THE EFFECT OF SEAL OIL ON PACLITAXEL INDUCED CYTOTOXICITY AND APOPTOSIS IN BREAST CARCINOMA MCF-7 AND MDA-MB-231 CELL LINES

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The Effect of Seal Oil on Paclitaxel Induced Cytotoxicity and Apoptosis in Breast Carcinoma MCF-7 and MDA-MB-231 Cell Lines

By

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To my Dad and Mom with love and respect

Abstract

Studies suggest that ω-3 polyunsaturated fatty acids (PUFAs) are beneficial in inhibiting the growth of cancer cells and may enhance the cytotoxicity induced by certain chemotherapeutic agents. It is known that seal oil is rich in ω-3 PUFAs. The effect of seal oil and its role on the cytotoxicity and apoptosis induced by paclitaxel was, therefore, investigated in breast cancer cell lines, MCF-7 and MDA-MB-231.

MCF-7 and MDA-MB-231 cells were respectively treated with seal oil, paclitaxel, and paclitaxel in combination with seal oil. Cytotoxicity was evaluated by MTT assay. Apoptosis was investigated by morphological changes and DNA strand break assay. Western blot assay was used to assess the expression of p53 and Bcl-2 proteins. The lipid peroxide products were measured by thiobarbituric acid reactive substances (TBARS) assay and the intracellular lipid composition was determined by gas chromatography (GC).

MTT assay showed that seal oil induced cell death and enhanced paclitaxel cytotoxicity in both cell lines. The IC_{50} values of paclitaxel in MCF-7 and MDA-MB-231 cells were reduced 3.3- and 2.4-fold, respectively, by the inclusion of 0.016% seal oil. Morphological assessment and DNA strand break assay indicated that more cells treated with paclitaxel in combination with seal oil underwent apoptosis than that with paclitaxel alone treatment. Seal oil alone was also found to induce apoptosis in both cell lines. Western blot showed that the expression of apoptosis inhibitor Bcl-2 protein in both cell lines was down-regulated by seal oil and was inhibited further when cells were treated with paclitaxel in combination with seal oil. GC assay determined that the total

PUFAs increased significantly in the intracellular lipid composition following incubation with seal oil, which may be responsible for the increased lipid peroxides found in cells incubated under the same conditions.

In addition to seal oil, Intralipid®, which is a soybean oil emulsion mainly containing ω-6 PUFAs, was also studied for its cytotoxicity and apoptosis induction. It was found that soybean oil induced cell death in the MCF-7 cell line but promoted cell growth in the MDA-MB-231 cell line. Soybean oil did not induce apoptosis as characterized by morphological assessment.

In conclusion, seal oil was found to induce apoptosis, and to enhance the cytotoxicity and apoptosis induced by paclitaxel in both breast cancer cell lines, which may be due to its ability to down-regulate the expression of Bcl-2 protein and to produce lipid peroxides. The beneficial effect exerted by seal oil suggests that seal oil may have a great potential in the management of cancer.

Key words: polyunsaturated fatty acids (PUFAs); seal oil emulsion; Intralipid®; paclitaxel; breast cancer; MCF-7 cell line; MDA-MB-231 cell line; apoptosis; cytotoxicity; MTT assay; Hoechst staining; TUNEL assay; Annexin-V-FLOUS staining; Western blotting assay; TBARS assay; GC assay; p53; Bcl-2; lipid peroxide products

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AA	arachidonic acid
AIF	apoptosis-inducing factors
APAF-1	apoptotic protease-activating factor-1
AR	androgen receptor
Bcl-2	B cell lymphoma/leukemia-2
BH	Bcl-2 homology
BSA	bovine serum albumin
Caspase	cysteinyl-aspartate specific proteases
COX	cyclooxygenase
CS_2	carbon bisulfide
DHA	docosahexaenoic acid
dH_2O	distilled H_2O
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DOX	doxorubicin
DPA	docosapentaenoic acid
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EPA	eicosapentaenoic acid
ER	estrogen receptors
FA-CoA	fatty acid acyl-coenzyme A
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate conjugate
GC	gas chromatography
HETEs	hydrooxyeicosatetraenoic acids
IC_{50}	50% of inhibitory concentration
IGF	insulin-like growth factors
LA	linoleic acid
α-LA	α-linolenic acid
γ-LA	γ-linolenic acid
LDL-R	low-density lipoprotein receptor
LOX	lipoxygenase
LTs	leukotrienes
mAb	monoclonal antibody
MDA	malondialdehyde
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
O.D.	optical density

PBS	phosphate-buffered saline
PGs	prostaglandins
PI	propidium iodide
PMSF	phenylmethylsulfonyl fluoride
PR	progesterone receptors
PS	phosphatidylserine
PTP	permeability transition pore
PUFAs	polyunsaturated fatty acids
ROS	reactive oxygen species
SD	standard deviation
SDS	sodium dodecyl sulphate
TBARS	thiobarbituric acid reactive substances
TBST	tris buffer solution with Tween-20
TdT	terminal deoxynucleotidyl transfererase
TEMED	N, N, N', N'- tetramethylethylenediamine
TGF-α	transforming growth factor-α
TUNEL	terminal deoxynucleotidyltransferase-mediated bio-dUTP nick-end labelling
TXA_2	thromboxane A_2
TXs	thromboxanes
VLDL	very low-density lipoprotein

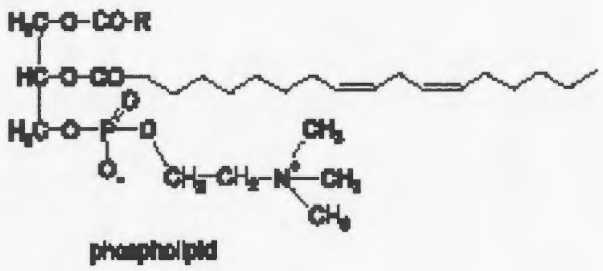
Chapter 1 Introduction

1.1 Polyunsaturated fatty acids (PUFAs) and cancer

1.1.1 Biochemistry of PUFAs

PUFAs are a series of unsaturated fatty acids with even numbers of carbon atoms, between 18 and 22, and 1 to 6 double bonds in the carbon skeleton. PUFAs and their metabolites have a variety of physiological roles including energy provision, membrane construction, and synthesis of cell signalling molecules and regulation of gene expression (Jump *et al.*, 1996 and Diggle, 2002). The hydrophobic biological membrane made by fatty acyl chains acts as a barrier and communication boundary to regulate movements and metabolic products across the membrane. PUFAs in the membrane can also interact with proteins located in the membrane such as enzymes, receptors and transporters to conduct cellular signals.

PUFAs comprise two forms: non-esterified fatty acids or free fatty acids and esterified fatty acids. Esterified PUFAs exist as glycerol esters, such as phospholipids and triglycerides, which are the major composition of cell membrane. Figure 1.1 shows an example of a phospholipid in which the second hydroxyl group of the glycerol is esterified with a PUFA and the other two hydroxyl groups are esterified to a phosphate and either to a saturated or monounsaturated fatty acid or an aldehyde in its enolic form, respectively (Spiteller, 2003) (Figure 1.1).



R: a saturated or monounsaturated fatty acid or an aldehyde

Figure 1.1 **Structure of an esterified polyunsaturated fatty acid**

(Adapted from Spiteller, 2003)

The designation of PUFAs is characterized by the number of carbon atoms, the number of double bonds and the position of the first double bond starting from the methyl terminal of the molecule. For example, 18:2ω-6 refers to linoleic acid (LA) with a chain length of 18 carbon atoms, 2 double bonds, and with the first double bond being at the sixth carbon atom from the methyl terminal. Based on the position of the first double bond starting from the methyl terminal of the carbon chain, PUFAs can be classified into two main classes, ω-6 class and ω-3 class, in which the first double bond starts at the sixth and third carbon atom from the methyl terminal, respectively. The ω-6 PUFAs exist mainly in plant sources such as soybean oil, including LA, γ-linolenic acid (γ-LA) (18:3ω-6) and arachidonic acid (AA) (20:4ω-6). The ω-3 PUFAs are rich in marine sources such as fish oil. They commence with α-linolenic acid (α-LA) (18:3ω-3) and mainly include eicosapentaenoic acid (EPA) (20:5ω-3) and docosahexaenoic acid (DHA) (22:6ω-3).

The first member of ω-6 class, LA, which is the most abundant PUFA found in plant seeds and oil, is synthesized by terrestrial plants; however, the first member of ω-3 class, α-LA, is synthesized by cold-water vegetation, and fish and sea animals that feed on these organisms convert it into the two most abundant members in the class, EPA and DHA. In animals and humans, LA and α-LA must be obtained from their diet because mammalian organisms lack Δ-12 and Δ-15 desaturase enzymes that are required for the synthesis of LA and α-LA (Spector and Burns, 1987). Therefore, both ω-6 and ω-3 PUFAs are considered as essential fatty acids. Mammalian cells can convert the PUFAs within each class, but they cannot convert ω-3 class into ω-6 class or vice versa because they do not possess the necessary enzymes (Spector and Burns, 1987, Noguchi *et al.*, 1995 and Simopoulos, 2002). In biological systems, LA or α-LA subjects to elongation, desaturation and conversion by elongases and desaturases to yield longer and more unsaturated fatty acids. In humans, LA is readily converted to AA via γ-LA; however, α-LA is not efficiently converted to EPA and DHA. Both AA and EPA are the substrates of eicosanoid metabolism, which produces prostaglandins (PGs) and thromboxanes (TXs) by cyclooxygenases (COXs), and leukotrienes (LTs) and hydroxyeicosatetraenoic acids (HETEs) by lipoxygenases (LOXs) and cytochrome P450, respectively. These eicosanoids behave as "local hormones" and chemical transmitters for a variety of intercellular and intracellular signals. Figure 1.2 shows the structures and relationship of the main fatty acids in each class and their metabolic pathways.

