondria could be attributed to the appearance of aggregated mitochondria. Cytoplasmic lipid droplets appeared. Blebbing was a characteristic feature and some of the cells were undergoing apoptosis. The cell nuclei were variable in size; some were as large as those of normal cells but others were more compact and densely staining.

Some cells were noted to have developed additional, progressive morphological changes: the nuclei of these cells were fragmented into a cluster of vesicles; and the massive cytoplasm-filled blebs began to pinch-off and detach (Figure 3.26). Loss of cytoplasm by bleb detachment may partly explain the observed cytoplasmic shrinkage.

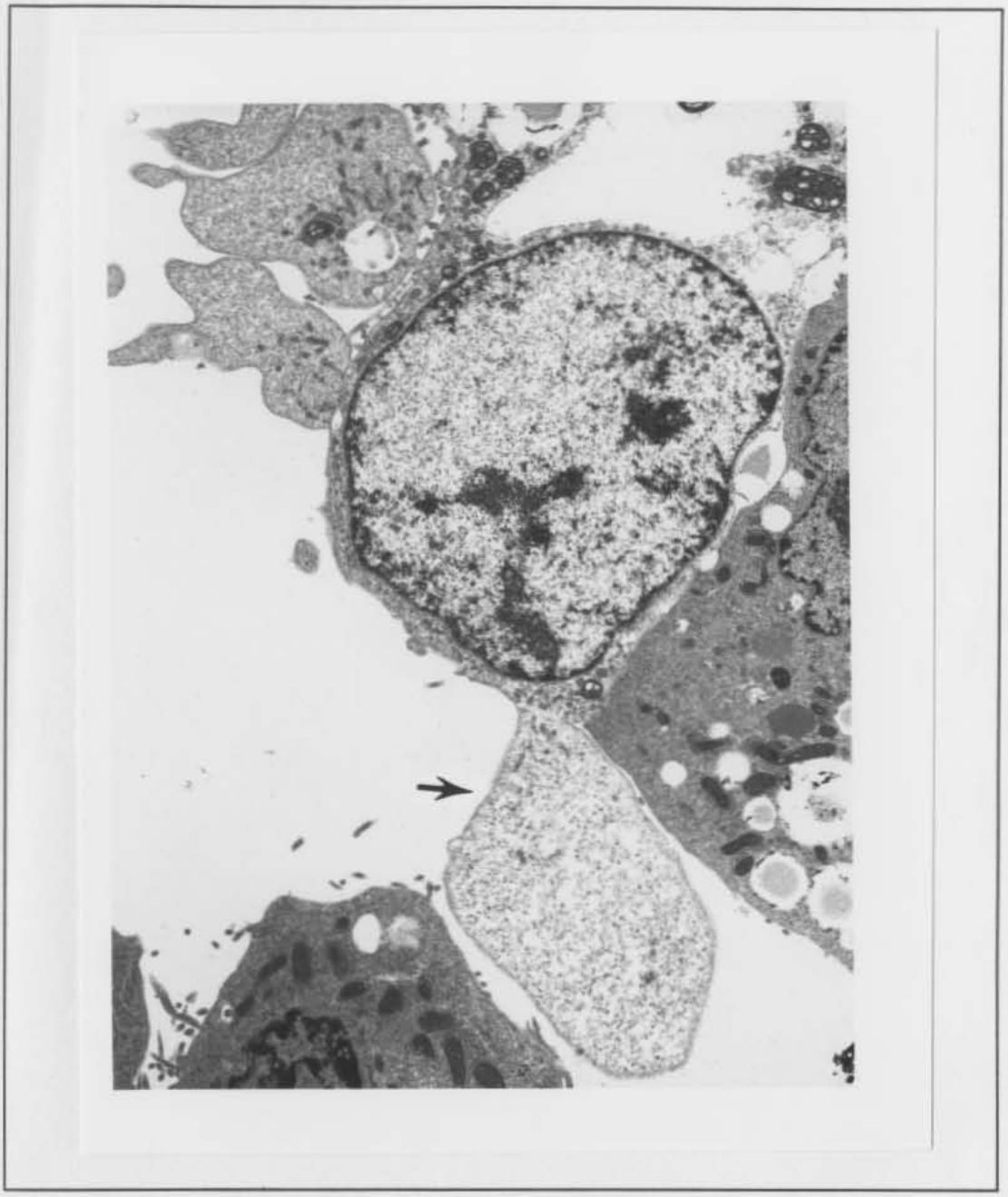

Fig.3.26. Electron micrograph showing cytoplasm-filled blebs beginning to pinch-off and detach (arrow) (100 μM lovastatin after 24 hours) (Original magnification × 9,000).

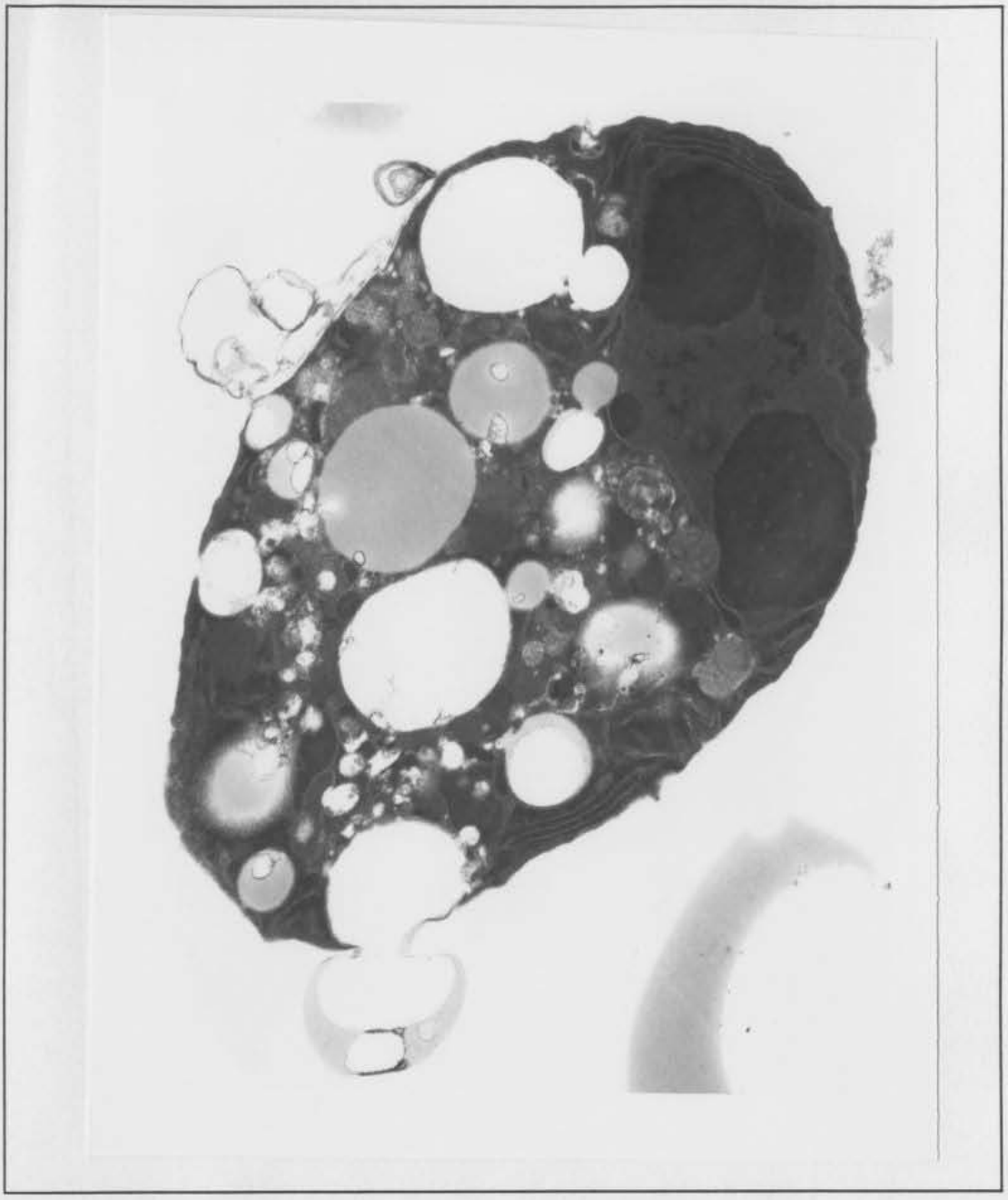

Fig.3.27. Electron micrograph showing apoptotic body with mixed features (100 μM lovastatin after 3 days). (Original magnification × 12,600).

The final state in this degenerative apoptotic process is shown in Figures 3.24 and 3.27. By 48 hours necrotic cells and a few apoptotic bodies were observed. After 72 hours lipid droplets were huge and the mitochondria though still high in number were swollen and cristae had completely disappeared. Cytoplasmic membranes were disintegrating.

Upon further incubation with 100 μM lovastatin for 2-6 days it was observed that some of the cells, although damaged, were not progressing to cell death (typical apoptotic bodies, secondary necrosis ,and necrosis) as quickly as the others. As will be discussed under flow cytometry, the reason for this phenomenon might have been that lovastatin-induced cell death does not take place at all stages in the cell cycle. Cells in S phase (interphase) are resistant. Death takes place during the transition to G2/M (induction of mitosis). In rat liver induction of both apoptosis and necrosis has been described for hepatotoxic agents *in vivo* (Ledda-Columbano et al., 1991) and T call *in vitro* (Aten et al., 1995).

3.3.2 Effects of lovastatin and oleic acid

The structural consequences of supplementing cultures with 0.8 mM oleic acid/BSA were dramatic, even after 24 hours (Table 3.2.b). Cells cultured with oleic acid/BSA without lovastatin exhibited 'mild' structural damage (Table 3.1). This became 'moderate' at lovastatin concentrations between 5 μM and 10 μM lovastatin and 'severe' with 50 μM lovastatin.

Characteristic damage after 24-hour exposure to oleic acid/BSA alone included changes to the mitochondria (increased in number with reduction in number of cristae, appearance of intramitochondrial vacuoles and granules), cytosolic lipid droplets, and swelling of the endoplasmic reticulum. Addition of lovastatin up to a concentration of 5μM did not significantly increase the structural damage after 24 hours incubation.

When the cells were incubated with 10 μM lovastatin and 0.8 mM oleic acid/BSA for 24 hours, the number and the size of lipid droplets was increased further, mitochondrial cristae disappeared, and chromatin started to clump. Some of the cells showed necrotic features. Cell damage with 50 μM lovastatin and oleic acid/BSA was more severe than at 10 μM. Myelin figures and cell blebbing was evident. Some of the cells showed advanced apoptosis. With 100 μM lovastatin and oleic acid/BSA, cells exhibited rounding-up or shrinkage and non-refringent cells floating in the medium (i.e no longer attached to the culture dish surface monolayer) were observed. The remaining adherent monolayer cells exhibited mitochondrial changes, huge lipid droplets, disruption of ribosomes, and apoptotic bodies. A level of damage was achieved in 24 hours with 100 μM lovastatin and 0.8 mM oleic acid/BSA comparable to that of 100 μM lovastatin alone in 3-4 days.

Transmission electron microscopy of the floating cells recovered from the medium demonstrated typical apoptotic bodies with clear features of secondary necrosis (i.e mixed

features of apoptosis (Figure 3.27) and necrosis (Figure 3.25). That these cells were in fact dead was verified independently by SRB assay.

3.4 Results of Flow Cytometry

In order to assess the extent of lovastatin-induced apoptosis and to observe whether this process was selective to any phase of the cell cycle, I studied the cell cycle distribution of HepG2 cells treated with 10, 50, and 100 μM lovastatin with and without 0.8 mM OA/BSA and untreated control (Tables 3.3 and 3.4, Figures 3.28 and 3.29). Flow cytometric analysis of propidium iodide-stained nuclei was performed for 24 and 48 hours (average of three experiments). The appearance of cells with a DNA content less than G1, characteristic of early apoptotic cells (sub-G1 M1), could be observed after the addition of 100 μM lovastatin for 24 hours (Figure 3.28 D). Cell arrest in G1 mixed with 25.3 % apoptotic cells was seen with 50 μM after 48 hour with lovastatin alone (Figure 3.28 G). In case of OA/BSA, the M1 region appeared with 50 μM after 24 hour and with 100 μM lovastatin after 48 hour (Figure 3.29 C & H).

3.4.1 Effects of lovastatin alone

Cells with 10 μM lovastatin after 48 hours showed arrest in the G1 phase with a decrease in the S phase and a pronounced G2/M arrest. Treatment with 10 μM produced no significant change during the first 24 hours. Although there was no obvious change in the total number of S phase cells after 24 hours, there was a decrease in the number

TABLE 3.3 Effect of Lovastatin on HepG2 Cell Cycle phase distribution after 24 and 48 hours incubation*.

Treatment (μM)	time (hr)	G1	S	G2/M	Apoptotic cells (M1%)
0	24	73.8±0.4	5.4±1.0	20.3±1.4	0.25±0.3
10	24	76.0±0.7	6.6±0.6	19.4±0.8	1.90±0.2
50	24	76.0±0.6	9.0±0.3	15±0.7	8.50±1.3
100	24	61.3±0.2	9.5±0.5	29.2±0.5	51.1±0.9
0	48	73.5±0.7	5.6±0.3	20.9±0.5	0.5±0.7
10	48	83.0±0.5	3.3±0.4	13.7±5.0	49.0±0.4
50	48	80.7±0.8	8.9±0.6	10.4±0.8	27.3±0.9
100	48	41.9±0.6	34.4±0.6	23.7±0.9	8.7±1.1

TABLE 3.4 Effect of Lovastatin supplemented with 0.8 mM OA/BSA on HepG2 Cell Cycle phase distribution after 24 and 48 hours incubation*.

Treatment (μM)	time (hr)	G1	S	G2/M	Apoptotic cells (M1%)
0	24	71.8±0.4	4.5±1.1	24.5±0.2	2.3±0.5
10	24	71.8±0.3	6.7±0.8	21.5±2.6	2.6±0.6
50	24	74.2±0.7	12.0±0.1	13.8±0.6	35.1±0.9
100	24	56.2±0.3	27.1±0.1	16.7±0.2	5.9±0.4
0	48	70.5±0.9	4.3±0.2	25.2±0.9	8.9±0.6
10	48	62.5±0.7	17.3±0.1	20.2±0.6	7.5±0.4
50	48	64.4±1.3	25.1±0.8	9.5±0.4	9.1±0.9
100	48	40.2±0.6	5.0±1.5	54.8±0.8	26.5±0.5

* The results are the mean ± SD of three experiments.

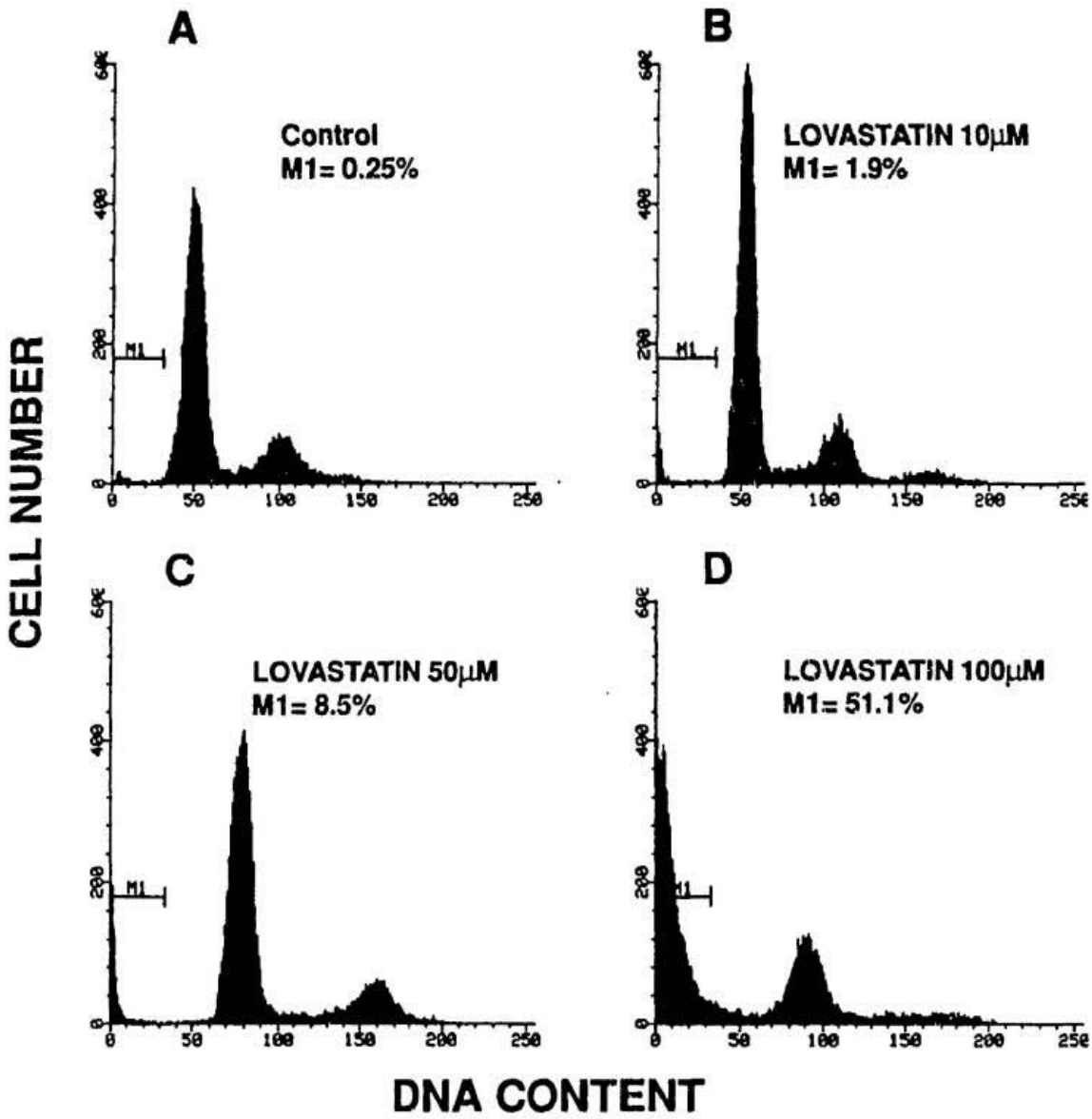

Fig. 3.28 (Part)

DNA content histogram of HepG2 cells treated with different concentrations of lovastatin.

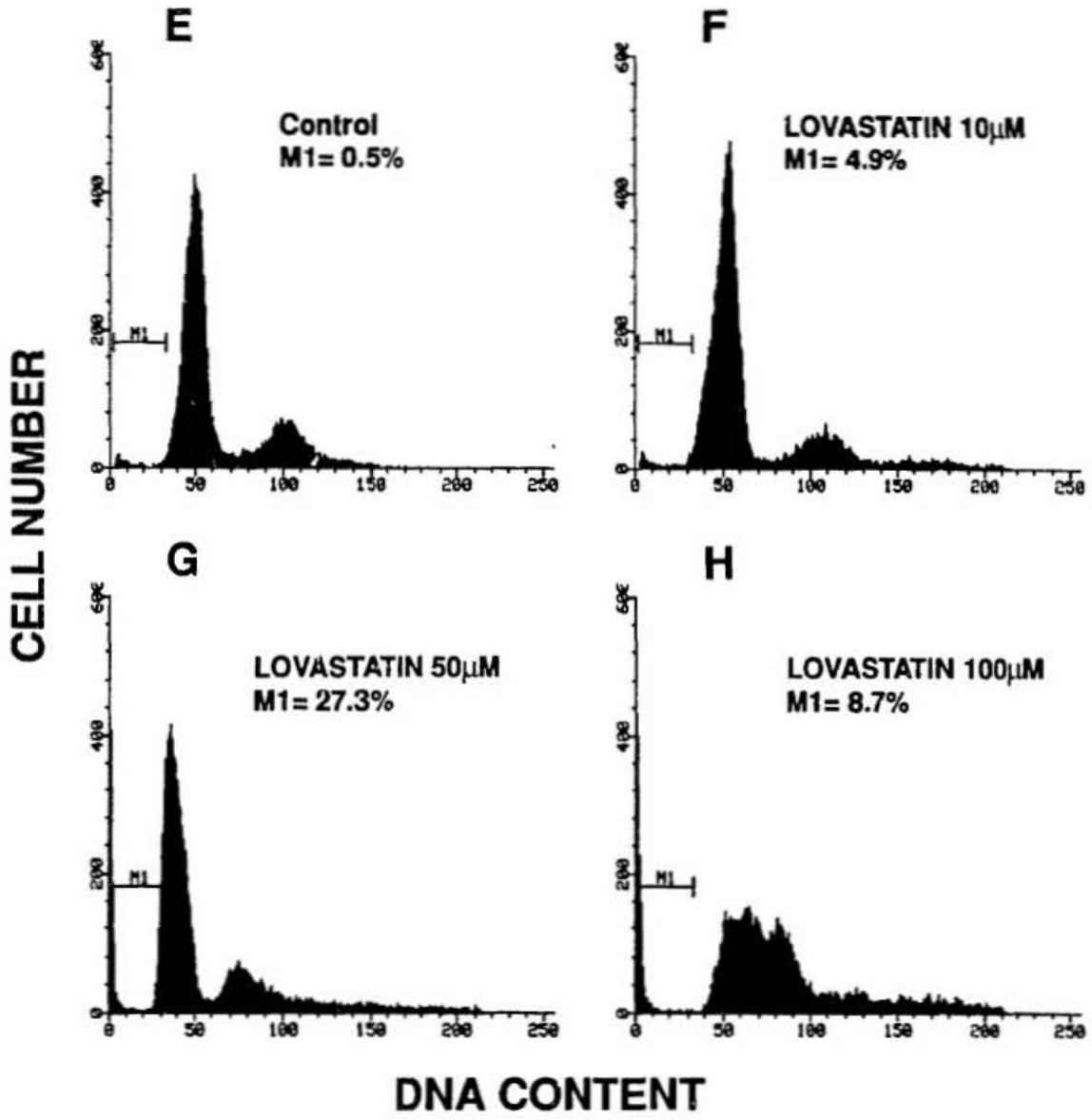

Fig. 3.28 (Continue)

DNA content histogram of HepG2 cells treated with different concentrations of lovastatin.

HepG2 cells were incubated with 10, 50 and 100 μM lovastatin for 24 and 48 hours and were analyzed for relative DNA content by flow cytometry. The cultures received no treatment (control A and E), or were treated with 10, 50 and 100 μM lovastatin for 24 hours (B, C and D) and for 48 hours (F, G and H). The percentage of cells with reduced DNA content due to apoptosis are presented in the M1 region.

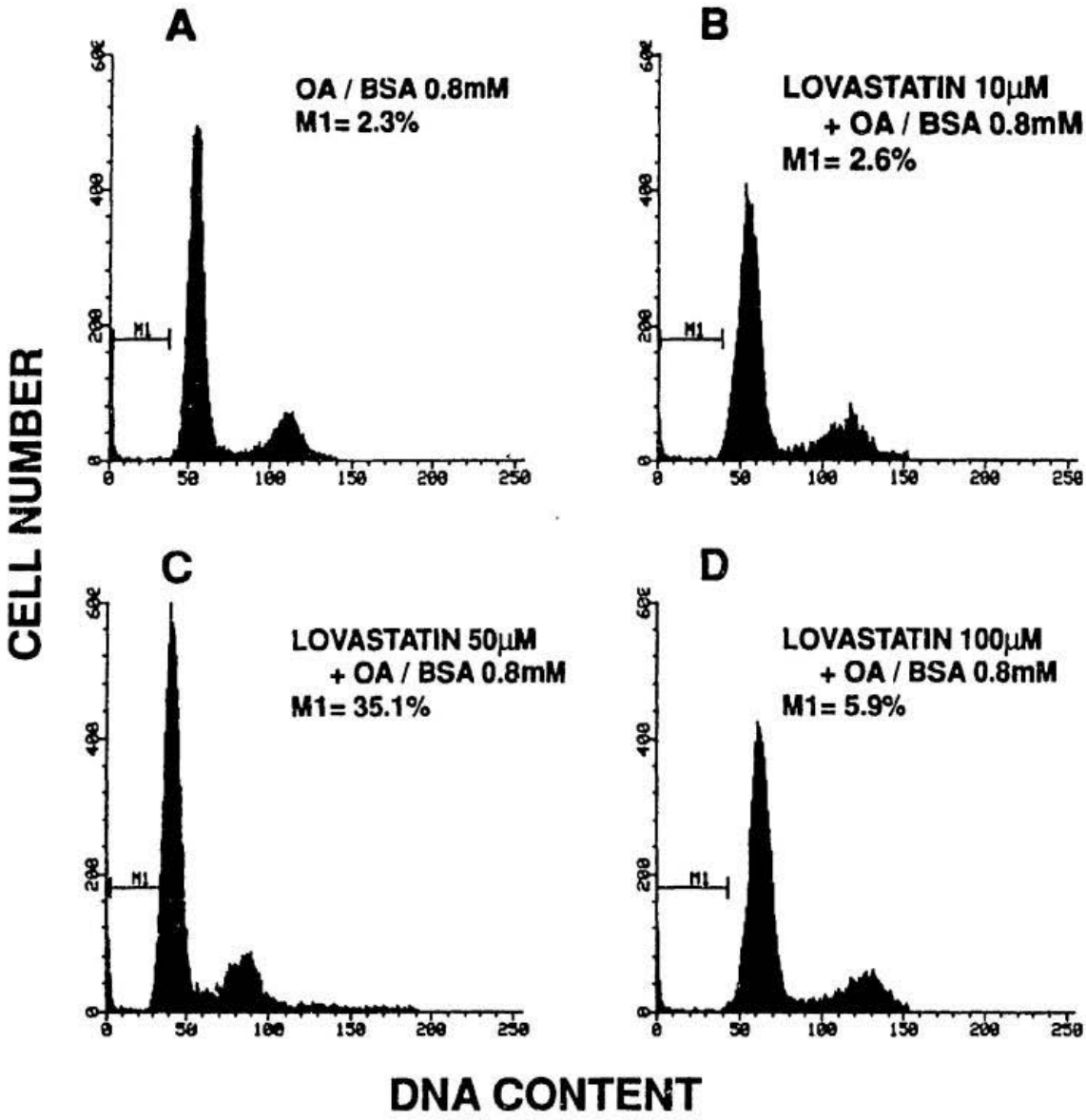

Fig. 3.29 (Part)
DNA content histogram of HepG2 cells treated with 0.8 mM OA/BSA and different concentrations of lovastatin.

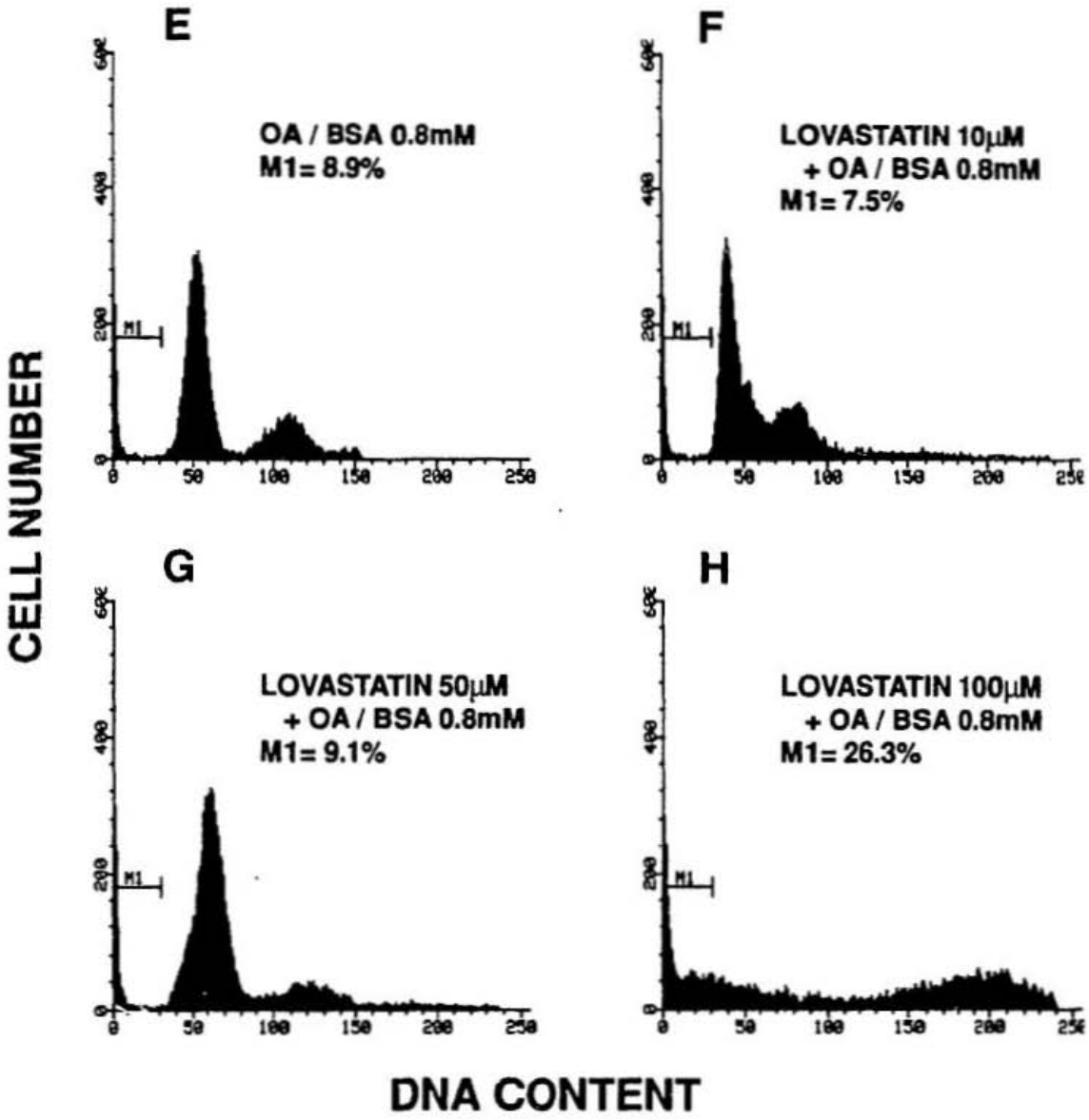

Fig. 3.29 (Continue)
DNA content histogram of HepG2 cells treated with 0.8 mM OA/BSA and different concentrations of lovastatin.