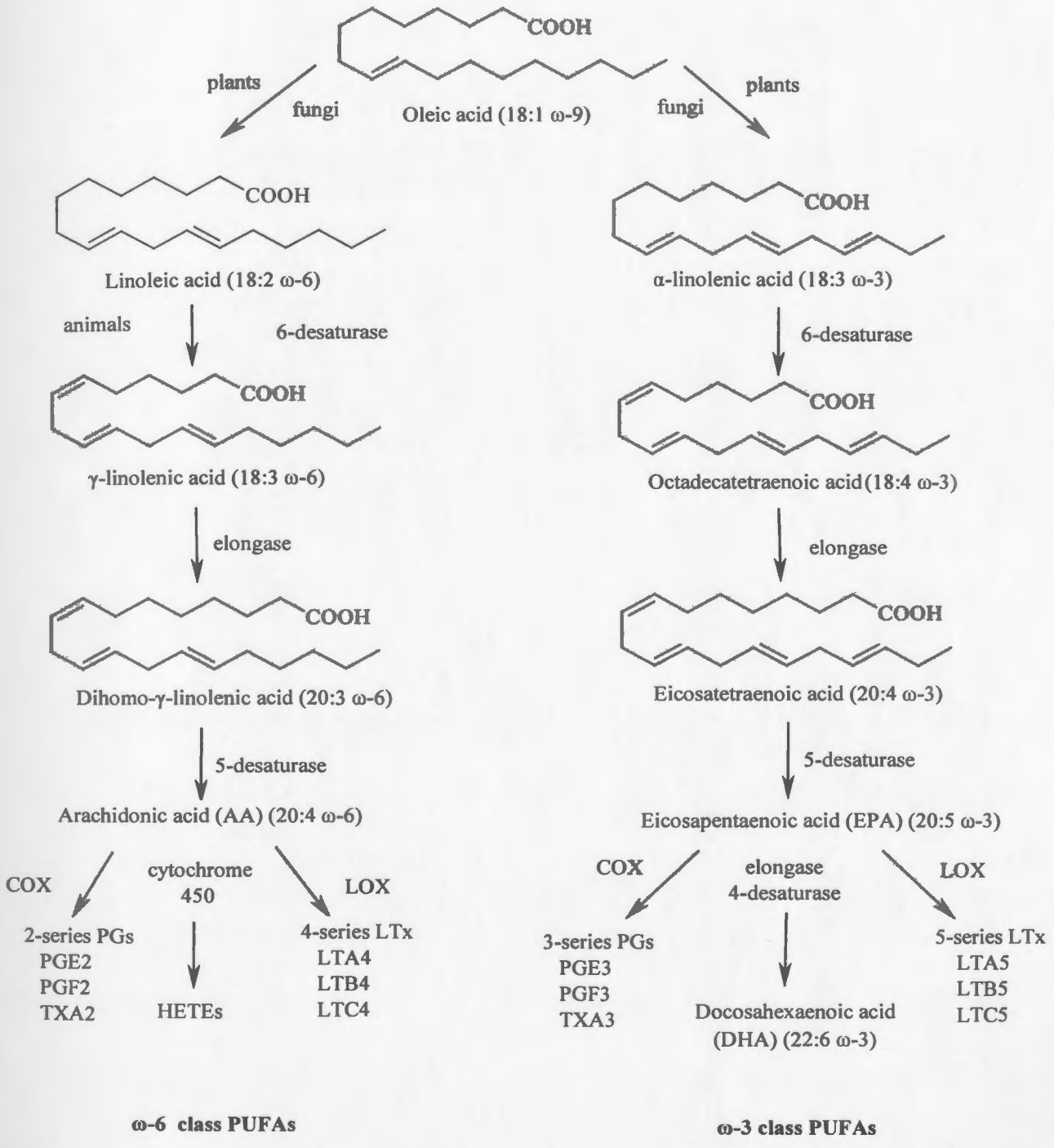


Figure 1.2 **Pathways of elongation, desaturation and metabolism of dietary fatty acids** (Adapted from Noguchi et al., 1995 and Tapiero et al., 2002)

In humans, fatty acids obtained from the diet or extrahepatic tissues enter hepatocytes and are rapidly converted to fatty acid acyl-coenzyme A (FA-CoA) thioesters that are substrates for further elongation, desaturation, lipid synthesis, β-oxidation, mediator synthesis and protein acylation reactions (Stulnig, 2003). They are processed to form complex lipids, such as triacylglycerols and phospholipids, or are diverted to oxidation in mitochondria, peroxisomes and the endoplasmic reticulum. Complex lipids are assembled into lipoprotein complexes and secreted in the form of very low-density lipoproteins (VLDL) to extrahepatic tissues, such as adipose tissue, where fatty acids are assembled into triacylglycerols for storage (Jump *et al.*, 1996).

1.1.2 Effect of PUFAs on cancers

1.1.2.1 ω-3 PUFAs versus ω-6 PUFAs

PUFAs not only play various essential roles in the physiological processes but also have pathophysiological activities on nervous system, cardiovascular disease, inflammatory disease, immune system and especially some cancers. Exogenous PUFAs have been investigated in different kinds of cancer including breast, colon and prostate cancer. With breast cancers, hormone dependent cell lines, MCF-7, ZR-75 and T-47-D, and hormone independent cell lines, MDA-MB-231, HBL-100, have been treated with ω-6 and ω-3 PUFAs. The results showed that the cell growth was inhibited by ω-3 PUFAs, and EPA and DHA were the most effective fatty acids in the arresting of cell growth (Chajes *et al.*, 1995 and Germain *et al.*, 1998). When athymic nude mice bearing with MCF-7 or MDA-MB-231 cancer cells were fed with fish oil or corn oil diet for 6-8 weeks, it was found that cancer growth was suppressed in mice consuming

fish oil diet as compared with mice fed with corn oil, which is rich in ω-6 PUFAs. The data showed that the greater the amount of dietary fish oil consumed, the greater the cancer suppression observed. At the end of the experiments, it was found that the mean tumour volume of mice in the fish oil group was the least and that in the corn oil group was the largest (Pritchard *et al.*, 1989, Gonzalez *et al.*, 1991 and Gonzalez *et al.*, 1993). These results suggested that diet high in ω-3 PUFAs inhibited tumour cell growth but ω-6 PUFAs were associated with tumour cell proliferation. Similar results were also found in humans where a higher ratio of ω-6 to ω-3 consumed in the diet was associated with a higher risk of breast cancer occurrence in women (Bagga *et al.*, 1997 and Klein *et al.*, 2000).

Several studies have also shown that ω-3 PUFAs inhibit the growth of some other cancers while ω-6 PUFAs promote cancer development. For example, it has been reported that diet high in ω-6 PUFAs could stimulate prostate cancer development. On the contrary, long chain ω-3 PUFAs such as DHA and EPA could inhibit prostate cell growth *in vitro* and *in vivo* (Giovannucci *et al.*, 1993; Motaung, *et al.*, 1999 and Chung *et al.*, 2001). The cytotoxicity of PUFAs investigated in HL-60 leukaemic cell line (Arita *et al.*, 2003) and HT-29 colon cancer cells (Chen *et al.*, 2000) revealed that ω-3 PUFAs had an inhibitory effect on the growth of these cancer cells. When cytotoxicity caused by various PUFAs was examined by flow cytometric analysis, results showed that the cytotoxicity was related to the chain length of the PUFAs and the number of double bonds in them, and DHA and EPA were shown to be most potent (Lima *et al.*, 2002).

1.1.2.2 PUFAs in combination with chemotherapy and radiotherapy

A number of studies have shown positive effect of exogenous PUFAs on chemotherapy and radiotherapy both in cell culture and in tumour-bearing animals (Burns *et al.*, 1986; Zulstra *et al.*, 1987; Petersen *et al.*, 1992; Shao *et al.*, 1995; Germain *et al.*, 1998 and Conklin, 2002). Chemotherapeutic drugs investigated include the anthracyclines such as doxorubicin (DOX), the epipodophyllotoxins, the camptothecins, the platinum coordination complexes and alkylating agents. Cancer types investigated included breast cancer, ovarian cancer, and leukemia and lymphoma cells. It was found that in comparison with chemotherapeutic drug alone, the cytotoxicity of chemotherapeutic drugs was enhanced when PUFAs such as DHA, EPA, γ-LA, AA and LA were used in combination with the chemotherapeutic drugs (Germain *et al.*, 1998, Yamamoto *et al.*, 1999 and Conklin, 2002). In athymic mice with human mammary or lung carcinoma, these anti-cancer drug treatments resulted in a greater inhibition of tumour growth when mice were fed fish oil (varying from 4-20 %) enriched diet (Conklin, 2002). In addition to chemotherapy, PUFAs can also enhance the sensitivity of cancer cells to radiation therapy (Vartak *et al.*, 1997). It was reported that an improved response to radiotherapy as well as chemotherapeutic agents was found in patients who received 5-7 g/day of ω-3 PUFAs (Barozio *et al.*, 1998).

1.1.3 Possible mechanisms of PUFAs activities on cancers

1.1.3.1 Modulation of PUFAs in cell membrane

Since PUFAs participate in membrane construction and cell proliferation, the enrichment of PUFAs in membrane has potential impacts on the normal barrier function and the physical and

biochemical characteristics of the membrane. It has been reported that plasma membranes enriched in PUFAs have lower order parameters, or greater fluidity and higher permeability (Spector and Burns, 1987 and William *et al.*, 1993). These changes might cause loss of surrounding lipid matrix, change of the accessibility of binding site, alteration of the tightness of binding ligand, the size of the transmembrane channel and the signal transduction of receptors. In addition, because a number of transport and receptor proteins locate in the membrane and across the lipid bilayer, changing in membrane composition may affect the properties of these proteins. Such effects have more important consequences for tumour cells by influencing the transport and uptake of nutrients and chemotherapeutic drugs, which enter into cells by carrier-mediated and energy-requiring transporters (Spector and Burns, 1987). When PUFAs are used in combination with anti-cancer drugs, the composition of fatty acids in the tumour cell membrane may be altered as a result of incorporation of PUFAs. This change may assist drugs to flux across the cell plasma membrane and increase the intracellular drug concentration leading to a reduced resistance and increased sensitivity to chemotherapy (Ikushima *et al.*, 1991; Germain *et al.*, 1998 and Conklin, 2002).

1.1.3.2 Generation of lipid peroxidation products from PUFAs

It is conceivable that as the first defence line PUFAs in the cell membrane are highly susceptible to oxidative stress in biological systems, producing reactive oxygen species (ROS) (Table 1.1). The transformation of these products can be used as an economical way to transit the communication signals generated in the membrane to cytoplasm. ROS and their derivatives can react with and attack almost every critical cellular macromolecule including DNA, lipids,

proteins and carbohydrates, and cause structural as well as functional alteration, leading to cell membrane damage, cellular composition and molecular architecture changes, and signalling disruptions (Gonzalez *et al.*, 1993 and Conklin, 2002). For example, various potential compounds found among ROS can form adduct with amino acids and DNA. These compounds include several reactive α, β-unsaturated aldehydes such as malondialdehyde (MDA) (Bartsch *et al.*, 1999). They are strong electrophiles and can bind to the sulfhydryl groups and cysteine residues of proteins and DNA bases, showing both genotoxic and mutagenic actions (Luczaj *et al.*, 2003). Low levels of these compounds were suggested to inhibit the transition of cells from the G0 phase to the G1 phase, prolong the G1 phase and slow the progression through the S phase by inhibiting the activity of DNA polymerases for DNA synthesis (Conklin, 2002 and Rex *et al.*, 2002). The affected cells may repair such damage. However, extensive damage leads to apoptosis and cell death.

Table1.1 Lipid peroxide products of oxidative stress (Adapted from Kenneth *et al*, 2002)

Free Radicals	**Nonradicals**	**Lipid Peroxidation Products**	**Secondary Products**
Hydroxyl radical ($HO^{\cdot}$)	Hydrogen peroxide (H_2O_2)	Peroxyl radical ($ROO^{\cdot}$)	Malondialdehyde
Peroxide radical ($O_2^{\cdot -}$)	Singlet oxygen ($^1O_2$)	Alkoxyl radical ($RO^{\cdot}$)	4-Hydroxyalkenals

It has been reported that tumour cells are highly susceptible to free radical-induced cytotoxicity because they have decreased activity of fatty acid desaturase enzymes, decreased antioxidant content and decreased capacity to generate free radicals and lipid peroxides due to

the lower amount of cytochrome P450, which normally can participate in the initiation of lipid peroxidation (Begin *et al.*, 1986; Cheeseman *et al.*, 1986 and Das 1990, 1999). Therefore, it is expected that tumour cells may be more sensitive than normal cells to the attack of ROS generated from PUFAs. In addition, compared with normal cells, tumour cells have a higher tendency to ingest excessive PUFAs from the environment for the membrane construction to meet their high proliferation and migration. Thus, tumour cells may have more potential than normal cells to be targeted by ROS from PUFAs.

1.1.3.3 Metabolism pathways of ω-6 and ω-3 PUFAs

Substantial experimental evidence suggests that ω-6 PUFAs enhance the risks of cancer development, while increased intake of ω-3 PUFAs has inhibitory effects. Such a difference is likely due to the different metabolic pathways and metabolites of ω-6 PUFAs vs. ω-3 PUFAs as shown in Figure 1.2. ω-6 PUFAs promote tumorigenesis and tumour cell proliferation directly and indirectly via increased synthesis of eicosanoids, the COX- and LOX-catalyzed products of AA (Noguchi *et al.*, 1995). COX, which has two isoforms, COX-1 and COX-2, is a key enzyme responsible for the oxidation of AA to PGs. COX-1 enzyme is expressed constitutively in most cells and tissues, and stays constant under either physiological or pathological conditions to control the synthesis of PGs primarily involved in the regulation of homeostatic functions. In contrast, COX-2 is absent in normal cells but highly inducible under the stimulation by inflammatory, tumour promoters, cytokines and growth factors, resulting in enhanced PGs release (Bakhle *et al.*, 1996 and Smith *et al.*, 2000). The PGs produced from COX-catalyzed pathways possess a broad spectrum of biological activities. They primarily mediate pain and

inflammation but also favour tumorigenesis. The production of PGs is controlled in most normal tissues but tumour cells or the pathological tissue may produce larger amounts of PGs and eicosanoids than their normal counterparts, as it has been found that the levels of COX-2 and PGs are more abundant in cancers than those in normal tissues from which the cancers arise, influencing the carcinogenic process (Spector and Burns, 1987). The PGs and other eicosanoids generated in tumour cells can act as tumour promoters, stimulate tumour growth or influence tumour migration and the metastatic potential. In human breast cancers and animal models, for example, it has been determined that COX-2 up-regulation and elevated PGE_2 levels are involved in breast cancer growth and invasion as COX-induced PGs enhance estrogen synthesis in mammary tissue thereby stimulating growth in hormone dependent tumours (Tapiero *et al.*, 2002).

In the metabolic pathway of ω-6 PUFAs, AA is the precursor of PGs and can be converted to 2-series of PGs by COX-2 enzymes. The 2-series of PGs include PGH_2, a common precursor for the biosynthesis of other 2-series PGs such as PGD_2, PGE_2, $PGF_2\alpha$, PGI_2, and thromboxane A_2 (TXA_2). Eicosanoids such as PGI_2 and TXA_2 are important in tumour metastasis. PGI_2 is synthesized primarily by vascular endothelial cells and inhibits platelet activation and aggregation, while TXA_2, a product of COX from PGH_2 in platelets, has opposing actions to PGI_2, which is to promote platelet aggregation. They are involved in tumour angiogenesis by affecting the tumour cell-vascular endothelial cell and tumour cell-platelet interactions and tumour vascular formation.

The 2-series of PGs and 4-series of LTs produced from LOX-pathway can also stimulate the

growth of malignant cells by suppressing host immunity as immunosuppressive and mutagenic substances (Das, 1990 and Noguchi *et al.*, 1995). PGE_2 can suppress the production of TNF-α, IL-1 and IL-6 by monocytes and macrophages (Stulnig, 2003), while $PGF_2\alpha$ is required for the invasive and metastatic activity of tumour cells (Reich *et al.*, 1989). Therefore, it is thought that by suppressing the host immune response, eicosanoid products produced from AA help tumours avoiding immunologic inhibition and promote tumour initiation, division, proliferation, tissue invasion and metastatic spread.

However, ω-3 PUFAs can interfere with AA to produce certain eicosanoids and lead to less effective generation of these messenger molecules. Compared with ω-6 PUFAs, ω-3 PUFAs have a greater affinity for desaturase and elongase enzymes (Rose *et al.*, 1999). Incorporation of ω-3 PUFAs can inhibit early steps in the long-chain fatty acid biosynthesis by competing for the enzymes necessary for desaturation and elongation, causing the depletion of ω-6 PUFAs including AA. In addition, as shown in Figure 1.2, both EPA and AA are the precursors of PGs in the eicosanoid metabolic pathway. EPA can compete with AA as the substrate for eicosanoid metabolism thereby blocking the AA metabolic pathway, giving rise to the 3-series of PGs with 3 double bonds and 5-series LTs with 5 double bonds, which exert less biological activities in comparison to AA-derived eicosanoids (Koller *et al.*, 2003), leading to a decline in the conversion to 2-series PGs and 4-series LTs which promote tumour origin, growth and metastasis. Thus, ω-3 PUFAs may offset the effect of ω-6 PUFAs on cancer cells.

In conclusion, PUFAs may play their roles on tumour cells by affecting the composition of the cell membrane, creating lipid peroxide products and producing eicosanoids through their

metabolic pathways.

1.2 Apoptosis

1.2.1 Necrosis and apoptosis

There are two distinct modes of cell death, necrosis and apoptosis. Necrosis ("accidental" cell death) is a pathological process and a result of acute cellular dysfunction which occurs when cells respond to a serious physical or chemical insult. It is progressive and results in irreversible damage and complete degradation of affected cells. Morphologically, necrosis is characterized by a dramatic increase in cell volume and rupture of the plasma membrane, with spilling of the cellular contents into the intracellular milieu. The release of the contents of necrotic cells can cause further tissue damage by affecting neighbouring cells, resulting in extensive inflammation response (Chandra *et al.*, 2000).