HepG2 cells were incubated with 0.8 mM OA/BSA in addition to 10 , 50 and 100 μM lovastatin for 24 and 48 hours and were analyzed for relative DNA content by flow cytometry. The cultures received 0.8 mM OA/BSA (0.8 mM OA/BSA A and E), or were treated with 10, 50 and 100 μM lovastatin with 0.8 mM OA/BSA for 24 hours (B, C and D) and for 48 hours (F, G and H). The percentage of cells with reduced DNA content due to apoptosis are presented in the M1 region.

of S phase cells after 48 hours, which was the same time when cells appeared with arrested G1 cells. This suggests that the late S phase cells may be the subpopulation of cells undergoing cell arrest rapidly following exposure to lovastatin. These cells were probably in early S phase at the time they were incubated with lovastatin.

With exposure of HepG2 cells for 24 hours with 50 μM lovastatin, cells began to appear with DNA content of less than G1. There was an increase in the cells entering S phase and decrease in G2/M, which suggested the occurrence of a transient G1 arrest. By 48 hours, the G1 arrest was evident with relative increase in apoptotic cells in the hypodiploidy region, further decrease in G2/M and almost no change in S phase. These observations suggest that the cells were arrested in the G1/S phase and with extended time the number of G2 arrested cells gradually decreased at the same time that cells appeared with DNA content less than G1 (M1 region).

In HepG2 treated cells at 24 hours with 100 μM there was a massive increase in hypodiploidy (M1%) with decrease in the G1 phase and concomitant increase in the S and G2/M phases, indicating an arrest in the S-G2 phase with increase in the proportion of hyperdiploid cells(Figure 3.28 D). The continuous loss of cells in G1 and arrest of the cells in the late S phase and G2 mitosis could be seen at 48 hours but with much higher cells in S phase and less cells in G2/M and with increase in the hyperdiploid cells (Figure 3.28 H). The percentage of apoptotic cells in M1 region was dramatically decreased. The

majority of cells which did not undergo apoptosis became arrested in the S-G2 phase and, with extended time, the number of G2 arrested cells gradually decreased without reentering the normal cell cycle.

3.4.2 Effects of lovastatin and oleic acid

Cells treated with 0.8 μM OA/BSA alone after 24 hours only showed increase in the G2/M (Table 3.4). Continuous incubation for 48 hours showed an increase in the hypodiploid cells and almost no change from the 24 hours cycle.

The addition of 10, 50, and 100 μM lovastatin to OA/BSA for 24 hours showed almost the same behaviour as with lovastatin alone except apoptotic cells appeared with 50 μM, similar to exposure to 50 μM lovastatin alone for 48 hours. Also, exposure to 100 μM lovastatin with OA/BSA showed an increase in S-phase cells and decrease in the G/M phases as compared to the control cells which is similar to the cell cycle after 48 hours with lovastatin alone at the same concentration.

By 48 hours, exposure to 10 and 50 μM showed cells decrease in the G1 phase, increase in S phase, and G2/M as compared to control but to differing degree. The decrease in G1 was almost the same in both concentrations. The increase in S phase in 50 μM was much more than in 10 μM, and the decrease in G2/M was much less than control. Hyperdiploid cells could be seen in each histogram. The percentage of apoptotic

cells was around the same as for the control.

Extended incubation with 100 μM lovastatin for 48 hours resulted in DNA fragmentation. The DNA cell cycle distribution was no longer detectable; as shown in (Figure 3.29 H).

3.5 Lipid Metabolism

The levels of triacylglycerol, cholesteryl ester, apoB, and apo A were measured following 0.8 mM OA/BSA to investigate the effects of lovastatin on synthesis and secretion of the major lipid fraction in nutritionally-supplemented cells. Although the free fatty acid to albumin mole ratio used was unphysiologically high, this allowed the addition of the smallest amount of albumin and least volume while still delivering adequate free fatty acid (Ellsworth et al., 1986). The use of medium containing oleic acid/BSA on HepG2 cells, should facilitate the synthesis and formation of ApoB, cholesterol, cholesteryl ester, and triglyceride and demonstrate the effects of lovastatin lipid metabolism (Pullinger et al., 1989; Cianflone et al., 1990).

3.5.1 Effects of lovastatin and oleic acid/BSA on synthesis and secretion of cholesterol and cholesteryl ester.

For quantitative assay of lipids within cells and in the culture medium, extracts were separated by TLC, the cholesteryl ester spot was visualized with iodine vapour, and

scraped off. Three independent methods were used for quantitation:

- radiometric using a [^{14}C]-oleic acid dilution method.
- gas chromatography/mass spectroscopy.
- spectrophotometric analysis following derivitization with o-phthaldehyde.

Figures 3.30 and 3.31 show a dose-response relationship between lovastatin and both intracellular and extracellular cholesteryl ester concentrations as assayed by the radiometric method. With 1μM lovastatin in the culture medium the endogenous cholesteryl ester decreased by 70 ± 3.5% ($p < 0.0025$) and the extracellular cholesteryl ester by 65 ± 7% ($p < 0.0001$), both relative to the control cultures (The actual control values were 147 ± 3.1 and 94.7 ± 6.3 nmol/mg protein within cells and in the medium, respectively). No further decreases in cholesteryl ester were noted when the lovastatin concentration was increased above 1 μM, indicating that endogenous cholesterol synthesis and secretion into the medium had been completely suppressed. Comparing radiometry, GC/MS and spectroscopy; all three methods gave essentially the same results (Table 3.5). Cholesterol inhibitions by lovastatin which are also shown in the table almost parallel cholestryl ester.

3.5.2 Effects of lovastatin and oleic acid/BSA on synthesis and secretion of triacylglycerols.

Triacylglycerols were determined radiometrically by a [^{14}C] dilution method

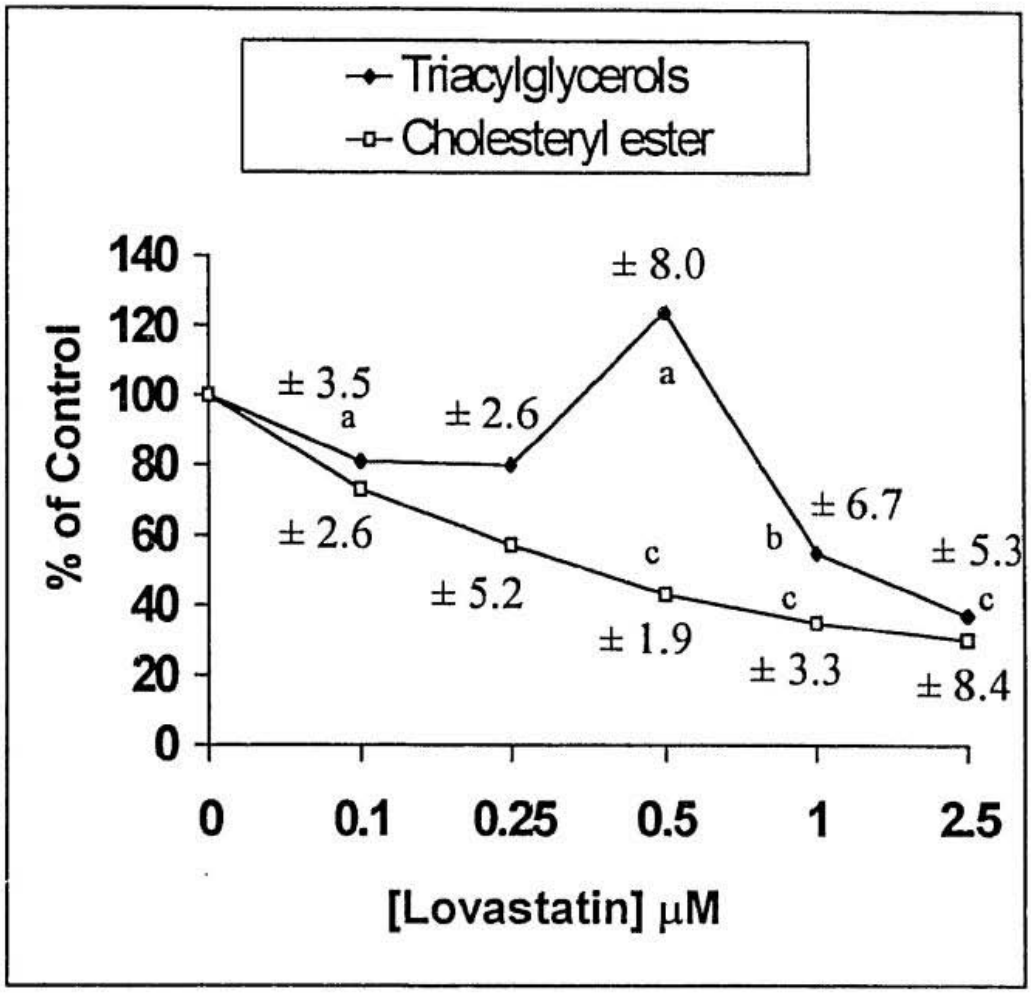

Fig. 3.30. Effect of lovastatin on secretion of lipids into the extracellular medium

Cells were incubated with 0 - 2.5 μM lovastatin in 0.8 mM [^{14}C] OA/BSA for 24 hours. The results were the average of 10 experiments. Medium [^{14}C] triglycerides and cholesteryl ester expressed as percentage of control ± SD. The basal concentration for triglyceride was 41.7 ± 2.8 nmol/mg cell protein and for cholesteryl ester was 94.7 ± 6.3 nmol/mg cell protein.

a $P < 0.005$

b $P < 0.001$

c $P < 0.0001$

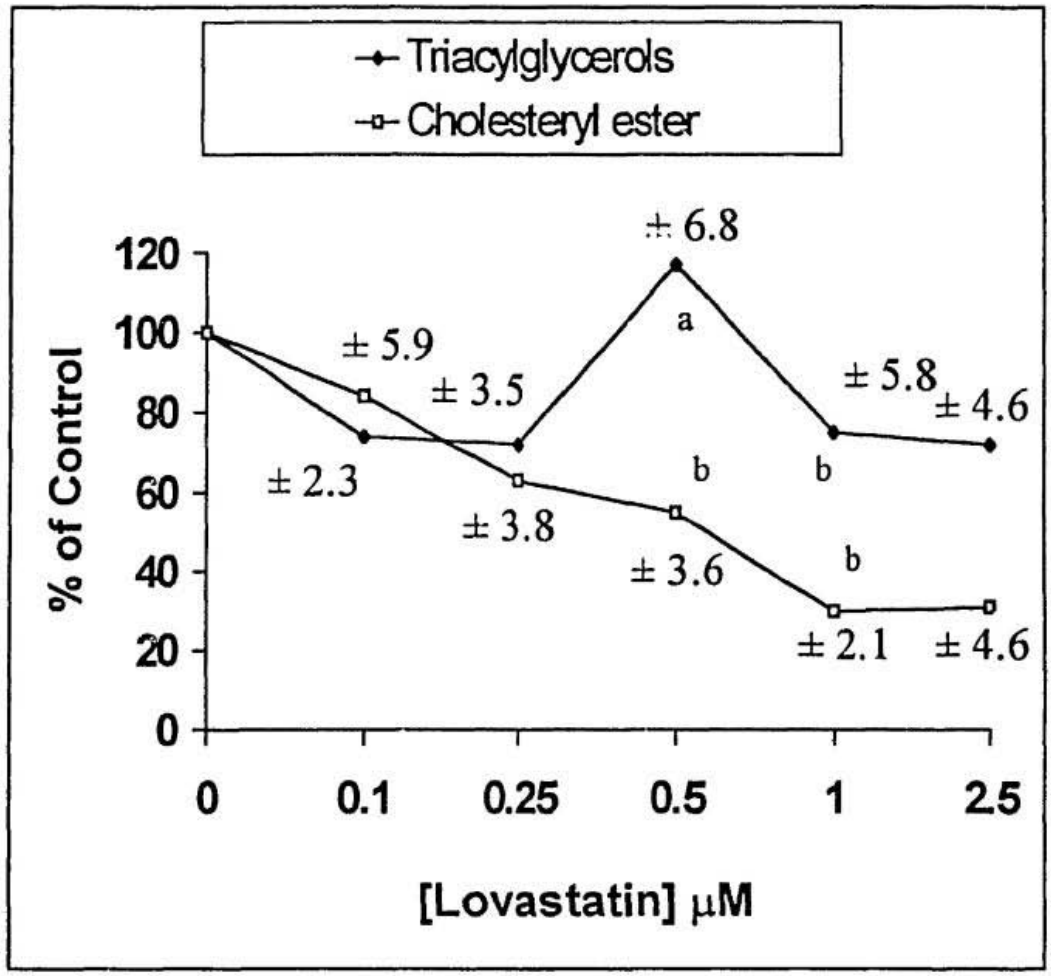

Fig. 3.31. Effect of lovastatin on HepG2 intracellular lipid synthesis.

Cells were incubated with 0 - 2.5 μM lovastatin in 0.8 mM [^{14}C] OA/BSA for 24 hours. The results were the average of 10 experiments. Cellular [^{14}C] triglycerides and cholesteryl ester expressed as percentage of control ± SD. The basal concentration for triglyceride was 294.3 ± 10.1 nmol/mg cell protein and for cholesteryl ester was 147.3 ± 3.1 nmol/mg cell protein.

a $P < 0.005$

b $P < 0.0025$

Table 3.5 Comparison between HepG2 cell cholesterol and cholesteryl ester, cellular and extracellular, as measured by three different methods after treatment with lovastatin.

Treatment	[^{14}C]				MS/GC				o-phthalaldehyde			
	Cells		Med		Cells		Med		Cells		Med	
	Ch.	CE	Ch.	CE	Ch.	CE	Ch.	CE	Ch.	CE	Ch.	CE
0.8mM OA/BSC	65.5 ±3.3	147.3 ±3.1	52.6 ±2.5	94.7 ±6.2	89.3 ±5.1	178.6 ±2.3	65.9 ±8.2	119.9 ±7.8	73.8 ±5.4	174.8 ±4.0	-	-
0.5μM Lovastatin	28.8 ±6.6	66.7 ±5.5	26.2 ±4.6	42.9 ±8.5	60.7 ±4.9	120.8 ±6.7	43.8 ±2.4	70.4 ±6.3	47.3 ±5.2	111.0 ±4.7	-	-
1.0μM Lovastatin	21.5 ±7.9	54.4 ±5.8	19.1 ±6.1	33.4 ±6.1	50.9 ±4.4	68.8 ±1.2	20.7 ±6.2	42.4 ±2.7	31.9 ±7.3	63.8 ±3.9	-	-

HepG2 cells were incubated with lovastatin in 0.8 mM OA/BSA for 24 hours. Cholesterol (Ch) and cholesteryl ester (CE) content were determined by the appropriate method as described in experimental procedures. The results reported as the mean ±SD nmol/mg cell protein of at least 6 different experiments.

(Figures 3.30 and 3.31). The effect of lovastatin on triacylglycerol synthesis and secretion over this range of concentrations was observed. In the absence of lovastatin, intracellular triacylglycerol levels were 294.3 ± 10.1 nmol/mg cell protein, but were decreased by 25.5% ($p < 0.0025$) in the presence of 1μM lovastatin. No further decrease of intracellular triacylglycerol levels was observed if the lovastatin concentration was increased beyond 1μM. However, for secretion of triacylglycerols into the medium the response to lovastatin was different: at 1 μM lovastatin the decrease from control values (41.7 ± 2.8 nmol/mg protein) was 45% ($p < 0.001$) and at 2.5 μM lovastatin the decrease was 63% ($p < 0.0001$). The intracellular synthesis of triacylglycerols was much less responsive to inhibition by lovastatin than is that of cholesteryl esters. A very interesting observation is that at 0.5 μM lovastatin, triacylglycerol synthesis is apparently increased. The triacylglycerol concentrations, relative to controls, were 117% ($p < 0.005$) and 134% ($p < 0.005$) in the cells and in the medium, respectively.

3.6 Effect of Lovastatin on the Secretion of Apolipoproteins A and B.

Culture medium recovered from 24 hour incubation of HepG2 cells supplemented with 0.8 mM oleic acid/BSA and 0 - 2.5 μM lovastatin was assayed for apolipoprotein-A (apo-A) and apolipoprotein-B (apo-B). Control values for cells cultured with oleic acid/BSA were: 18.4 ± 2.7 μg/mg cell protein for apo-A; 10.5 ± 1.5 μg/mg cell protein for apo-B.

In the case of medium unsupplemented with oleic acid/BSA, lovastatin had no effect on apoB and apoA secretion, the control levels of apo-A and apo-B were 17.45 ± 2.3 μg/mg cell protein and 3.6 ± 1.6 μg/mg cell protein, respec . . ly. Addition of 2.5 μM lovastatin had minimal effect: 17.74 μg/mg for apo-A and 3.8±2.1 μg/mg cell protein for apo-B. Different sets of subculture experiment were used to compare values and express the results as percent of control (with 0.8 mM OA/BSA without lovastatin).

Figure 3.32 shows the effect of lovastatin on apo-B secretion in medium supplemented with 0.8mM OA/BSA over a range of lovastatin concentrations after 24 hours exposure. The control apo-B concentration was 10.51 ± 2.80 μg/mg cell protein and decreased to 9.84 - 6.99 μg/mg cell protein with lovastatin concentrations 0.1 -2.5 μM, respectively. The significant reduction in apo-B into the medium is associated with the dramatic decline in cholesteryl ester synthesis ie. 1 μM lovastatin, 31.7% less apo-B ($p < 0.05$) and 70% decrease in cholesteryl ester ($p < 0.0025$).

There was no significant effect on apo-A secretion. The apo-A control concentration was 18.4 ± 2.7 μg/mg cell protein and it was 18.79 - 20.01 μg/mg cell protein exposed to lovastatin (0.1 -2.5 μM).

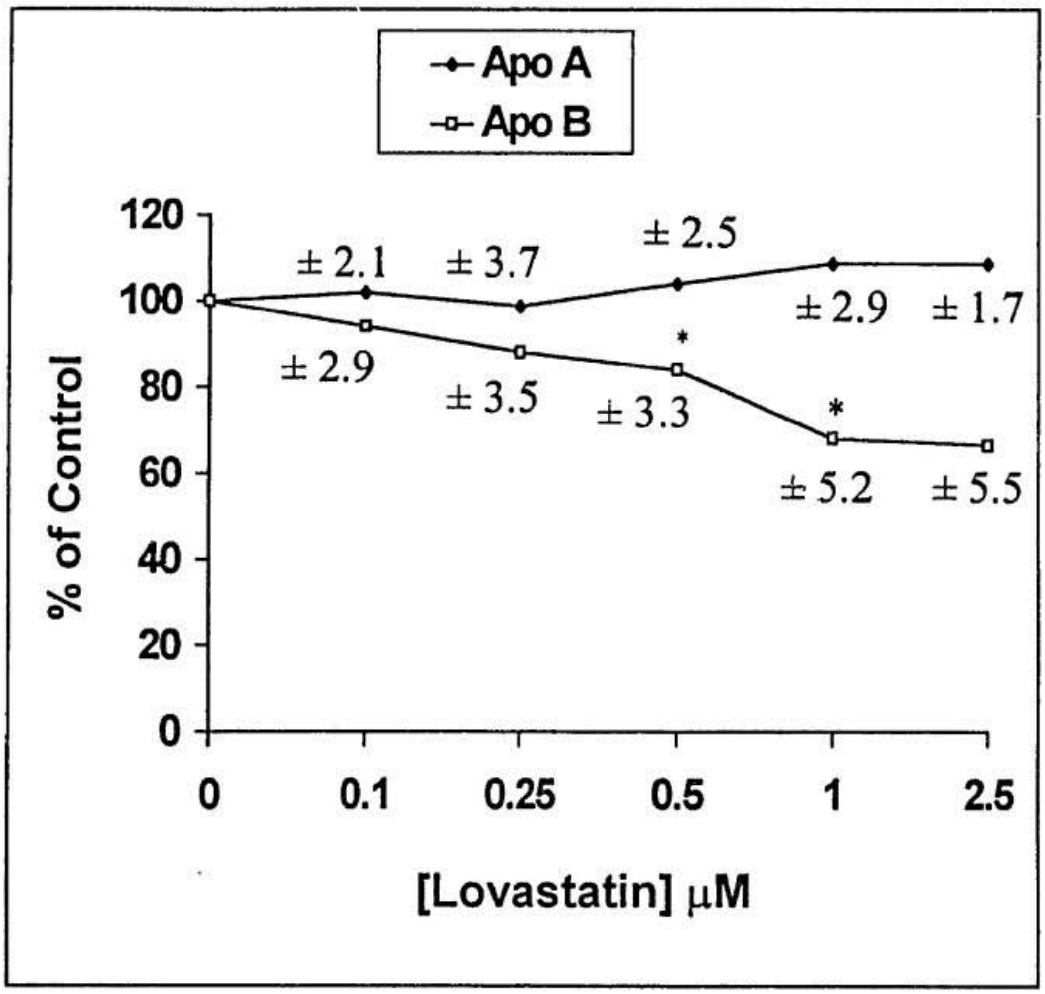

Fig. 3.32. Effect of lovastatin on HepG2 apolipoprotein accumulation.

Cells were incubated with 0 - 2.5 μM lovastatin in 0.8 mM [^{14}C] OA/BSA for 24 hours. The results were the average of 6 experiments. Culture medium apo A and apo B levels were expressed as percentage of control ± SD. The basal concentration for Apo A was 18.4 ± 3.7 μg/mg cell protein and for Apo B was 10.5 ± 2.8 μg /mg cell protein.

* $P < 0.05$

CHAPTER 4

DISCUSSION

Cultured human HepG2 cells were used to investigate the acute and chronic effects of the drug lovastatin, which is used clinically to treat hypercholesterolemia. Lovastatin is important clinically because it is an effective inhibitor of HMG-CoA reductase, the rate-limiting enzyme in the biosynthesis of cholesterol. The immediate product of HMG-CoA reductase is mevalonate, a key substance not only in cholesterol biosynthesis but also in posttranslational modification of protein by prenylation.

The investigations reported here highlight the cytotoxic effect of HMG-CoA reductase inhibition by lovastatin on the cultured human hepatoma cell line, HepG2. Previous studies on lovastatin toxicity with cultured human cells used either a pheochromocytoma cell line, PC-12, (Marom et al., 1994) or an enterocyte line, CaCo-2 (Herold et al., 1995). As liver cells in culture might behave differently because lovastatin, as used clinically, is a pro-drug requiring prior liver metabolism, it was decided to investigate the effects of lovastatin on a hepatoma cell line.

This investigation focused on the toxic effects of lovastatin at exposure levels at and above those currently used clinically. The laboratory approaches used included electron microscopy and cell cycle analysis by flow cytometry, investigation of peripheral cell damage by enzyme and other protein studies, and analysis of intracellular and extracellular lipids at various exposure concentrations and times. The experiments were also extended to consider the effects of supplementation of cell cultures with oleic acid.

The findings are discussed in light of current understanding on the significance of protein prenylation on the cell cycle and the mechanisms of cell death by necrosis and apoptosis.

To determine the acute and chronic toxicity of lovastatin to HepG2 cells, a cell survival study was conducted using two different analytical approaches, the SRB test and LDH leakage. It was found that 50 μM, but not lower, concentrations of lovastatin in the culture medium markedly decreased survival after an exposure of only 24 hours (α=0.05). The lower lovastatin concentrations used in this study (0.1 - 2.5 μM) did not kill cells, even after exposure for 8 days. But after 2-3 days, cells cultured at lovastatin concentrations of 5 and 10 μM showed a significant decrease in viability as compared to control specially in the first three days (α=0.05).

Albumin is generally considered to have nine high-affinity binding sites for oleic acid. Moberly et al. (1990) were unable to detect cytotoxicity to HepG2 cells with up to 1.0 mM oleic acid at a 9:1 molar ratio of oleic acid/BSA. Cell damage was assessed by LDH release into the culture medium or by changes in cell proteins. The absence of observable cytotoxicity of fatty acids on cultured HepG2 cells was also reported by Ellsworth et al.(1986). On the basis of these two reports that 0.8 mM oleic acid in a saturated complex with albumin does not cause cell death and is within the physiological free fatty acid range for human serum, this concentration was chosen for the experiments reported in this investigation.

It was not anticipated that oleic acid/BSA would engender any significant cytotoxicity as a nutritional supplement and it was found, in agreement with the authors cited above, that LDH leakage and intracellular protein content were normal for the first 24 hours. On the other hand, the degree of ALT and AST release and the morphological changes suggested strongly that pathological changes had been initiated.

In addition, if cell cultures containing otherwise nontoxic lovastatin concentrations were supplemented with oleic acid/BSA, cell viability was significantly reduced at all lovastatin concentrations (α=0.05): Oleic acid and lovastatin act synergistically in their cytotoxic effects. It has been shown (McKenney et al., 1995) in clinical studies that dietary fat enhances the bioavailability of lovastatin. Patients whose blood cholesterol levels were reduced with lovastatin and a low fat diet experienced an additional reduction in levels when they increased their intake of fats. It did not matter if the supplementary fats were saturated or unsaturated. This observation was explained by either increased availability of lovastatin due to coadministration of fats or by lovastatin promoting up-regulation of LDL receptor synthesis. These findings are interesting in light of an observation in rats (Skomedal et al., 1994) that enhancement of diets with lipids caused increased osmotic erythrocyte fragility and heart muscle response to catecholamines. When lovastatin was coadministered the effects of a high-fat diet were enhanced. These effects of lovastatin were dependent upon the type of dietary fat as they were prevented by the presence of cod liver oil. Our data showed that in addition to lipid reduction, in the

presence of oleic acid/BSA the cytotoxicity of lovastatin was increased. These observations can best be explained by suggesting that oleic acid acts as a carrier vehicle for lovastatin, or vice-versa. The clinical significance of this explanation is that the hepatic uptake of the drug is dependent on adequate serum lipid levels: this includes, of course, not only the clinical efficacy of the drug but also cytotoxic effects.

The presence of AST and ALT in the medium is indicative of cell leakage. These enzymes were detected after 24 hours in oleic acid/BSA-free medium with 10 μM lovastatin, or with oleic acid/BSA alone. Even control cells were demonstrated to be leaking AST and ALT by the sixth day. These two enzymes were found to be more sensitive early indicators of plasma membrane damage than the SRB test but less sensitive than LDH after 24 hours (α=0.05). But, after three days the situation became reversed: AST and ALT leakage provided a clearer indication of cellular damage than LDH leakage ($p < 0.0001$).

Morphologic evidence of lovastatin toxicity was assessed by transmission electron microscopy. Exposure of HepG2 cells to lovastatin resulted in the development of a variety of morphologic alterations with two distinct endpoints, namely necrosis and apoptosis. A complex pattern of changes involving cell membrane, organelles, cytosol and nuclear chromatin was observed. This spectrum of ultrastructural abnormalities started with simple cellular swelling and ended with frank necrosis with loss of cellular

structural details. The earliest effects were produced by a 3-day exposure to 2.5 μM of lovastatin alone. The magnitude of these changes was both dose- and time-dependent. Abnormalities resulting from exposures for less than 6 days to 50 μM or for 2 days to 100 μM lovastatin were mostly limited to the mitochondria, rough endoplasmic reticulum and cytosol. Toxic degeneration to this extent is probably reversible (Cui et al., 1995). Frank necrosis as indicated by complete disruption of cell membrane, chromatin clumping and loss of structural details was produced by exposure to 50 μM lovastatin alone for 6 days. Although necrosis also resulted from exposure to 100 μM lovastatin for 1-2 days, it was noted that longer exposures to this relatively high concentration did not necessarily increase the proportion of necrotic cells. Studies have shown that many cytotoxic agents "target" cells in the late G2 and M phases since the interphase cells (S phase) are generally resistant (Reipert et al., 1995). If this principle applies to lovastatin-induced cytotoxicity, susceptibility would not be universal to all tested cells, but would rather be limited to those in the G2/M phase. This hypothesis may provide an explanation for the observation that longer exposure to 100 μM lovastatin did not result in increased necrosis.

Supplementing the cultures with 0.8 mM oleic acid/BSA boosted the extent of lovastatin-induced morphological damage. Cells incubated with oleic acid, even in the absence of lovastatin, showed mild degenerative changes after 24 hours. Morphologic changes produced by a 24-hour exposure to 100 μM lovastatin and 0.8 mM oleic acid/BSA were similar to those resulted from the exposure to the same concentration of

lovastatin alone for 2-3 days.

In apoptosis, also referred to as programmed cell death, fragmentation of DNA occurs under the effect of calcium-dependent endonucleases (Buja et al., 1993; Barbiero et al., 1995). This active process specifically targets nuclear chromatin where it produces characteristic morphologic changes. Unlike other conventional mechanisms of cell death, apoptosis is not initiated by alterations in the cell membrane permeability or cell respiration. Accordingly, apoptotic cells exhibit characteristic condensation of the chromatin in tight apposition to the nuclear membrane with relatively preserved integrity of the cell membrane (Lizard et al., 1995). Organelles, particularly mitochondria, are generally preserved. Apoptosis is also characterized by a loss of mitochondrial function. It has been reported that mitochondria may have an important role in regulating apoptosis (Vayssiere et al., 1994; Reipert et al., 1995). These features represent the basis for the morphologic distinction of apoptosis from necrosis by electron microscopy. Since the key mechanism of necrosis is cell membrane damage, necrotic cells tend to exhibit cell membrane disruption, mitochondrial derangement and other organelle damage as described earlier.