However, apoptosis ("normal" or "programmed" cell death) affects scattered single cells rather than tracts of contiguous cells. It is a spontaneous physiological process by which unwanted or useless cells are eliminated during development and other normal biological processes. Apoptosis is most often found during normal cell turnover and tissue homeostasis, embryogenesis, induction and maintenance of immune tolerance, development of the nervous system and endocrine-dependent tissue atrophy. As a complementary but opposite role to mitosis to keep the balance between cell production and loss, apoptosis regulates cell population by controlling cell deletion under both physiological and pathological conditions (Kerr *et al.*, 1972).

The morphological changes occurring in cells upon apoptosis include cell membrane

alteration, cell shrinkage, nuclear condensation with the chromatin forming clumps that gather adjacent to the nuclear membrane, and nuclear fragmentation together with mono- and oligonucleosomal fragments of DNA breaks with 180-200 base pairs, as well as some apoptosis-related protein expression changes. During apoptosis, cytoplasm condenses, causing contraction of the cell, loss of volume and adhesion to surrounding cells. The cell surface then becomes irregular with numerous surface protrusions that eventually round up and break off as membrane-bound apoptotic bodies (apoptotic bodies), in which the integrity of various subcellular organelles is initially maintained. In biological systems, the apoptotic bodies are recognized, engulfed and digested by macrophages or phagocytosed by adjacent epithelial cells so no inflammatory response is elicited. Since the fragments of apoptotic cells are disposed by nearby intact cells, apoptosis is economical in terms of re-utilization of cell components (Kerr *et al.*, 1972 and Wyllie, *et al.*, 1980). Figure 1.3 shows the morphological features associated with necrosis and apoptosis. Table 1.2 summarizes the characteristics of necrosis and apoptosis.

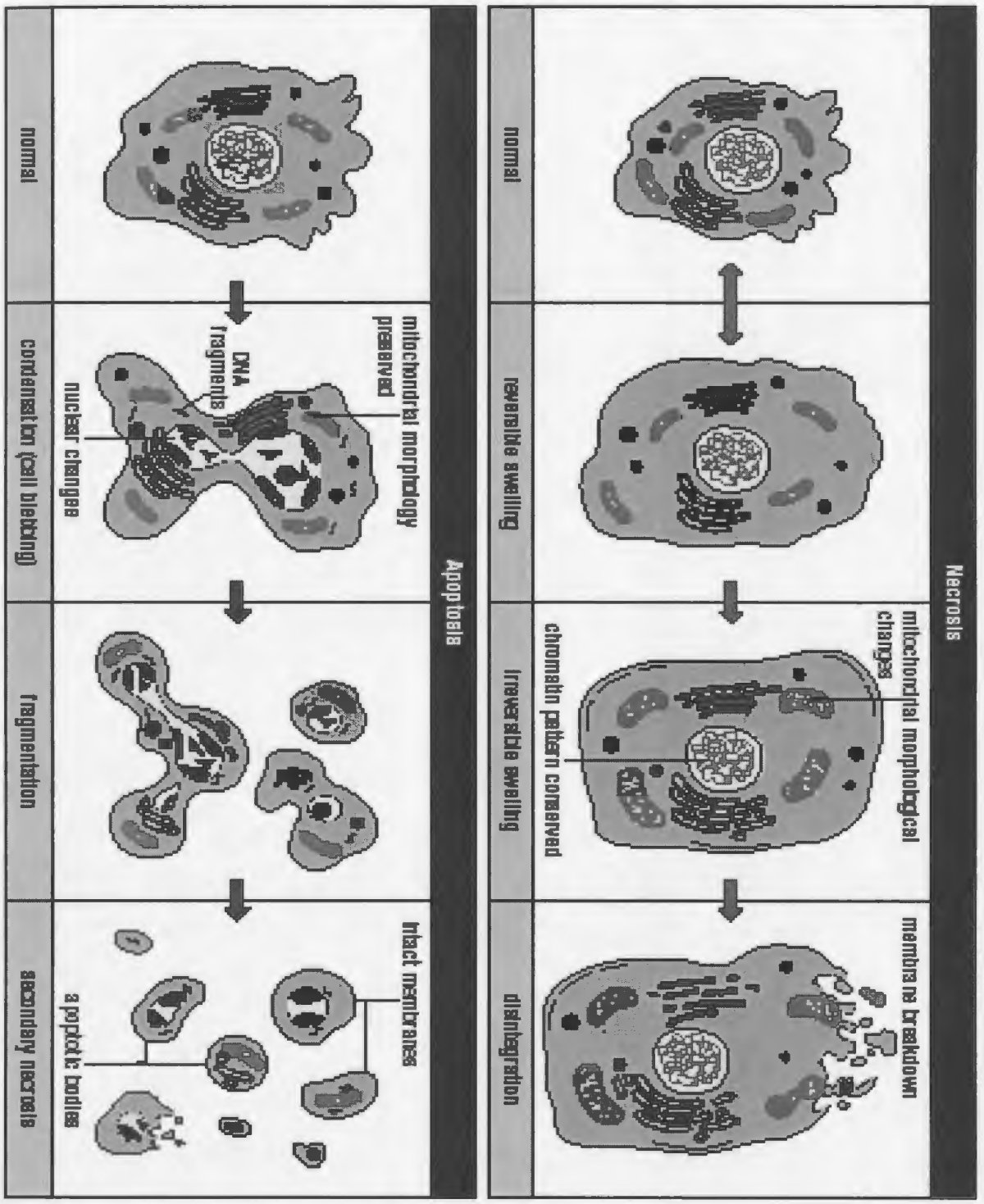


Figure 1.3 Schematic diagram of morphological changes associated with necrosis and apoptosis

(Adapted from http://www.roche-applied-science.com)

Table 1.2 Differential features of necrosis and apoptosis

(Adapted from http://www.roche-applied-science.com)

Necrosis	Apoptosis
Morphological features	
Loss of membrane integrity	Membrane blebbing, no loss of integrity
Begins with swelling of cytoplasm and mitochondria	Aggregation of chromatin at the nuclear membrane
Ends with total cell lysis	Begins with shrinking of cytoplasm and condensation of nucleus
No vesicle formation, complete lysis	Ends with fragmentation of cell into smaller bodies
Disintegration (swelling) of organelles	Formation of membrane bound vesicles (apoptotic bodies)
	Mitochondria become leaky due to pore formation involving proteins of the Bcl-2 family
Biochemical features	
No energy requirement	Tightly regulated process involving activation and enzymatic steps
Random digestion of DNA	Energy (ATP)-dependent
	Non-random mono- and oligonucleosomal length fragmentation of DNA
	Release of various factors (cytochrome C, AIF) into cytoplasm by mitochondria
	Activation of caspase cascade
	Alteration in membrane asymmetry (i.e., translocation of phosphatidylserine from cytoplasmic to the extracellular side of the membrane)
Physiological features	
Affects groups of contiguous cells	Affects individual cells
Evoked by non-physiological disturbance (complement attack, lytic viruses, hypothermia and metabolic poisons)	Induced by physiological stimuli (lack of growth factors, changes in hormonal environment)
Phagocytosis by macrophages	Phagocytosis by adjacent cells or macrophages
Significant inflammatory response	No inflammatory response

1.2.2 Cascade of apoptosis

Unlike necrosis, apoptosis is complicated but precisely ordered for self-destruction rather than degeneration. It is a complex set of multi-step, multi-branched pathways with many checkpoints and balances. The whole process requires energy, protein synthesis and activation of series of subcellular components and factors for its execution. It can be triggered by a variety of physiological and chemical stimulation either from intracellular or extracellular environments, which initiate one or several distinct cross linking pathways (Figure 1.4). Intracellular triggers include DNA damage, telomere malfunction and inappropriate proliferative signals produced by oncogenic mutation. Extracellular triggers include chemical and radiation stimulation, growth factor depletion, hypoxia and loss of cell matrix. The activation pathways are dependent on the cell types and stimulation, but the general apoptotic cascade manifests itself in two major execution downstream pathways of the initiating signals: the caspase (cysteinyl-aspartate specific proteases, which dismantle cells by cleaving a subset of cellular proteins after aspartic acid residues) pathway and the organelle dysfunction. The caspase pathway is involved with many proteolytic enzymes subdivided into classes which initiate the process, propagate and amplify the signals. This pro-apoptotic machinery interacts with cellular survival mechanisms at different levels, including related proteins such as p53 protein and Bcl-2 family members. The organelle pathway is best characterized by mitochondrial dysfunction.