Indications of apoptosis started to appear with 50 μM lovastatin after a 24-hour exposure, and fully developed apoptotic bodies were seen after 6 days. It was also noted that the degree of apoptosis seen by electron microscopy after a 6-day exposure to 50 μM

lovastatin was comparable to that of a 24-48-hour exposure to 100 μM. It is concluded that the ability of lovastatin to induce apoptosis is both dose- and time-dependent. This aspect of lovastatin toxicity on HepG2 cells was similar to its necrosis-inducing capacity discussed earlier. It is noteworthy that with longer exposure time (> 48 hours) to 100 μM lovastatin alone, not many apoptotic bodies were seen. *In vito* studies of chemotherapeutic agents that are purely cytotoxic reported similar results (Zaleskis et al., 1994). Apoptosis was shown to be a time-dependent phenomenon that disappears at high concentrations of the drug. It was suggested that relatively high drug concentrations applied over extended periods of time probably produces necrosis via different metabolic mechanisms and overrides apoptotic processes (Zaleskis et al, 1994; Ormerod et al., 1994). This characteristic 48-hour response to 50 or 100 μM lovastatin was achieved within 24 hours in the presence of 0.8 mM oleic acid/BSA. Exposure for longer intervals to lovastatin in the presence of 0.8 mM oleic acid/BSA probably had a higher necrotic effect that eliminated cells before apoptosis could be induced. Apoptotic bodies that were formed at earlier stages of the exposure most likely disintegrated and become inconspicuous when the sample was examined after a 4-6 day exposure to 100 μM lovastatin.

When not dividing or preparing for division, cells are in a quiescent state known as G0. When a cell is actively engaged in the act of mitosis, the stage is known as M. Proliferating cells progress through a sequence of several phases as they progress from

G0 to M. These phases are referred to as G1, S, and G2.

During G1, a cell undergoes RNA and protein synthesis. As DNA has not replicated, G1 cells can not be distinguished from G0 cells on the basis of DNA content. At the beginning of S phase, a diploid cell contains two copies of each chromosome (2N) and by the end of the S phase this has doubled (4N). The following G2 phase is the period of RNA and protein synthesis in preparation for mitosis and cell division (cytokinesis). The mitotic M phase is usually so short that cells in this phase cannot be distinguished from G2 phase cells on the basis of DNA content, since both are 4N. The process rapidly proceeds to cytokinesis and the resulting daughter cells have reestablished the diploid karyotype. They can continue for one or more cycles of replication, or reenter the G0 resting state for indefinite periods.

As discussed previously, the percentage of cells in each stage of the cell cycle in a mixed population of dividing cells can be determined efficiently by flow cytometry; a technique which labels DNA with a fluorescent dye and analyses the relative fluorescence, and thus the DNA content, of individual cells as they are caused to pass across a detector. The value of flow cytometry, that it can analyze the DNA content of individual cells, is also its limitation because it cannot distinguish between stages in the cell cycle that share the same DNA/cytoplasm ratio.

The normal DNA histogram generated by flow cytometry is characterized by a dominant (G1) peak, a second smaller (G2/M) peak, and a low S phase lying between the G1 and the G2/M peaks (Zarbo, 1994). Flow cytometry can also detect cells containing less than the normal diploid DNA content (subdiploid; M1): the presence of such cells is, as will be discussed below, characteristic of cells undergoing apoptotic DNA degradation (Aten et al., 1995). Flow cytometry also detects hyperdiploid cells that contain amounts of DNA in excess of the normal 4N typical of S phase cells.

Determination of the percentage of cells in each phase is an important key to understanding the biology and underlying biochemistry of cell replication; particularly if the analysis is coupled with experimental treatment of dividing cells with drugs that act as specific inhibitors of mitosis and cell division (Ormerod 1990; Keren et al., 1994). Such analyses permit the elucidation of biochemical processes responsible for cell replication, particularly in the case of drugs such as lovastatin that act on specific stages within the cell cycle. This approach carries the potential of identifying cell-cycle-specific inhibitors that may be of use in the management of cancer.

In general, cultured cells treated with toxic levels of lovastatin are arrested at stage G1 in the cell cycle and as cell death occurs by apoptosis a subdiploid peak is generated (Nicoletti et al., 1991; Jones and Lafrenz, 1992).

Flow cytometry was used to study the effect of lovastatin on cultured HepG2 cells at exposure levels of 10, 50, and 100 μM; and with 24 and 48 hour incubation periods.

High numbers of frankly apoptotic cells were only seen with 100 μM lovastatin. After 24 hours incubation, subdiploid cells represented 51.1% of the total population. This corresponded to the percentage of dead cells estimated by the SRB test (56.9%). Electron microscopic examination of these cells confirmed the presence of morphological features of apoptosis: cell membrane blebbing, chromatin aggregation, and densely-staining cytoplasm. In addition to the high proportion of subdiploid (M1) cells, the percentage of cells in S phase was almost doubled and that of G2/M was almost 17% higher, relative to the controls. Decrease in G1 cells with increase in apoptotic cells in sub-G1 and a concomitant increase of S and G2/M have been reported elsewhere (Gorczyca et al., 1993; Hirano et al., 1995a; Shiff et al., 1996). The general conclusion is that cells treated with 100 μM lovastatin for 24 hours were only undergoing apoptosis and that necrosis was insignificant.

After 48 hours of incubation, electron microscopy showed that most of the characteristic features seen after 24 hours with 100 μM lovastatin had disappeared. By this time the characteristic feature was necrosis rather than apoptosis. However, some apparently normal diploid cells remained intact, as demonstrated by the flow cytometry histogram, which also showed that the distribution of cells in the cell cycle was

significantly different from the controls. The M1 peak after 48 hours was 8.7%. This was much lower in comparison with the 56% peak detected 24 hours earlier. The occurrence of S phase arrest was confirmed by the high peak (32.4%) as compared to control (9.5%). The G2/M peak was 25.7% compared with 34.2% for 24 hours and with 15.5% for the control. The absolute number of cells counted by trypan blue and SRB test was very low, indicating that most of the cells had disintegrated.

Considering the results of 24 and 48 hour exposure to 100 μM lovastatin, it is apparent that the majority of cells which did not undergo apoptosis became arrested in the S and G2/M phases within 24 hours. With extended exposure time, the number of G2/M-arrested cells gradually decreased. G2 arrest prior to cell death has been reported elsewhere (Barry et al., 1993; Enoch and Norbury, 1995). Both studies reported that delayed cell death with DNA degradation may be due to apoptosis.

The results with electron microscopy confirm that there is more than one type of cell death taking place: apoptosis and necrosis are evident. In rat liver, induction of both apoptosis and necrosis has been described for hepatotoxic agents *in vivo* (Ledda-Columbano et al., 1991) and in cultured T cells by mercuric chloride (Aten et al., 1995).

A significant proportion of the cells remained resistant to apoptosis after 48 hours. This could be explained by cell arrest in the S-phase. The apoptosis-inducing effect of

lovastatin was most pronounced in G1 and least pronounced in the S phase. It could also be due to increase of mitochondrial number as a positive adaptive response to toxic agents (Reipert et al., 1995). Increase in mitochondrial numbers did not occur when cells were grown at low lovastatin concentrations.

Apoptosis started within the first 24 hours of incubation and by 48 hours the apoptotic cells had either disintegrated, floated to the surface of the culture medium, or became necrotic. Necrotic cells were readily identified by electron microscopy, which demonstrated the presence of a mixed population of apoptotic and necrotic cells after 48 hours incubation. It has been shown that cells maintain the typical apoptotic morphology for only a short period of time and then undergo secondary necrosis if they escape phagocytosis (Borner et al., 1995; Wyllie et al., 1980; Majno and Joris, 1995).

With 50 μM lovastatin for 24 hours, the M1 component was 9.8% and G2/M had decreased by 26% relative to the controls, a finding which was consistent with the SRB test (23% cell death). Electron microscopy showed changes in cell membranes and very early indication of apoptosis. Either cell death was taking place in the G2/M phase, a phenomenon that has been observed in other similar studies (Fearnhead et al., 1994; Engelke and Hacker, 1994; Pittman et al., 1994; Borner et al., 1995; Srikant, 1995; Barbiero et al., 1995); or the cells were arrested at G2/M (Barry et al., 1993).

With 48 hours of incubation with 50 μM lovastatin, the proportion of apoptotic cells was 35.5% as determined by flow cytometry, electron microscopy, and the SRB test; all three approaches giving essentially the same result. Compared with 24 hours, the proportion of cells in the G1 phase peak had increased, the S phase was unchanged, and G2/M cells decreased by 50%.

Treatment with 10 μM lovastatin for 24 hours did not induce cell death. However, the S phase peak was two times greater than the control. This indicated that the cells had become arrested in the cell cycle after DNA replication but before mitosis. G1 cell arrest was evident with 10 μM lovastatin for 48 hours. Flow cytometry analysis showed an M1 of 4.9%. Other studies have shown that cell arrest in the G1 phase is characteristic of lovastatin toxicity towards cultured normal or tumour cells (Engeleke and Hacker, 1994; Borner et al., 1995; Reedquist et al., 1995).

The apoptosis-resistant phenotype of HepG2 cells in S phase and G2/M most likely explains the relatively greater cell survival at 100 μM lovastatin upon prolonged incubation. At lower concentrations apoptosis resistance was also seen but with different cell cycle phases and incubation times. The cells that became apoptotic were predominantly from the G1, and to a lesser extent from the G2/M phase, of the cell cycle. It is a valid generalization that the transition to apoptosis happened when cells were in the quiescent phase of the cell cycle.

Three populations of pathological cells were seen: necrotic cells; cycling, pre-apoptotic cells; and cells blocked in the G2/M phase. The relative increase in the proportion of M1 cells and the apparent stabilization of cell viability can be explained by the concomitant presence of two populations of cells; one population was dying and the other dividing.

The findings of this investigation are strongly consistent with the premise that each time a group of cells progressed through the G2/M phase a proportion would become arrested and the remainder would proceed through another division cycle. Thus, the number of apoptotic cells at any given time would depend on the balance between the rate of their appearance and rate of their loss. In other words, if the rate of induction of apoptosis was lower than the rate of disintegration of apoptotic cells, then few of the latter would be observed; or vice-versa. In practice, apoptotic cells were sufficiently stable to permit their observation after 24 hours with 100 μM lovastatin, or 48 hours with 50 μM lovastatin.

This explanation is equally applicable to cells incubated with lovastatin and 0.8 mM oleic acid/BSA but the presence of oleic acid advanced the appearance of abnormal features to an extent corresponding more-or-less to an additional incubation of 24 hours with lovastatin alone. For example with 100 μM lovastatin and 0.8 mM oleic acid/BSA and 24 hours incubation, the induced pathology was essentially similar to 100 μM

lovastatin alone for 48 hours. However, less terminally-apoptotic cells (apoptotic bodies) were detected by flow cytometry than by electron microscopy. This probably means that some cells progressed through another cell cycle before succumbing to apoptotic death, as has been reported in other studies (Barry et al., 1993; Shiff et al., 1996).

For cells treated with 10 and 50 μM lovastatin and oleic acid/BSA, the relative increase of cells entering S phase from 24-48 hours and the corresponding decrease in G2/M confirms the occurrence of a transient G1 arrest. The majority of the cells which did not undergo apoptosis became arrested in the G2 phase within 24 hours. With extended incubation time the number of G2/M arrested cells gradually decreased without reentering the normal cell cycle. Following prolonged incubation necrosis became more significant than apoptosis: with 100 μM lovasatatin DNA fragmentation occurred and the cell cycle distribution was no longer detectable.

The rate of triacylglycerol, cholesteryl ester and apoB synthesis in cultured liver cells is a function of the fatty acid concentration in the medium (Rash et al. 1981; Bostrom et al. 1988; Cianflone et al. 1990, Brissette and Falstrault, 1994). Supplementation of the culture medium with 0.8 mM oleic acid/BSA permitted an analysis of the effects of fatty acid nutritional enhancement on cholesterol, cholesteryl ester and triacylglycerol synthesis and secretion by HepG2 cells. The impact of lipid supplementation on apoB and ApoA secretion was also determined. Since lovastatin is

a strong inhibitor of primary cholesterol synthesis it will also inhibit cholesteryl ester and apo B production indirectly (Cianflone et al., 1994). Oleic acid\BSA was added to the culture medium in the expectation that this would enhance secretion of apolipoprotein B-containing particles; and so magnify the differences between control cultures and cultures containing lovastatin (Moberly et al., 1990; Cianflone et al., 1990.; Dixon et al., 1991).

Lovastatin concentrations up to 2.5 μM were used because this concentration range included the serum levels achieved in normal clinical practice and has been shown in this study not to engender significant morphological damage after 24 hours. The intracellular synthesis and extracellular secretion of triacylglycerols was much less responsive to inhibition by lovastatin than was that of cholesteryl esters. Intracellular synthesis of triacylglycerol and cholesteryl ester were essentially independent. During experimental interventions, levels of apo-B changed in concert with intracellular and extracellular cholesteryl ester and triacylglycerol, but intracellular triacylglycerol remained unchanged.

Apolipoprotein B (apoB) is the major protein component of plasma VLDL and LDL. Increased concentration of plasma LDL cholesterol and apoB have been implicated as risk factors for the development of atherosclerosis (Sharrett et al., 1994; Carmena et al., 1996). Exposure of HepG2 cells to oleic acid/BSA elevates intracellular cholesterol levels, stimulates apoB secretion and reduces receptor-mediated uptake of LDL (Fuki et al., 1989). Cianflone et al. (1990) demonstrated that oleate stimulates ApoB secretion

from cultured HepG2 cells and that the effect was completely suppressed by simultaneous incubation with lovastatin. As lovastatin inhibits cholesterol ester biosynthesis, and cholesterol ester biosynthesis is tightly coupled to the synthesis and secretion of apoB (Cianflone et al., 1990), these observations imply that the stimulatory effect of oleate on apoB secretion may in fact be due to enhanced cholesterol ester synthesis from oleate. These *in vitro* findings appear to complement the experimental observations of Arad, Ramakrishnan and Ginsberg (1990) who demonstrated that when patients with combined hyperlipidaemia were treated with lovastatin, their apoB production decreased but the catabolic rate of the LDL apoB was unchanged. On the other hand, the work of Furukawa and Hirano (1993) suggests that, for at least short-term incubation, experimental modulation of cholesterol ester biosynthesis, which was inhibited by fluvastatin, does not alter apoB kinetics in HepG2 cells. These authors concluded that rapid stimulation of apoB secretion by oleate is not associated with cholesterol ester biosynthesis. Our findings are consistent with Cianflone and would suggest that the discrepancy with Furukawa and Hirano could be the short period of incubation which they used (180 minutes) which would be inadequate for induction of morphological damage by OA/BSA. Also, it is demonstrated that 24 hours of incubation with 1 μM lovastatin were required to demonstrate 70% suppression of cholesteryl ester synthesis, but even after 24 hours apo B synthesis was only suppressed by 31.7%. Also, Furakama and Hirano used 0.4 mM oleic acid/BSA, half the concentration used by Cianflone and in this present investigation.

The safety of lovastatin for clinical purposes is attributed to its ability to block cholesterol biosynthesis at a concentration that is insufficient to inhibit protein prenylation significantly (Sinensky et al., 1990). The results presented in this report demonstrate that even relatively high concentrations of lovastatin are not toxic to the well-differentiated hepatoma cell line HepG2. It could be that their action on these processes are linked to the modulation of the mevalonate pathway. This could add another dimension to the antiatherosclerotic properties of this drug, by providing a pharmacological tool to understand the role of isoprenoids in the modulation of cellular growth and biology (Corsini et al., 1995). Therefore this cell line is a convenient model for investigation of mevalonate-dependent cellular mechanisms. However, this study demonstrates that prolonged exposure of cells to quite low concentrations of lovastatin in combination with fatty acid can cause cell death.

It is concluded that below acutely-toxic lovastatin concentrations, the resistance of HepG2 cells to chronic exposure may be explained as a consequence of the cells having stopped dividing. Such division-arrested cells would have reduced demand for cholesterol for membrane synthesis and the residual slow flux of mevalonate-derived isoprene units diverted into non-sterol branches of the mevalonate pathway such as essential protein prenylations (Marom et al.,1994; Corsini et al., 1995). The findings of our study are consistent with suggestions (Buchwald, 1992) that lovastatin and other similar drugs have utility in cancer chemotherapy as inhibitors of cell division, provided that the general

cytotoxicity of this class of drugs could be overcome by selective targeting (Corsini et al., 1995). Targeting malignant cells by lovastatin in specific segments of their progression through the cell cycle could permit the therapeutic induction of apoptosis and the selective deletion of undesirable cells.

These results, taken together with clinical observations in the literature, confirm the necessity for a stringent combination of drugs and diets for treatment of hypercholesterolemia: a low dose of lovastatin with strict diet control would appear a safe choice. Attempts to control hypercholesterolemia solely by drug intervention without adequate control of dietary fat would necessitate much higher drug administration to achieve an adequate level of serum cholesterol and might expose patients to the cytotoxic effects of inhibited protein prenylation, such as we have demonstrated in this study. Although the observations presented above are based on *in vitro* data, it is clear that careful studies on the *in vivo* chronic toxic side effects of lovastatin and other HMG-CoA reductase inhibitors are required.

CHAPTER 5

SUMMARY

1. The lower lovastatin concentrations used in this study (0.1 - 2.5 μM) do not kill cells, even after exposure for 8 days. However, if cell cultures containing otherwise nontoxic lovastatin concentrations are supplemented with oleic acid (as an oleic acid/ bovine serum albumin complex), cellular morphology and function are deranged and cell viability is significantly reduced.

2. The results of this work suggest that cultured HepG2 cells provides a valuable practical model to investigate mevalonate-dependent cellular mechanisms; and also as a general experimental system for *in vivo* investigations of cell death.

3. The cytotoxicity of oleic acid on its own is apparent. Thus it is difficult to consider this compound as simply a non-toxic nutritional supplement for long-term cell culture. As lipids appear to act as transcellular carriers for lovastatin, both the clinical efficacy and any potential cytotoxic effects will be dependent on nutritional status.

4. Lovastatin-induced apoptosis is characterized by an increase in mitochondrial numbers and abnormal morphology. Lovastatin-induced apoptosis has potential as a tool to investigate the role of the mitochondrion in induction and regulation of apoptosis.

5. This study has demonstrated that apoptosis and necrosis occur sequentially in lovastatin-induced cell death over a prolonged period in HepG2 cells, unlike other systems where cell death takes place quickly. Therefore, HepG2/lovastatin provides a model to study the mechanism of cell death.

6. Cell death was shown to take place at a specific stage in the cell cycle.

7. The results of this study on specific cytotoxic effects of lovastatin provide experimental evidence in support of suggestions in the literature that lovastatin, by virtue of its effects on cholesterol-dependent processes in cell division, may have a role in cancer therapy.

REFERENCES

Acosta, D.; Sorensen, E.M.B.; Anuforo, D.C.; Mitchell, D.B.; Ramos, K.; Santone, K.S.; Smith, M.A., An *in vitro* approach to the study of target organ toxicity of drugs and chemicals. In Vitro Cell. Dev. Biol. 1985, 21:495-504.

Aden, D.P.; Fogel, A.; Plotkin, S.; Damjanov, I.; Knowles, B., Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. Nature 1979, 282:615-616.

Alberts, A., HMG-CoA reductase inhibitions the development. Atherosclerosis Rev. 1988, 18:123-131.

Alberts, A.W.; Chen, J.; Kuron, G.; Hunt, V.; Huff, J.; Hoffman, C.; Rothrock, J.; Lopez, M.; Joshua, H.; Harris, E.; Patchett, A.; Monaghan, R.; Currie, S.; Stapley, E.; Albers-Schonberg, G.; Hensens, O.; Hirshfield, J.; Hoogsteen, K.; Liesch, J.; Springer, J., Mevinolin: A highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol lowering agent. Proc. Natl. Acad. Sci. USA 1980, 77:3957-3961.

Amenta, J.; Sargus, M.; Baccino, F.; Sacchi, C.; Bonelli, G., Cell death induced in L-cell by treatment with thymidine: staging of the process and relationship to apoptosis. In Vitro Cell. Dev. Biol. 1993, 29A:855-861.

Arad, Y.; Ramakrishnan, R.; Ginsberg, H., Lovastatin therapy reduces low density lipoprotein apoB levels in subjects with combined hyperlipidemia by reducing the production of apoB-containing lipoproteins:implication for the pathophysiology of ApoB production. J. Lipid Res. 1990, 31:567-582.

Aten, J.; Prigent, P.; Poncet, P.; Blanpied, C.; Claessen, N.; Druet, P.; Hirsch, F., Mercuric chloride-induced programmed cell death of a murine T cell hybridoma. Cell. Immunol. 1995, 161:98-106.

Avogaro, P.; Cazzolato, G.; Bitolo Bon, J.; Quinci, B., Are apolipoproteins better discriminators than lipids for atherosclerosis?. Lancet 1979, 1:901.

Axelson, M.; Mork, B.; Everson, G.T., Bile acid synthesis in cultured human hepatoblastoma cells. J. Biol. Chem. 1991, 266:17770-17777.

Babich, H.; Stern, A.; Borenfreund, E., Eugenol cytotoxicity evaluated with continuous cell lines. Toxic. in Vitro 1993, 7:105-109.

Ball, S.; Scatina, J.; Sisenwine, S.; Fisher, G., The application of *in vitro* models of drugs' metabolism and toxicity in drug discovery and drug development. Drug Chem. Toxicol. 1995, 18:1-28.

Barbiero, G.; Duranti, F.; Bonelli, G.; Amenta, J.; Baccino, F., Intracellular ionic variations in the apoptotic death of L cells by inhibitors of cell cycle progression. Exp. Cell Res. 1995, 217:410-418.

Barry, M.; Reynolds, J.E.; Eastman, A., Etoposide-induced apoptosis in human HL-60 cells is associated with intracellular acidification. Cancer Res. 1993, 53:2349-2357.

Beaver, J.; Waring, P., Lack of correlation between early intracellular calcium ion rises and the onset of apoptosis in the thymocytes. Immunol. Cell Biol. 1994, 72:489-499.

Bensch, W.R.; Ingebritsen, T.S.; Diller, E.R., Lack of correlation between the rate of cholesterol biosynthesis and the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase in rats and in fibroblasts treated with ML-236B. Biochem. Biophys. Res. Commun. 1978, 82:247-254.

Blum, C., Comparison of properties of four inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. Am. J. Cardiol. 1994, 73(S):3D-11D.

Bondesson, I.; Ekwall, B.; Hellberg, S.; Romert, L.; Stenberg, K.; Walum, E., MEIC-A new international multicenter project to evaluate the relevance to human toxicity of *in vitro* cytotoxicity tests. Cell Biol. Toxicol. 1989, 5:331-348.

Borner, M.; Myers, C.; Sartor, O.; Sei, Y.; Toko, T.; Trepel, J. and Schneider, E., Drug-induced Apoptosis is not neccessarily dependent on macromolecular synthesis or proliferation in the p53-negative human prostate cancer cell line PC-3. Cancer Res. 1995, 55:2122-2128.

Bostrom, K.; Boren, J.; Wetteseten, M.; Sjoberg, A.; Bondjers, G.; Willund, W.;Carlesson, P. and Olofsson, O., Studies on the assembly of apoB-100-containing lipoproteins in HepG2 cells. J. Biol. Chem. 1988, 263:4434-4442.

Bradford, R.H.; Shear, C.L.; Chremos, A.N.; , C.; Downton, M.; Franklin, F.A.; Gould, A.L.; Hesney, M.; Higgins, J.; Hurley, D.P.; Langendorfer, A.; Nash, D.T.; Pool, J.L.; Schnaper, H., Expanded clinical evaluation of lovastatin (EXCEL) study results. Arch. Inter. Med. 1991, 151:43-49.

Brissette, L.; Falstrault, L., Analysis of the selective uptake of the cholesteryl ester of human intermediate density lipoproteins by HepG2 cells. Biochem. Biophys. Acta. 1994, 1213:5-13.

Brown, M.S.; Goldstein, J.L., Drugs used in treatment of hyperlipoproteinemias. In *The Pharmacological Basis of Therapeutics*. ed.: Gilman, A.G.; Goodman, L.S.; Rull, T.W.; Murad, F. New York, MacMillan, 1985, 827-845.

Bouma, M.; Rogier, E.; Verthier, N.; Labarre, C.; Feldman, G., Further cellular investigation of the human hepatoblastoma derived cell line HepG2: morphology and immucytochemical studies of hepatic-secreted proteins. *In Vitro* Cell. Devel. Biol. 1989, 25: 267-275.

Buchwald, H., Cholesterol inhibition, cancer, and chemotherapy. Lancet. 1992, 339:1154-1156.

Buja, L.M.; Eigenbrodt, M.L.; Eigenbrodt, E.H., Apoptosis; necrosis. Basic types and mechanisms of cell death. Arch. Pathol. Lab. Med. 1993, 117:1208-1214.

Carmena, R.; Lussier-Cacan, S.; Roy, M.; Minnich, A.; Lingenhei, A.; Kronenberg, F.; Davignon, J., Lp(a) levels and arterosclerotic vascular disease in a sample of patients with familial hypercholesterolemia sharing the same gene defect. Arterioscler. Thromb. Vasc. Biol. 1996, 16(1):129-138.

Chao, Y.; Chen, Y.S.; Hunt, V.M.; Kuron, G.W.; Karkas, J.D.; Liou, R.; Alberts, A.W., Lowering of plasma cholesterol levels in animals by lovastatin and simvastatin. Eur. J. Clin. Pharmacol. 1991, 40:S11-S14.

Chin, J.; Dart, A., Therapeutic restoration of endothelial function in hypercholesterolaemic subjects:effect of fish oils. Clin. Exp. Pharmacol. Physiol. 1994, 21:749-755.

Cianflone, K.; Dahan, S.; Monge, J.C.; Sniderman, A.D., Pathogenesis of carbohydrate-induced hypertriglyceridemia using HepG2 cells as a model system. Arterioscler. Thromb. 1992, 12:271-277.

Cianflone, K.; Vu, H.; Zhang, Z.; Sniderman, A., Effects of albumin on lipid synthesis, Apo B-100 secretion and LDL catabolism in HepG2 cells. Atherosclerosis 1994, 107:125-135.

Cianflone, K.M.; Yasruel, Z.; Rodriguez, M.A.; Vas, D.; Sniderman, A.D., Regulation of apo B secretion from HepG2 cells: Evidence for a critical role for cholesterol ester synthesis in the response to a fatty acid challenge. J. Lipid Res. 1990, 31:2045-2055.

Clayton, D.F.; Harrelson, A.L.; Darnell, J.E. Jr., Dependence of liver-specific transcription on tissue organization. Mol. Cell. Biol. 1985, 5:2623-2632.

Cobb, M.; Teitelbaum, H.; Breslow, J. Lovastatin efficacy in reducing low-density lipoprotein cholesterol levels on high- vs low-fat diets. JAMA. 1991, 265:997-1001.

Cooper, A.D.; Craig, W.Y.; Taniguchi, T.; Everson, G.T., Characteristics and regulation of bile salt synthesis and secretion by human hepatoma HepG2 cells. Hepatology 1994, 20:1522-1531.

Corsini, A.; Maggi, F.; Catapano, A., Pharmacology of competitive inhibitors of HMG-CoA reductase. Pharma. Res. 1995, 31:9-27.

Cui, L.; Yoon, S.; Schinazi, R.; Sommadossi, J-P., Cellular and molecular events leading to mitochondrial toxicity of 1-(2-deoxy-2-fluoro-1-β-d-arabinofuranosyl)-5-iodouracil in human liver cells. J. Clin. Invest. 1995, 95:555-563.

Dashti, N.; Williams, D.L.; Alaupovic, P., Effects of oleate and insulin on the production rates and cellular mRNA concentrations of apolipoproteins in HepG2 cells. J. Lipid Res. 1989, 30:1365-1373.

Dashti, N.; Wolfbauer, G., Secretion of lipids, apolipo- proteins and lipoproteins by human hepatoma cell line, HepG2: effects of oleic acid and insulin. J. Lipid Res. 1987, 28:423-436.

Dixon, J..; Furukawa, S.; Ginsberg, H., Oleate stimulates secretion of apolipoprotein B-containing lipoproteins from HepG2 cells by inhibiting early intracelelluar degradation of apolipoprotein B. J. Biol. Chem. 1991, 266:5080-5086.

Dixon, J.L.; Ginsberg, H.N., Regulation of hepatic secretion of apolipoprotein B-containing lipoproteins: information obtained from cultured liver cells. J. Lipid Res. 1993, 34:167-179.

Doostdar, H.; Duthie, S.J.; Burke, M.D.; Melvin, W.T.; Grant, M.H., The influence of culture medium composition on drug metabolising enzyme activities of the human liver derived HepG2 cell line. FEBS Lett. 1988, 241:15-18.

Dujovne, C.; Chremos, A.;Pool, J.; Schnaper, H.; Bradford, R.; Shear, C., Expanded clinical evaluation of lovastatin (EXCEL) study results.IV. Additional perspectives on the tolerability of lovastatin. Am. J. Med. 1991, 91(S):25S-30S.

Duthie, S.,; Grant, M., The toxicity of menadione and mitoxantrone in human liver-drived HepG2 hepatoma cells. Biochem. Pharmacol. 1989, 38:1247-1255.