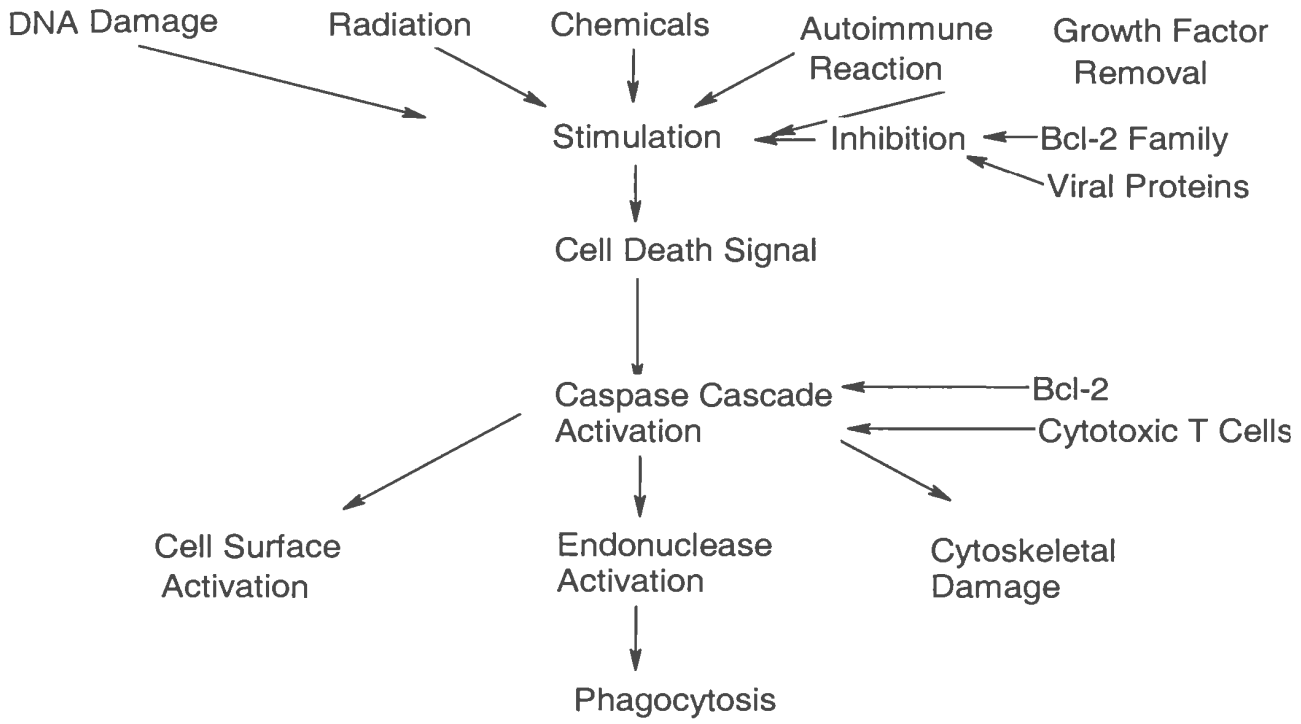


Figure 1.4 Schematic illustrations of various phases of apoptosis
(Adapted from Gross *et al.*, 1999)

1.2.3 Some important apoptotic markers

1.2.3.1 p53 protein

Preserving genomic integrity by the repair or removal of damaged DNA is essential for cell survival as the persistence of genomic damage could potentially lead to neoplasia. p53 protein acts as a tumour suppressor involved in damage recognition and DNA repair. It can induce cell growth arrest, senescence, or apoptosis in response to a variety of cellular stresses including exposure to DNA damage, hypoxia, nucleotide depletion and oncogene activation (Sionov *et al.*, 1999). p53 protein performs as a checkpoint protein during the G1 and G2 phases of the cell cycle to prevent cells with damaged genomes from undergoing DNA replication and mitosis

(Sionov *et al.*, 1999 and Haupt *et al.*, 2003). When cells encounter DNA damage such as double-strand breaks, the level of p53 increases rapidly and induces G1 arrest, stopping DNA replication, which allows DNA repair to take place. If this is not possible or DNA repair mechanisms fail, then p53 triggers apoptosis to eliminate damaged cells permanently. This growth inhibitory activity prevents the proliferation of cells with damaged DNA or with a potential neoplastic transformation.

p53 protein induces extrinsic and intrinsic apoptotic signalling pathways that lead to the activation of the caspases to execute apoptosis. The extrinsic pathway involves engagement of particular death receptors that belong to the tumour necrosis factor receptor family. Through the formation of the death-inducing-signalling-complex, this pathway leads to a cascade of activation of caspases, which in turn induce apoptosis. The intrinsic pathway is triggered in response to DNA damage and is associated with mitochondrial depolarization, the release of cytochrome c and apoptotic protease-activating factor-1 (APAF-1) from mitochondria into cytoplasm, and the shift of balance in the Bcl-2 family towards to the pro-apoptotic members, promoting the formation of the apoptosome, and consequently caspase-mediated apoptosis (Haupt *et al.*, 2003).

1.2.3.2 Bcl-2 protein family

The Bcl-2 protein family is another crucial group of proteins related to apoptosis. The first found member, Bcl-2 oncogene, was discovered present at the interchromosomal breakpoint of the t(14;18), the molecular hallmark of follicular B cell lymphoma/leukemia-2 (Chao *et al.*, 1998). According to the effect on apoptosis, the Bcl-2 protein family is divided into

pro-apoptotic proteins (agonist) such as Bax, Bak, Bcl-Xs, Bad and anti-apoptotic proteins (antagonist) including Bcl-2 and Bcl-X_L (Table 1.3).

Table 1.3 Bcl-2 family proteins

Anti-apoptotic	**Pro-apoptotic**
Bcl-2	Bcl-X_S
Bcl-X_L	Bax
Bcl-w	Bak
Mcl-1	Bid
A1	Bad

Bcl-2 family proteins execute their functions by dimerizing with homologous proteins, associating with non-homologous proteins, and forming ion channels and pores or integral membrane proteins to affect the organelles where they reside (Gross *et al.*, 1999). Bcl-2 family members possess up to four conserved regions, namely Bcl-2 homology (BH) domains. The BH domains that individual proteins have in common appear to be crucial in the ability of these proteins to bind with each other and enhance or attenuate the action of the others. All Bcl-2 family proteins share at least one of BH domains that are important for homodimer or heterodimer formation. For example, many of the anti-apoptotic members display sequence conservation in all four domains, whereas the pro-apoptotic molecules usually lack BH4 but preserve BH3 domain, which is critical for induction of apoptosis (Gross *et al.*, 1999). Evidence suggests that by binding at the BH domains, Bax: Bax homodimers tend to promote apoptosis and Bcl-2: Bcl-2 homodimers can act to anti-apoptosis; however, Bax can form heterodimers with Bcl-2 or Bcl-X_L to abolish their anti-apoptotic activity. Therefore, the dominant dimerized molecules of Bcl-2 protein members determine cell fate for survival or apoptosis (Sheikh *et al.*,

2000).

Some of the Bcl-2 family proteins can be regulated by p53 protein. Pro-apoptotic protein Bax, for example, is a known target of p53 protein and can be up-regulated by p53 in some cell types by γ-radiation, chemotherapeutic drugs and other forms of genotoxic stress (Zhan *et al.*, 1994), while anti-apoptotic protein Bcl-2 was reported to be able to be down-regulated by p53 (Miyashita *et al.*, 1995 and Haldar *et al.*, 1994). Thus, induction of Bax expression and down-regulation of Bcl-2 after p53 activation leads to an altered Bcl-2/Bax ratio which may be important in inducing apoptosis. On the other hand, Bcl-X_L, an anti-apoptotic protein, is reported to be able to inhibit p53-mediated apoptosis in certain breast cancers (Bronchud *et al.*, 2000), suggesting that Bcl-2 family proteins can also regulate apoptosis by influencing p53 protein.

Bcl-2 family members may insert into intracellular membranes and function as ion channels. Anti-apoptotic Bcl-2 family members are integral membrane proteins in the mitochondria, endoplasmic reticulum and nuclear membrane (Rose *et al.*, 1993). However, a substantial fraction of such pro-apoptotic members as Bax localizes in cytosol or cytoskeleton. Following a death signal, activation of the cytosolic pro-apoptotic members appears to involve subcellular translocation and dimerization. They target and integrate into membranes, especially the mitochondrial outer membrane, where they become integral membrane proteins and cross-link dimers (Gross *et al.*, 1998 and Gross *et al.*, 1999). Bax homodimers and Bax: Bcl-2 heterodimers can form ion-conductive pores in mitochondrial membrane. They control the release of apoptotic factors from mitochondrial intermembrane into cytoplasm by the regulation

of mitochondrial membrane permeability. Under apoptotic stimuli, the mitochondrial transmembrane potential ($\Delta\psi_m$) is decreased and the pore is open. The opening pore allows the intra-mitochondrial cytochtome c, apoptosis-inducing factors (AIF) and some caspases to be released into the cytosol. Once cytochrome c reaches the cytoplasm, it binds to apoptotic protease-activating factor-1 (APAF-1), which is a cytoplasmic protein, to produce an ATP-dependent complex to recruit and activate the caspase cascade; the post-mitochondrial apoptotic pathway thereby is activated (Haupt *et al.*, 2003) (Figure 1.5).

Bcl-2 family proteins can also act as a checkpoint at the upstream of caspases. Pro-apoptotic members such as Bax and Bad connect survival signals, while anti-apoptotic members of Bcl-2 family can shut off the apoptotic signal transduction pathway that is located in the upstream of caspase activation (Chao *et al.*, 1998).

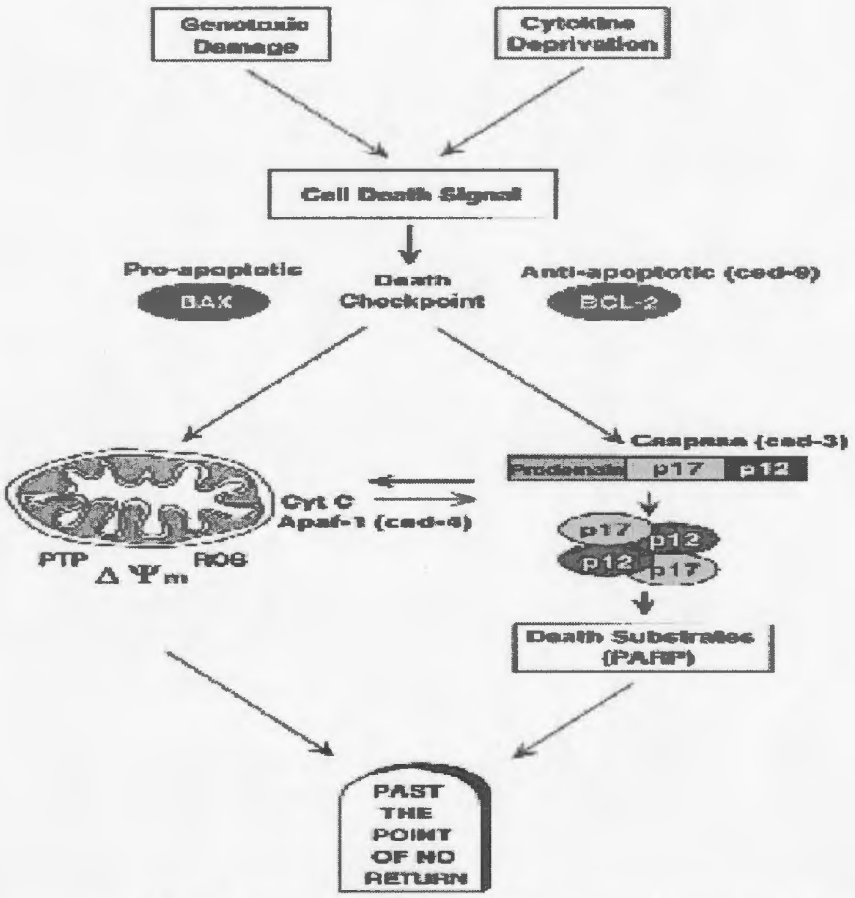


Figure 1.5 Schematic model of apoptosis pathway (Adapted from Gross *et al.*, 1999)

1.2.3.3 Mitochondria and cytochrome c

Mitochondria are cellular powerhouses and respiration organelles. They play a decisive role in apoptotic pathway. The organelle apoptotic pathway, which is mitochondrial dysfunction under apoptosis, is characterized by a loss of membrane barrier functions including opening of the permeability transition pore (PTP), collapse of the membrane potential, and a release of various classes of molecules from the intermembrane space via the outer mitochondrial membrane. The PTP is a large conductance pore that is permeable to solutes with molecular mass of around 1500 Daltons. The molecules released upon opening the PTP include at least: 1)

cytochrome c, which participates in the activation of caspases; 2) procaspases, in particular procaspase-2, -3, and -9, which may facilitate the activation upon release from the intermembrane space; 3) apoptosis-inducing factor, a flavoprotein which induces large scale of chromatin fragmentation (Jacobson *et al.*, 2002). As a result of the release of these factors, the downstream caspases are activated and come into action, leading to the biochemical and morphological hallmarks of apoptosis. Opening the PTP also induces loss of ion gradient and mitochondrial depolarization, causing influx of water into mitochondria and swelling of the mitochondria (Gross *et al.*, 1999). Expanding of the matrix space eventually results in organelle rupture and consequent elimination by apoptosis.

Cytochrome c normally resides between the inner and outer mitochondrial membranes and functions as a part of the respiratory chain in mitochondria. When apoptosis happens, apoptotic signals promote the translocation of Bax from cytosol to the outer layer of mitochondrial membrane, forming the channels to allow cytochrome c to escape into the cytoplasm. When released into the cytosol, cytochrome c becomes a critical component of the apoptosis execution machinery, where it activates caspases. Cytochrome c needs another cytosolic factor to initiate apoptosis. This factor is designated APAF-1. Cytochrome c combines with APAF-1 in conjunction with ATP to activate pro-caspase-9 to caspase-9, which then activates caspase-3 and -7. Caspase-3 can activate caspase-2 and -6, while caspase-6 can activate caspase-8 and -10 (Palmer *et al.*, 2000). In turn, these caspases cleave and activate a variety of substrates and key cellular regulators to enable collapse of cells and give rise to characteristic morphological changes of apoptosis such as membrane bleb, chromatin condensation, and DNA fragmentation.

Moreover, caspase-3 can activate caspase-9 again, and provides a positive feedback loop to some of the Bcl-2 proteins, for example, pro-apoptotic protein Bid, leading to the amplification of apoptotic signals (Figure 1.6).

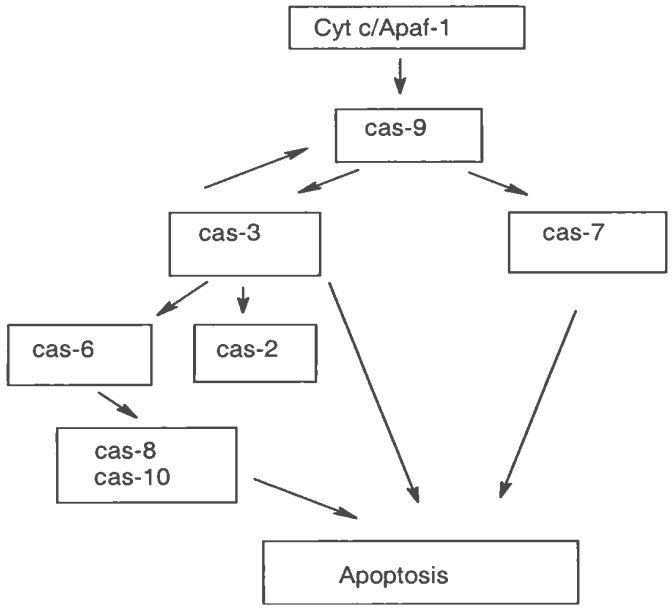


Figure 1.6 Order of caspase activation events downstream of Apaf-1/cytochrome c complex (Adapted from Palmer *et al.*, 2000)

1.2.4 Apoptosis in cancers

The size of a cell population depends on the balance between cell production and cell loss. Apoptosis is an important mode of controlled cell death, which contributes to the regulation of cell populations. Cancers occur through unregulated cell division, enhanced proliferation, diminished cell turnover and abnormal apoptotic machinery. Loss of apoptosis can impact on carcinogenic processes to extend tumour life span and resistance to chemo- and radiotherapy. One way to avoid apoptosis by cancer cells is through oncogenic mutations, leading to a change of expression of apoptotic proteins.

1.2.4.1 Change of the expression of Bcl-2 family members in cancers

Overexpression of anti-apoptotic Bcl-2 family members has been widely observed in human tumour cells (Reed, 1998). It is believed to be associated with increased resistance of the cancers to treatments, and results in a poorer prognosis. These anti-apoptotic proteins appear to prolong the survival of cells and allow additional mutation or genetic changes so the propensity of cancer development is increased. They can also exert their effects by blocking cytochrome c release from mitochondria and consequently to inhibit the activation of caspases cascade. In addition, abnormally high Bcl-2 expression in cancers has been shown ultimate resistance of cells to diverse apoptosis stimulation, including chemotherapeutic drugs and radiation, growth factor deprivation, hypoxia, loss of cell attachment to extracellular matrix (relevance to tumour metastasis) and lysis by cytolytic T cells (Reed *et al.*, 1996).

1.2.4.2 Dysfunction of p53 and lack of caspase-3 in cancers

The mutated p53 gene has been found in over 50% of tumours. It has been estimated that the vast majority of tumours have a disruption in the p53 pathway either by mutation of p53 or inhibition of p53 function (Burns *et al.*, 1999 and Haupt *et al.*, 2003). Tumours with mutated p53 can be more anaplastic, metastatic and aggressive, and have a higher rate of proliferation than those with wild-type p53 (Brown *et al.*, 1999). They can survive from those stimuli that normally activate wild-type p53 to promote apoptosis.

The expression of caspase-3, the central protein in the execution of apoptosis, has been found altered in most breast cancer cases as well. Approximately 75% of the breast cancer tissue samples lack in the caspase-3 transcript and expression, and the remaining samples show

substantial decrease of the caspase-3 expression (Devarajan *et al.*, 2002). For example, resistance to apoptotic stimuli and decreased sensitivity to such chemotherapeutic agents as cisplatin, doxorubicin and etoposide have been reported in MCF-7 human breast cancer cells that lack the expression of caspase-3 (Janicke *et al.*, 1998). However, reconstitution with caspase-3 rendered MCF-7 cells sensitive to these stimuli, suggesting that the loss of caspase-3 expression represent an important mechanism of cell survival and chemo-resistance by breast cancer cells (Poznak *et al.*, 2002 and Devarajan *et al.*, 2002).

Since genetic alteration that disables the apoptotic pathway is presented in carcinoma and produces multi-drug resistance, restoring and activating apoptosis should be a critical strategy in cancer treatments.

1.3 Breast cancer

1.3.1 Breast cancer and hormone

Breast cancer is the most common cancer in North American society and is the second cause of cancer-related death in women (Poznak *et al.*, 2002). It is characterized and affected by female sex steroid hormones, namely estrogen and progesterone. The biological activities of steroid hormones are mediated by acting with their specific receptor proteins. With hormone binding, the ligand-receptor complex acquires high affinity for specific chromosomal binding-sites and interacts to modulate transcription of specific genes to regulate the secretion of proteins with growth-factor-like activities and the specific protein biosynthesis such as increased progesterone receptor production. The proteins with growth-factor-like activities

include the epidermal growth factor (EGF), transforming growth factor-α (TGF-α) and other TGF-α-like proteins, insulin-like growth factors (IGF) and fibroblast growth factors, which can trigger cell division (Dickson *et al.*, 1986; Berkenstam *et al.*, 1989 and Leonessa *et al.*, 1992).