Duthie, S.J.; Melvin, W.T.; Burke, M.D., Bromobenzene detoxification in the human liver-derived HepG2 cell line. Xenobiotica 1994, 24:265-279.

Ekwall, B.; Bondesson, I.; Castell, J.V.; Gomez-Lechon, M.J.; Hellberg, S.; Hogberg, J.; Jover, R.; Ponsoda, X.; Romert, L.; Stenberg, K.; Walum, E., Cytotoxicity evaluation of the first ten MEIC Chemicals: Acute lethal toxicity in man predicted by cytotoxicity in five cellular assays and by oral LD50 tests in rodents., ATLA. 1989, 17:83-100.

Ekwall, B.; Bondesson, I.; Hellberg, S.; Hogberg, J.; Romert, L.; Stenberg, K.; Walum, E., Validation of *in vitro* cytotoxicity tests: past and present strategies. ATLA. 1991, 19:226-233.

Ekwall, B.; Ekwall, K. Comments on the use of diverse cell systems in toxicity testing. ATLA 1988, 15:193-201.

Ekwall, B.; Silano, V.; Paganuzzi-Stammati, A.; Zucco, F., Toxicity tests with mammalian cell cultures. In: *Short-Term Toxicity Tests for Non-Genotoxic Effects.* ed.: P. Bourdeau et al., John Wiley & Sons, New York. 1988, pp. 75-97.

Ellis, R.; Yuan, J.; Horviz, H., Mechanism and function of cell death. Ann. Rev. Cell Biol. 1991, 7:663-698.

Ellsworth, J.L.; Erickson, S.K.; Cooper, A.D., Very low and low density lipoprotein synthesis and secretion by the human hepatoma cell line Hep-G2: effects of free fatty acid. J. Lipid Res. 1986, 27:858-872.

Endo, A.; Tsujita, Y.; Kuroda, M.; Tanzawa, K., Effects of ML-236B on cholesterol metabolism in mice and rats: lack of hypocholesterolemic activity in normal animals. Biochem. Biophys. Acta. 1979, 575:266-276.

Engelke, K.; Hacker, M., Anoncharacteristic response of L1210 cells to lovastatin. Biochem. Biophys. Res. Commun. 1994, 203:400-407.

Enoch, T.; Norbury, C. Cellular responses to DNA damage: cell-cycle checkpoints, apoptosis and the roles of p53 and ATM. TIBS. 1995, 20(10):426-430.

Fairbanks, K.; Witte, L.; Goodman, D., Relationship between mevalonate and mitogenesis in human fibroblasts stimulated with platelet-drived growth factor. J. Biol. Chem. 1984, 259:1546-1551.

Fears, R.; Richards, D.; Ferres, H., The effect of compactin, a potant inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity, on cholesterogenesis and serum cholesterol levels in rats and chicks. Atherosclerosis. 1980, 35:439-449.

Forrester, L.M.; Henderson, C.J.; Glancey, M.J.; Black, D.J.; Park, B.K.; Ball, S.E.; Kitteringham, N.R.; McLaren, A.W.; Miles, J.S.; Skett, P.; Wolf, C.R., Relative expression of cytochrome P-450 isoenzymes in human liver and association with the metabolism of drugs and xenobiotics. Biochem. J. 1992, 281:359-368.

Fuki, I.; Preobrazhensky, S.; Misharin, A.; Bushmakina, N.; Menschikov, G.; Repin, V.; Karpov, R., Effect of cell cholesterol content on apolipoprotein B secretion LDL receptor activity in the human hepatoma cell line HepG2. Biochem. Biophys. Acta., 1989, 1001:235-238.

Furukawa, S.; Hirano, T., Rapid stimulation of apolipoprotein B secretion by oleate is not associated with cholesteryl ester biosynthesis in HepG2 cella. Biochem. Biophys. Acta. 1993, 1170:32-37.

Furukawa, S.; Sakata, N.; Ginsberg, H.N.; Dixon, J.L., Studies of the sites of intracellular degradation of apo-lipoprotein B in HepG2 cells. J. Biol. Chem. 1992, 267:22630-22638.

Goldstein, J.; Brown, M., Regulation of the mevalonate pathway. Nature. 1990, 343:425-430.

Gorczyca, W.; Gong, J.; Ardelt, B.; Traganos, F.; Darzynkiewicz, Z., The cell cycle related differences in susceptibility of HL-60 cells to apoptosis induced by various antitumor agents. Cancer Res. 1993, 53:3186-3192.

Gotto, A.M., Jr.; LaRosa, J.C.; Hunninghake, D.; Grundy, S.M.; Wilson, P.W.; Clarkson, T.B.; Hay, J.W.; Goodman, D.S., The cholesterol facts. A summary of the evidence relating dietary fats, serum cholesterol and coronary heart disease. A joint statement by the American Heart Association and the National Heart, Lung and Blood Institute. Circulation 1990, 81:1721-1733.

Grant, M.H.; Duthie, S.J.; Gray, A.G.; Burke, M.D., Mixed-function oxidase and UDP-glucuronyltransferase activities in the human HepG2 hepatoma cell line. Biochem. Pharmacol. 1988, 37:4111-4116.

Gugen-Guillouzo, C.; Gripon, P.; Vandenberghe, Y.; Lamballe, F.; Ratanasavanh, D.; Guillouzo, A., Hepatotoxicity and molecular aspects of hepatocyte function in primary culture. Xenobiotica 1988, 18:773-783.

Habenich, A.; Glomset, J., Ross, R., Relation of cholesterol and mevalonic acid to the cell cycle in smooth muscle and Swiss 3T3 cells stimulated to divide by platelet-derived growth factor. J. Biol. Chem. 1980, 255:5134-5140.

Hall, T.J.; Cambridge, G.; James, P.R., Development of a co-culture system with induced HepG2 cells and K562 cells for examining drug metabolism *in vitro*. Studies with cyclophosphamide, ondansetron and cisplatin. Res. Commun. Chem. Pathol. Pharmacol. 1991, 72:161-168.

Hall, T.J.; James, P.R.; Cambridge, G., Development of an *in vitro* hepatoxicity assay for assessing the effects of chronic drug exposure. Res. Commun. Chem. Pathol. Pharmcol. 1993, 79:249-256.

Halpin, R.M.; Ulm, E.H; Till, A.E.; Kari, P.H.; Vyas; Hunninghake, DB.; Duggan, D.E., Biotransformation of lovastatin. V. Species differences *in vivo* metabolites profiles of mouse, rat, dog, and human. Drug. Metab. Dispos. 1993, 21(6):1003-1011.

Havel, R.J.; Hunninghake, D.B.; Illingworth, D.R.; Lees, R.S.; Stein, E.A.; Tobert, J.A.; Bacon, S.R.; Bolognese, J.A.; Frost, P.H.; Lamkin, G.E.; Lees, A.M.; Leon, A.S.; Gardner, K.; Johnson, G.; Mellies, M.J.; Rhymer, P.A.; Tun, P., A multicenter study of lovastatin (mevinolin) in the treatment of heterozygous familial hypercholesterolemia. Ann. Intern. Med. 1987, 107:609-615.

Herold, G.; Jungwith, R.; Rogler, G.; Geerling, I.; Stange, E., Influence of cholesterol supply on cell growth and differentiation in cultured enterocytes (CaCo-2). Digestion 1995, 56:57-66.

Hirano, T.; Gotoh, M.; Oka, K., Citrus flavone tangeretin inhibits leukaemic HL-60 cell growth partially through induction of apoptosis with cytotoxicity on normal lymphocytes. Br. J. Cancer 1995-a, 72(6):1380-1388.

Hirano, T.; Furukawa, S.; Kurokawa, M.; Ebara, T.; Dixon, J.; Nagano, S., Intracellular apoprotein B degradation is suppressed by decreased albuimin concentration in HepG2 cells. Kidney Inter. 1995-b, 47:421-431.

Hucker, H.B., Species differences in drug metabolism. Ann. Rev. Pharmacol. Toxicol. 1970, 10:99-118.

Hunninghake, D., Clinical Trials of lovastatin and simvastatin versus cholestyramine. Atherosclerosis Rev. 1988, 18:133-138.

Hunninghake, D.; Stein, E.; Dujovne, C.; Harris, W.; Feldman,B.; Miller, V.; Tobert, J.; Laskarzewski, P.; Quitter, E.; Held, J.; Tylor, A.; Hopper, S.; Leonard, S.; Brewer, B., The efficacy of intensive dietary therapy alone or combined with lovastatin in outpatients with hypercholesterolemia. N. Engl. J. Med. 1993, 328(17):1213-1219.

Hsu, I.; Spinler, S.; Johnson. N., Comparative evalution of safety and efficacy of HMG-CoA reductase inhibition monotherapy in the treatment of primary hypercholesterolemia. Ann. Pharmacother. 1995, 29:743-759.

Illingworth, D.R.; Bacon, S.P.; Larson, K.K., Long-term experience with HMG-CoA reductase inhibitors in the therapy of hypercholesterolemia. Atherosclerosis Rev. 1988, 18:161-187.

Ishak, K.G.; Glunz, P.R., Hepatoblastoma and hepatocellular carcinoma in infancy and childhood. Cancer 1966, 20:396-422.

Jakobisisak, M.; Bruno, S.; Skierski, J.; Darzynkiewicz, Z., Cell cycle-specific effects of lovastatin. Proc. Natl. Acad. Sci. USA. 1991, 88(9):3628-3632.

Javitt, N.B., HepG2 cells as a resource for metabolic studies: lipoprotein, cholesterol, and bile acids. FASEB J. 1990, 4:161-168.

Jefferson, D.M.; Reid, L.M.; Giambrone, M-A.; Shafritz, D.A.; Zern, M.A., Effects of dexamethasone on albumin and collagen gene expression in primary cultures of adult rat hepatocytes. Hepatology 1985, 5:14-20.

Jones, K.D.; Couldwell, W.T.; Hinton, D.R.; Su, Y.; He, S.; Anker, L.; Law, R.E., Lovastatin induces growth inhibition and apoptosis in human malignant glioma cells. Biochem. Biophys. Res. Commun. 1994, 205(3):1681-1687.

Jones, T.L.; Lafrenz, D., Quantitative determination of the induction of apoptosis in a murine B cell line using flow cytometric bivariate cell cycle analysis. Cell Immunol. 1992, 142:348-360.

Jover, R.; Ponsoda, X.; Castell, J.V.; Gomez-Lechon, M.J., Evaluation of the cytotoxicity of ten chemicals on human cultured hepatocytes: predictability of human toxicity and comparison with rodent cell culture systems. Toxic. in Vitro 1992, 6:47-52.

Kathawala, F.G., HMG-CoA reductase inhibitors: an exciting development in the treatment of hyperlipoproteinemia. Med. Res. Rev. 1991, 11:121-146.

Kazumi, T.; Yoshino, G.; Ohki, A.; Matsuba, K.; Ino, T.; Amano, M.; Kasuga, M., Long-term effects of simvastatin in hypercholesterolemic patients with NIDDM and additional atherosclerotic risk factors. Hyogo Simvastatin Study Group. Horm. Metab. Res. 1995, 27:239-243.

Keepers, Y.P.; Piazao, P.E.; Peters, G.J.; Ark-Otte, J.; Winagard, B.; Pinedo, H., Comparison of the sulforhodamine B protein and tetrazolium (MTT) assays for *in vitro* chemosensitivity testing. Eur. J. Cancer 1991, 27:897-900.

Keilson, L.C.; Hunninghake, D.; Insull, W.; Knopp, R.; McKenney, J.; Stein, E.; Troendle, A.; Efficacy of the synthetic HMG-CoA reductase inhibitor fluvastatin. Arteriosclerosis 1990, 10:856a.

Keren, D.; Hanson, C.; Hutubise, P., *Flow Cytometry and Clinical Diagnosis.* 1994: American Society of Clinical Pathologists Chicago.

Kerr, J.; Wyllie, A.; Curie, A., Apoptosis: a basic biological phenomenon with widespread implications in tissue kinetics. Br. J. Cancer. 1972, 26:239-257.

Knott, T.J.; Pease, R.J.; Powell, L.M.; Wallis, S.C.; Rall, S.C. Jr.; Innerarity, T.L.; Blackhart, B.; Taylor, W.H.; Marcel, Y.; Milne, R. et al., Complete protein sequence and identification of structural domains of human apolipoprotein B. Nature. 1986, 323:734-738.

Knowles, B.B.; Howe, C.C.; Aden, D.P., Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science 1980, 209:497-499.

Kornbrust, D.J.; MacDonald, J.S.; Peter, C.P.; Duchai. D.M.; Stubbs, J.R.; Germershausen, J.I.; Alberts, A.W., Toxicity of the HMG-CoA reductase inhibitor, lovastatin, to rabbits. J. Pharmacol. Exp. Ther 1989, 248:498-505.

Kubota, T.; Takahara, T.; Nagata, M.; Furukawa, T.; Kase, S.; Tanino, H.; Ishibiki, K.; Kitajima, M., Colorimetric chemosensitivity testing using sulforhodamine B. J. Surg. Oncol. 1993, 52:83-88.

Langer, T.; Strober, W.; Levy, R.I., The metabolism of low density lipoprotein in familial type II hyperlipoproteinemia. J. Clin. Invest. 1972; 51:1528-1534.

Ledda-Columbano, G.; Coni, P.; Curto, M.; Giacomini, L.; Faa, G.; Oliverio, S.; Piacentini, M.; Columbano, A., Induction of two different modes of cell death, apoptosis and necrosis, in rat liver after a single dose of thioacetamide. Am. J. Pathol. 1991, 139:1099-1109.

Linnet, K., A HPLC-GC/MS reference method for serum total cholesterol with control for ester hydrolysis. Clin. Biochem. 1994, 27:177-182.

Liu, T.Y.; Chao, T.W.; Hiang, S.H.; Chi, C.W., Diffrential sensitivity of human hepatoma cell line and primary rat hepatocyte culture to benzo(A)pyrene-induced unscheduled DNA synthesis and adduct formation. Cell Biol. Inter. 1993, 174:441-447.

Lizard, G.; Fournel, S.; Genestier, L.; Dhedin, N.; Chaput, C.; Flatcher, M.; Mutin, M.; Panaye, G.; Revillad, J-P., Kinetics of plasma membrane and mitochondrial alterations in cells undergoing apoptosis. Cytometry 1995, 21:275-283.

Lovastatin Study Group II., Therapeutic response to lovastatin (mevinolin) in nonfamilial hypercholesterolemia. JAMA. 1986, 256:2829-2834.

Lovastatin Study Group III., A multicenter comparison of lovastatin and cholestyramine therapy for severe primary hypercholesterolemia. JAMA. 1988, 260:359-366.

Lovastatin Study Group IV., A multicenter comparison of lovastatin and probucol in the therapy for severe primary hypercholesterolemia, Am. J. Caradiol., 1990, 66:22b.

Lowry, O.; Rosebrough, N.; Farr, A.; Randall, R., Protein measurement with the Folin phenol reagent. J. Biol. Chem. 1951, 193:265-275.