There are two major phenotypes of breast cancer, hormone dependent and hormone independent breast cancer. In hormone dependent breast cancer, the tumour cells express high levels of estrogen receptors (ER) and/or progesterone receptors (PR), and have a low metastatic potential. In contrast, hormone independent breast cancer lacks hormone receptors (ER- and PR-), and hormones are not necessarily required for tumour growth. They do not respond to hormonal stimulation since they frequently exhibit a high level of constitution growth factor expression which is not regulated by hormones, e.g., high levels of EGF receptors (Leonessa *et al.*, 1992). These kinds of breast cancers are usually found in late stage breast cancers because they tend to be more aggressive and invasive, with higher proliferation. They are generally more difficult to be treated due to the altered expression of growth factor receptors and elevated expression of enzymes of drug metabolism.

1.3.2 Factors influencing breast cancer occurrence

Studies suggest that there are many risk factors responsible for the increased likelihood of developing breast cancer, including diets, family inheritance, early menarche, late menopause, and hormone replacement therapy (Gerber *et al.*, 2003). For example, early menarche and late menopause would increase the exposure time of breast tissue to estrogenic stimulation, resulting in increased breast cancer occurrence, while early first full-term pregnancy and breast feeding appear to have some protective effects against breast cancer as pregnancy and prolonged

lactation involve the differentiation of breast epithelium, and reduce the term and level of estrogenic stimulation (Leonessa *et al.*, 1992).

Obesity in women is believed to be a risk factor for breast cancer as obesity is related to estrogen production and metabolism (Kirschner *et al.*, 1982). Obesity results in abnormal hormone production. In obese individuals, the estrogen levels are elevated as a result of the increased production of estrone and estradiol from their precursors (androstenedione to estrone and testosterone to estrodiol) by the estrogen-forming enzymes. The increased estrone and estradiol accumulate in the fat cells because adipose tissue is the major site of conversion (Kirschner *et al.*, 1982 and Siiteri, 1987). In addition, since only the free or unbound fraction of steroid hormones in plasma is biologically active, whereas some proteins which have high affinity with estrogen such as sex-hormone-binding globulin and high-density lipoprotein are depressed in obese subjects, the greater amount of free estrogen than normal in obese individuals is therefore available to target tissues from the circulation (Siiteri, 1987). Hyperestrogenism from obesity leads to excessive chronic stimulation of estrogen responsive target organs leading to hyperplasia and subsequently neoplasia (Kirschner *et al.*, 1982).

Since the environment is the foundation for all health and disease, and food is the most important part taken from the environment, extensive attention must be paid to diet and lifestyle. Studies show that the type and the amount of dietary fats consumed are implicated in the breast cancer aetiology. It has been reported that the population suffering from cancers is very low in Greenland Eskimo populations whose traditional diet contains relatively substantial amounts of unsaturated fatty acids and low fat compared to reference populations in the West (Byers, 1996).

Japanese consume diets high in long chain ω-3 PUFAs as well; however, Japanese migrants to the U.S. who adopt an American diet typically experience rapid increases in breast cancer incidence compared to their counterparts in Japan (Gerber *et al.*, 2003). This indicates that diet and lifestyle dominate over genetic predisposition towards breast cancer occurrence. Epidemiological and animal studies suggest that the present western dietary supply of fatty acids is greater than a 10:1 ratio of ω-6 to ω-3 PUFAs instead of 1:1 prior to industrialised society, causing a deficiency of ω-3 PUFAs, a high proportion of calories and high fat consumption all of which are associated with an increased incidence and growth of breast cancer (Horrocks *et al.*, 1999, Simopoulos, 2002 and Gerber *et al.*, 2003). Therefore, nutrition and diet are important factors impacting breast cancer occurrence.

1.3.3 Treatments of breast cancer

As for treatments for breast cancer, the first choice is surgery. If the breast cancer has not undergone metastasis, the surgical approach is very successful (Buzdar, 2001). In addition to surgery, endocrine therapy, chemotherapy and radiotherapy are other treatment approaches. Whether a tumour is hormone receptor-positive or negative is served as a useful predictive marker and treatment target in the clinic. Hormone receptor-positive tumours such as ER+ and/or PR+ are often treated with endocrine therapies. For example, tamoxifen, an estrogen antagonist, has been used as the conventional first-line endocrine therapy for breast cancers in women with ER+ diseases. It competes with estrogen for ERs, thereby blocking them and interrupting estrogenic action on tumours (Mamounas *et al.*, 2001). However, tumours that initially respond to one type of endocrine therapy often become refractory to that therapy. Thus,

a sequential endocrine therapy with second-, third- and fourth-line endocrine therapies using different types of estrogen antagonist or androgen is offered as resistance develops and disease progresses. If all endocrine options have been exhausted, or patients present receptor-negative diseases, radio- or chemotherapy will be considered.

Today, breast cancer is usually considered as a systemic disease from its earliest stages because micrometastases may be present at the time of diagnosis due to the rich breast parenchyma, mammary ducts and breast lymphatics. Hence, systemic hormonal therapy and chemotherapy as adjuvant treatments have been used and have achieved significant therapeutic goals to increase survival (Buzdar, 2001).

1.4 Paclitaxel

1.4.1 Mechanisms of anti-tumour activity of paclitaxel

Paclitaxel (Taxol) (Figure 1.7) is one of the most active first-line broad-spectrum anti-cancer drugs, and is particularly effective against primary epithelial breast cancer, ovarian carcinoma, colon, head and neck, and non-small cell lung cancer. It was isolated from the bark of Pacific Yew (*Taxus brevifolia*) in the early 1960s (Singla *et al.*, 2002). Paclitaxel inhibits cell proliferation by promoting the polymerization of tubulin and inducing a sustained mitotic block in the late G2 or M phases of the cell cycle. The tubulin/microtubule system plays a significant role in mitosis, intracellular transport, cell motility and maintenance of cell shape. The spindle microtubule formed in the presence of paclitaxel is extraordinarily stable and dysfunctional, thereby preventing chromosome segregation and subsequent cellular division, causing cell

death by disrupting the normal tubule dynamics required for cell division and vital interphase process (Rowinsky *et al.*, 1997 and Singla *et al.*, 2002).

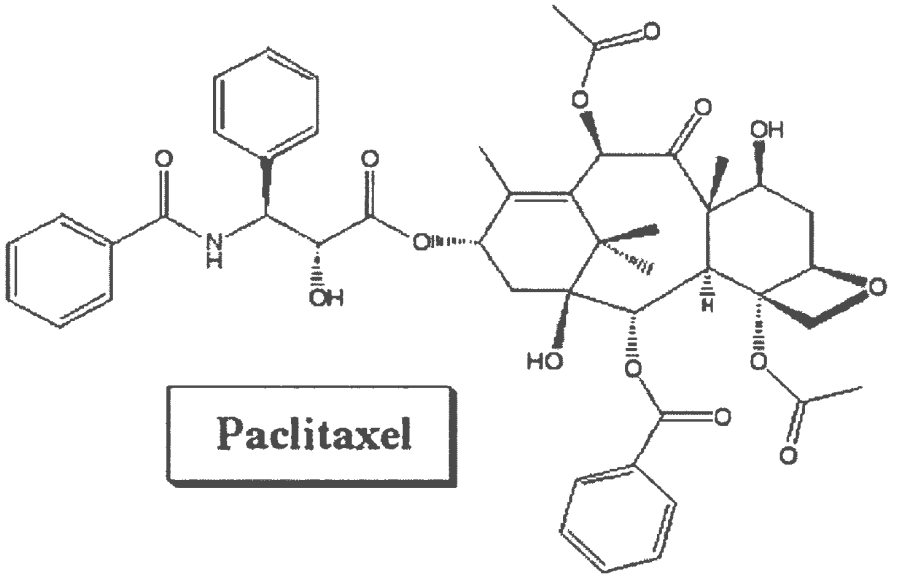


Figure 1.7 Structure of paclitaxel (Adapted from Singla *et al.*, 2002)

In addition to the disruptive action of cell mitosis by paclitaxel, the induction of apoptosis is another mechanism for the antineoplastic effects of paclitaxel. Several studies have reported that paclitaxel induces apoptosis in certain cancer cell types as breast cancer cells, leukemia cells and lung cancer cells by causing the production of cytokines and interleukin-1, Bcl-2 phophorylation, disruption of the balance between the dimerization of Bcl-2 and Bax protein, and the release of cytochrome c from mitochondria followed by activation of a caspase-9 cascade (Huang *et al.*, 1997; Torres, *et al.*, 1998; Yeung *et al.*, 1999 and Blajeski *et al.*, 1999). The mode of action of paclitaxel is multistep, concentration- and cell type-dependent. In breast cancer cell lines, the paclitaxel concentrations that induced apoptosis range from 5 – 1000 nM

(Rowinsky 1997, Yeung *et al.*, 1999 and Charles *et al.*, 2001), which are remarkably lower than the concentrations that cause non-specific necrotic cell death *in vitro* and also significantly below the attainable effective serum levels in patients (Saunders, 1997). It has been reported that when breast cancer cell lines, including hormone dependent MCF-7 cells and hormone independent MDA-MB-231 and MDA-MB-468 cells, were treated with paclitaxel at $\geq$ 10 nM for 3−24 hrs, cell growth inhibition, high molecular weight and oligonucleosomal DNA fragmentation and apoptosis-associated morphological changes were observed (McCloskey *et al.*,1996; Saunders *et al.*, 1996; Huang *et al.*, 1997 and Rowinsky *et al.*, 1998). Various apoptotic machineries have been reported to be involved in response to paclitaxel in breast cancers, including p53 pathway, phosphorylation of Bcl-2, mitogen-activated protein kinases cascade, ceramide generation and mediation of some gene expression (Young *et al.*, 1999; Bacus *et al.*, 2001; Charles *et al.*, 2001 and Blajeski *et al.*, 2001).