MacDonald, J.S.; Gerson, R.J.; Kornbrust, D.J.; Kloss, M.W.; Prahalada, S.; Berry, P.H.; Alberts, A.W.; Bokelman, D.L., Preclinical evaluation of lovastatin. Am. J. Cardiol. 1988, 62:16J-27J.

Majno, G.; Joris, I.; Apoptosis, O., Necrosis: An overview of cell death. Am. J. Pathol. 1995, 146(1):3-15.

Marom, M.; Ben-Baruch, G.; Roitelman, J.; Kloog, Y., Lack of correlation between 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and lovastatin resistance in nerve growth factor treated PC-12 cells. Cell. Mol. Neutobiol. 1994, 14:119-132.

McCloskey, TW.; Oyaizu, N.; Coronesi, M.; Savita, P., Use of flow cytomatric assay to quantitate apoptosis in human lymphocytes. Clin. Immunol. Immunopathol. 1994, 71:14-18.

McKenney J.M.; Proctor, J.D.; Wright, J.T.; Kolinski, R.J.; Elswick, R.K.; Coaker, J.S., The effect of supplemental dietary fat on plasma cholesterol levels in lovastatin-treated hypercholesterolemic patients. Pharmacotherapy 1995, 15:565-572.

Moberly, J.B.; Cole, T.G.; Alpers, D.H.; Schonfeld, G., Oleic acid stimulation of apolipoprotein B secretion from HepG2 and Caco-2 cells occurs post-transcriptionally. Biochem. Biophys. Acta 1990, 1042:70-80.

Molowa, D.T.; Cimis, G.M., Coordinate regulation of low-density-lipoprotein receptor and 3-hydroxy-3-methylglutaryl-CoA reductase and synthase gene expression in HepG2 cells. Biochem. J. 1989, 260:730-736.

Naji-Ali, F.; Hasspieler, B.M.; Haffner, D.; Adeli, K., Human bioassay to assess environmental genotoxicity: development of a DNA repair assay in HepG2 cells, Clin. Biochem., 1994, 27:441-448.

Neuman, M.G.; Koren, G.; Tirbelli, C., *In vitro* assessment of the ethanol-induced hepatotoxicity on HepG2 cell line. Biochem. Biophys. Res. Commun. 1993, 197:932-941.

Nicoletti, I.; Migliorati, G.; Pagliacci, MC.; Grignani, F.; Riccardi, C., Arapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J. Immunol. Methods 1991, 139:271-279.

Oates, J.; Wood, A., HMG-CoA reductase inhibitors for treatment of hypercholesterolemia. N. Eng. J. Med. 1988, 319:24-32.

Okey, A.B.; Roberts, E.A.; Harper, P.A.; Denison, M.S., Induction of drug-metabolizing enzymes: mechanisms and consequences. Clin. Biochem. 1986, 19:132-141.

Ormerod, M.G., *Flow cytometry. A practical approach.* 1990 IRL press, Oxford Univerisity Press, Oxford.

Ormerod, M.G.; Orr, R.M.; Peacock, J.K., The role of apoptosis in cell killing by cisplatin: a flow cytometric study. Br. J. Cancer 1994, 69:93-100.

Ortiz, M.B.; Goin, M.; de Alzaga, M.B.G.; Hammarstrom, S.; De Asua, L.J., Mevalonate dependency of the early cell cycle mitogenic response to epidermal growth factor and prostaglandin F2 in swiss mouse 3T3 cells. J. Cell. Phys. 1995, 162:139-146.

Pittman, S.M.; Strickland, D.; Ireland, C.M., Polymerization of tubulin in apoptosis cells is not cell cycle dependent. Exp. Cell Res. 1994, 215:263-272.

Pullinger, C.R.; North, J.D.; Teng, B.B.; Rifici, V.A.; Ronhild de Brito, A.E.; Scott, J., The apolipoprotein B gene is constitutively expressed in HepG2 cells: regulation of secretion by oleic acid, albumin, and insulin, and measurement of the mRNA half-life. J. Lipid Res. 1989, 30:1065-1077.

Quinn, G.P.; Axelrod, J.; Brodie, B.B., Species, strain and sex differences in metabolism of hexobarbitone, amidopyrine, antipyrine and aniline. Biochem. Pharmacol. 1958, 1:152-159.

Rash, J.M.; Rothblat, G.H.; Sparks, C.E., Lipoprotein apo-lipoprotein synthesis by human hepatoma cells in culture. Biochem. Biophys. Acta. 1981, 666:294-298.

Reedquist, K.; Pope, T.; Roess, D., Lovastatin inhibits proliferation and causes apoptosis in lipopolysacchride-stimulated murine B cells. Biochem. Biophys. Res. Commun. 1995, 211:665-670.

Reipert, S.; Berry, J.; Hughes, M.; Hickman. A.; Allen, T., Changes of mitochondrial mass in the hemopoietisster cell line EDCP-MIX after treatment with etoposide: a correlative study by multiparameter flow cytometry and confocal and electron microscopy. Exp. Cell Res. 1995, 221:281-288.

Robisno, R.; Suter, W.; Cox, R., Carcinogenicity and mutagenicity studies with fluvastatin, a new, entirely synthetic HMG-CoA reductase inhibitor. 1994, 23:9-20.

Rubinstein, L.V.; Shoemaker, R.H.; Paull, R.D.; Simon, R.M.; Tosini, S.; Shehan, D.A.; Scudiero, D.A.; Monks, A.; Boyd, M.R., Comparison of *in vitro* anticancer-drug screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. J. Natl. Cancer Inst. 1990, 82:1113-1118.

Rudel, L.L. and Moris, M.D., Determination of cholesterol using o-phthalaldehyde. J. Lipid Res. 1973, 14:364-336.

Sepp-Lorenzino, L.; Rao, S.; Coleman, P., Cell-cycle-dependent, differential prenylation of proteins. Eur. J. Biochem. 1991, 200 :579-590.

Serajuddin, A.; Ranadive, S.; Mahoney, E., Relative lipophilicities, solubilities, and structure-pharmacological considerations of 3-hydroxy-3-methylglutaryl-Coenzyme A(HMG-CoA) reductase inhibitors pravastatin, lovastatin, movastatin, and simvastatin. J. Pharmaceutical Sci. 1991, 80:830-834.

Sharrett, A.R.; Patsch, W.; Sorlie, P.D.; Heiss, G.; Bond, M.G.; Davis, C.E., Association of lipoprotein cholesterols, apolipoproteins A-1 and B, and triglycerides with carotid atherosclerosis and coronary heart disease. The Atherosclerosis Risk in Communities (ARIC) Study. Arterioscler. Thromb. 1994, 14(7):1098-104.

Shepherd, J., Fibrates and statins in the treatment of hyperlipidaemia: an appraisal of their efficacy and safety. Eur. Heart J. 1995, 16:5-13.

Shiff, S.J.; Koutsos, M.; Qiao, L.; Rigas, B., Nonsteroidal antiinflammatory drugs inhibit the proliferation of colon adenocarcinoma cells:effects on cell cycle and apoptosis. Exp. Cell Res. 1996, 222:179-188.

Silvers, K.J.; Eddy, E.P.; McCoy, E.C.; Rosenkranz, H.S.; Howard, P.C., Pathways for the mutagensis of 1-nitropyrene and diinitropyrenes in the human hepatoma cell HepG2. Environ. Health Persp. 1994, 102:195-200.

Sinesky, M,.; Beck, L.A.; Leonard, S.,; Evans, R., Differential inhibitory effects of lovastatin on protein isoprenylation and sterol synthesis. J. Biol. Chem., 1990, 165:19937-19941.

Singer, I.I.; Kawka, D.W.; Kazazis, D.M.; Alberts, A.W.; Chen, J.S.; Huff, J.W.; Ness, G.C., Hydroxymethylglutaryl -coenzyme A reductase-containing hepatocytes are distributed periportally in normal and mevinolin-treated rat livers. Proc. Natl. Acad. Sci. USA 1984, 81:5556-5560.

Singer, I.I.; Scott, S.; Kazazis, D.M.; Huff, J.W., Lovastatin, an inhibitor of cholesterol synthesis, induces hydroxymethylglutaryl-coenzyme A reductase directly on membranes of expanded smooth endoplasmic reticulum in rat hepatocytes. Proc. Natl. Acad. Sci. USA 1988, 85:5264-5268.

Sirtori, C.R., Tissue selectivity of hydroxymethylglutaryl coenzyme A (HMG CoA) reductase inhibitors. Pharmacol. Ther. 1993, 60:431-459.

Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J.T.; Bokesch, H.; Kenney, S.; Boyd, M., New colorimetric cytotoxicity assay for anticancer-drug screening. J. Natl. Cancer Inst. 1990, 82:1107-1112.

Skomedal, T.; Hostmark, A.T.;; Osnes, J.B., Fat diet, osmotic fragility of erythrocytes and inotropic response of rat heart papillary muscle to noradrenaline stimulation: early appearance of the diet effect by lovastatin. Pharmacol. Toxicol. 1994, 75:200-205.

Smith, R.L., HMG-CoA reductase inhibitors and lipid-lipoprotein metabolism. In: *Antilipidemic Drugs Medicinal, Chemical and Biochemical Aspects*, ed.: Witiak, D.T.; Newman, H.A.I. and Dr. Feller., Elsevier-Amsterdam-London-New York-Tokyo, 1991, 121-158.

Sniderman, A.D.; Shapiro, S.; Marpole, D.G.; Skinner, B.F.; Teng, B.; Kwiterovich, P.O. Jr., Association of coronary atherosclerosis with hyperapobetalipoproteinemia (increased protein but normal cholesterol levels in human plasma low-density (B) lipoproteins). Proc. Natl. Acad. Sci. USA, 1980, 77:604.

Sonc, R; Wick, G., Methods for the detection of apoptosis. Inter. Arch. Allerg Immunol. 1994, 105:327-332.

Srikant, C.B., Cell Cycle dependent induction of apoptosis by somatostatin analog SMS 201-995 in AtT-20 mouse pituitary cells. Biochem. Biophys. Res. Commun. 1995, 209:400-406.

Sumi, S.; Beauchamp, R.D.; Courtney, M.; Townsend, C.M. Jr.; Tatsuo, U.; Murakami, M.; Rajaraman, S.; Ishizuka, J.; Thompson, J., Inhibition of pancreatic adenocarcinoma cell growth by lovastatin. Gastroenterology. 1992, 103:982-989.

Sumi, S.; Beauchamp, R.D.; Townsend, C.M. Jr.; Pour, P.M.; Ishizuka, J.; Thompson, J.C., Lovastatin inhibits pancreatic cancer growth regardless of RAS mutation. Pancreas 1994, 9:657-61.

Tee, L.; Davies, D.S.; Seddon, C.E.; Boobis, A.R., Species differences in hepatotoxicity of paracetamol are due to differences in the rate of conversion to its cytotoxic metabolite. Biochem. Pharmacol. 1987, 36:1041-1047.

Thrift, R.N.; Forte, T.M.; Cahoon, B.E.; Shore, V.G., Characterization of lipoproteins produced by the human liver cell line, HepG2, under defined conditions. J. Lipid Res. 1986, 27:236-250.

Tobert, J.A., New developments in lipid-lowering therapy: the role of inhiitors of HMG-CoA reductase. Circulation 1987, 76: 534-538.

Tobert, J., Efficacy and long-term adverse effects of lovastatin. Am. J. Cardiol. 1988, 62:28-34.

Tsa, M.Y.; Yuan, J.; Hunninghake, D.B., Effects of fluvastatin, a HMG-CoA reductase inhibitor on the distribution and composition of low density lipoprotein subspecies in humans. Arteriosclerosis 1990, 10:860a.

Tse, F.L.S.; Smith, H.T.; Ballard, F.H.; Nicoletti, J., Disposition of fluvastatin, an inhibitor of HMG-CoA reductase, in mouse, rat, dog and monkey. Biopharm. Drug Dispos. 1990, 11:519-531.

Turley, S.D.; Dietschy, J.M., Cholesterol metabolism and excretion. In: *The Liver, Biology and Pathology,* ed.: Arias, I.; Popper, H.; Schachter, D.; Shafritz, D.A., Raven Press, NY, 1982, 467-492.

Uchiyama, Y., Apoptosis: The history and trends of its studies. Arch. Histol. Cytol. 1995, 58(2):127-137.

Vajreswari, A.; Narayanareddy, K., Effect of dietary fats on erythrocyte membrane lipid composition and membrane-bound enzyme activities. Metabolism 1992, 41(6):352-358.

Van Harken, D.; Dixon, C.; Heimberg, M., Hepatic lipid metabolism in experimental diabetes. J. Biol. Chem. 1969, 244:2279-2285.

Vayssiere, J-L.; Petit, P.; Risler, Y.; Mignotte, B., Commitment to apoptosis is associated with changes in mitochondrial biogenesis and activity in cell lines conditionally immortalized with simian virus 40. Proc. Natl. Acad. Sci. USA. 1994, 91:11752-11756.

Viau, C.J.; Curren, R.D.; Wallace, K., Cytotoxicity of tacrine and velnacrine metabolites in cultured rat, dog and human hepatocytes, Drug Chem. Toxicol. 1993, 16-3, 227-239.

Wang, S.R.; Pessah, M.; Infante, J.; Catala, D.; Salvat, C.; Infante, R., Lipid and lipoprotein metabolism in HepG2 cells. Biochem. Biophys. Acta. 1988, 961:351-363.

Waters, D.; Higginson, L.; Gladstone, P.; Boccuzzi, S.; Cook, T.; Lesperance, J., for the CCAIT Study Group. Effects of cholesterol lowering on the progression of coronary atherosclerosis in women. A Canadian Coronary Atherosclerosis Intervention Trial (CCAIT) Substudy. Circulation 1995 ,92:2404-2410.

Wyllie, A.H., Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activition. Nature. 1980, 284:555-556.

Wyllie, A., Cell death: A new classification separating apoptosis from necrosis. In: *Cell death in biology and pathology.* ed.: Bowen, I.; Lockshin, R.:. Chapman and Hall, London, 1981, 9-34.

Wyllie, A.; Kerr, J.F.; Currie, A., Cell death: the significance of apoptosis. Inter. Rev. Cytol. 1980, 68:251-306.

Yang, C.Y.; Chen, S.H.; Gianturco, S.H.; Bradley, W.A.; Sparrow, J.T ; Tanimura, M.; Li, W.H.; Sparrow, D.A.; DeLoof, H.; Rosseneu, M. et al, Sequence, structure, receptor-binding domains and internal repeats of human apolipoprotein B-100. Nature. 1986, 323:738-742.

Zaleskis, G.; Berleth, E.; Ehrke, V.; Mihich. E., Doxorubicin-induced DNA degradation in murine thymocytes. Mol. Pharmacol. 1994, 46:901-908.

Zarbo, R.J., Quality control issues and technical considerations in flow cytometric DNA ad cell cycle analysis of solid tumors. In: *Flow Cytometry and Clinical Diagnosis.* ed.: Keren, D.; Hanson, C.; Hutubise, P., American Socieyt of Clinical Pathologists Chicago. 1994 425-460.