1.4.2 Formulations of paclitaxel

Given the fact that paclitaxel undergoes extensive metabolism in the liver following oral administration, it is best administrated intravenously. For metastatic breast cancer, paclitaxel is usually given via infusion at 135-175 mg/m^2 every one to three weeks (Poznak *et al.*, 2002 and Singla *et al.*, 2002). Although paclitaxel is a promising anti-tumour agent, its poor water solubility is its greatest disadvantage. The current formulation for infusion employs a non-aqueous vehicle composed of Cremophor EL, a non-ionic surfactant, and ethanol at 1:1 ratio (v/v) which is diluted in normal saline or 5% dextrose solution prior to administration (Singla *et al.*, 2002). However, problems employing this vehicle have been reported. Firstly,

Cremophor EL causes histamine release and thereby leads to allergic reactions in patients. Fatal hypersensitivity reactions are the most prevalent. Therefore, histamine antagonists and immunosuppressant have to be given to patients prior to the paclitaxel treatment. Secondly, Cremophor EL is also found to be physically incompatible with the intravenous infusion sets (Singla *et al.*, 2002). It can result in the leaching of diethylhexylphtalate from PVC infusion sets, causing pain to patients. In practice, plasticizer-free containers or bags have to be used. Thirdly, paclitaxel can slowly precipitate out of the diluted solution during infusion. Hence, an in-line filter is recommended to remove the particles during intravenous administration, which causes inconvenience to medical staff. Moreover, the high toxicity of paclitaxel which includes neurotoxicity, myalgias/ arthralgias, bone marrow suppression (principally neutropenia), myocardial ischemia and atrial arrhythmias, complete alopecia, and hypersensitivity reactions also raises much clinical concern (Poznak *et al.*, 2002 and Markman, 2003).

Due to these factors there is a need to develop novel formulations of paclitaxel with improved aqueous solubility and at the same time decreased side effects. Various strategies have been investigated including the preparation of emulsions, liposomes, microspheres, nanocapsules and soluble semi-synthetic paclitaxel derivatives to avoid the use of Cremophor EL (Singla *et al.*, 2002). However, no apparent success has been achieved.

Chapter 2 Objective

ω-3 PUFAs have been reported to be beneficial in treating cancers. The objective of this study was to investigate the effects of seal oil rich in ω-3 PUFAs on the cytotoxicity and apoptosis caused by paclitaxel in breast cancer cell lines.

Breast cancers with different phenotypes, MCF-7, a hormone dependent cell line, and MDA-MB-231, a hormone independent cell line, were chosen as *in vitro* models. In the first step, cells were treated with varying concentrations of seal oil for different time intervals and the cytotoxicity was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Since MTT assay showed that seal oil was toxic to both cell lines and induced cell death, optional conditions for apoptosis induction by seal oil were then determined. Following incubation of cells with seal oil, paclitaxel alone and paclitaxel in combination with seal oil, respectively, cell viability was examined by MTT assay and apoptotic characteristics presented at different stages were investigated using various assays including Hoechst staining, TUNEL assay and Annexin-V-FLUOS staining. The expression of two apoptotic marker proteins, p53 and Bcl-2, was measured by Western blotting assay. To further illustrate the mechanisms of cytotoxicity and apoptosis induced by seal oil, the production of intracellular lipid peroxide products was examined, as the highly unsaturated PUFAs derived from seal oil were considered to be susceptible to oxidative stress. Gas chromatography was used to reveal changes of lipid composition in cells following seal oil treatments, to determine if there was a relationship between the changes with the lipid peroxide production.

In comparison to seal oil, Intralipid®, which is a soybean oil emulsion containing ω-6 PUFAs, was also studied for its cytotoxicity and apoptosis induction.

Chapter 3 Materials and Methods

3.1 Materials

Human breast cancer cell lines, MCF-7 and MDA-MB-231, were kindly provided by Dr. Alan Pater, Division of Basic Medical Sciences, Faculty of Medicine, Memorial University of Newfoundland, St. John's, NL, Canada.

Dulbecco's modified Eagle's medium (DMEM) and 1X trypsin-ethylenediamine tetraacetic acid (trypsin-EDTA) were purchased from Sigma-Aldrich Canada Ltd. Fetal bovine serum (FBS HyClone®) and penicillin-streptomycin (Cellgro®) were provided by HyClone Laboratories, Inc.

Paclitaxel was purchased from Shanghai Fudan Taxusal New Technology Corp., Shanghai, China. Harp seal oil was obtained from Caboto Seafoods Ltd., St.John's, NL, Canada and 20% Intralipid® was purchased from Baxter Corporation Toronto, Canada.

VWR International supplied 25 cm^2 and 75 cm^2 cell culture flasks, 6- and 96-well cell culture plates and 8-well Lab-Tek chamber sliders.

Hoechst 33342, MTT, tris-base, igepal CA-630, sodium azide, sodium dodecyl sulphate (SDS), deoxycholic acid sodium salt, phenylmethylsulfonyl fluoride (PMSF), aprotinin, acrylamide mix (29% of acrylamide and 1% N, N'- methylenebisacrylamide), ammonium persulphate, N, N, N', N'- tetramethylethylenediamine (TEMED), vitamin E, lecithin and hydroquinone were purchased from Sigma-Aldrich Ltd. Canada. Sigma was also the provider of optima grade dimethyl sulfoxide (DMSO), acetone, methanol, chloroform, hexane and carbon bisulfide (CS_2). Hydrochloric acid, potassium chloride, sodium chloride, sodium phosphate,

potassium phosphate, glycine and Tween-20 were provided by Fisher Scientific Canada.

PUFA-2 and PUFA-3 standards from Supelco, U.S.A. were kindly offered by Dr. Gene Herzberg, Department of Biochemistry, Faculty of Science, Memorial University of Newfoundland, St. John's, NL, Canada.

Mounting medium for fluorescence (VectaShield®) was purchased from Vector Laboratories, Inc. and Roche Diagnostics provided *In Situ* Cell Death Detection Kit and Annexin-V-Fluorescein Staining Kit.

Novocastra Laboratory was the provider of mouse anti-human monoclonal antibody (mAb) for p53 protein (specific for human p53 wild-type and mutant forms). Santa Cruz Biotechnology provided the mouse anti-human mAb for Bcl-2 protein. The mouse anti-human mAb for β-actin protein was obtained from Sigma. The secondary antibody, anti-mouse Ig horseradish peroxidase linked whole antibody (from sheep) and chemiluminescence detection solutions were purchased from Amersham Biosciences UK Ltd. Electrophoresis apparatus and electrophoretic transfer apparatus for Western blotting assay were purchased from Bio-Rad Laboratories, Inc.

Delsa-44 particle size analyzer was purchased from Beckman Coulter (Miami, FL, USA). Centra-CL 3-series ventilated centrifuge was purchased from Thermo IEC U.S.A. Microplate Reader (Bio-Rad Model 550) equipped with Manager 4.0 Bio-Rad software, bovine serum albumin (BSA) and DC protein assay kit were obtained from Bio-Rad Laboratories, Inc. and were used to analyze protein concentration.

Olympus BX50 40x0.65 fluorescence microscope was used for morphological assessment. Thiobarbituric acid reactive substances (TBARS) assay kit was purchased from ZeptoMetrix Corp.

and Beckman DU-70 spectrophotometer was used for the analysis of lipid peroxide products. Hewlett Packard 5890 Series II Gas Chromatograph was employed for the analysis of lipid composition.

3.2 Methods

3.2.1 Cell culture

MCF-7 and MDA-MB-231 cells were maintained as monolayers in 75 cm^2 flasks in DMEM containing 10% FBS and 1% penicillin-streptomycin, and routinely cultured at 37 ^{o}C in a humidified incubator with an atmosphere of 5% CO_2.

Once cells grew to 80-90% confluence, they were subcultured. In order to subculture, the medium was removed and the monolayer was washed with approximately 10 mL of phosphate buffered saline (PBS, pH 7.4, 2.67 mM KCl, 1.47 mM KH_2PO_4, 138 mM NaCl and 8.1 mM $Na_2HPO_4{\cdot}7H_2O$). The PBS was then removed and 3 mL of 1X trypsin-EDTA solution was added to each flask. The flasks were then placed back in the incubator for approximately 5 min. Once the majority of cells had detached from the flasks, approximately 7 mL of PBS was added to each flask and the cell suspension was transferred to a sterile 15 mL conical centrifuge tube. The cells were then precipitated by centrifuging at 3000 RPM for 5 min using a Thermo IEC tabletop centrifuge. Following centrifugation, the supernatant was carefully removed with disposable pipettes and the cell pellets were then re-suspended with DMEM and reseeded at 1:3 or 1:4 in each flask. Cells in the logarithmic phase of growth were used for all experiments.

3.2.2 Preparation of paclitaxel solutions, seal oil emulsions and soybean oil emulsions

One hundred mM and 100 μM of paclitaxel solutions were prepared in DMSO and sterilized using a 0.2 μm Corning® syringe filter. In order to determine the IC_{50} value of paclitaxel, 100 mM of sterilized paclitaxel in DMSO was diluted with DMEM to 400 μM, 100 μM, 25 μM, 6.25 μM, 1.56 μM and 0.39 μM by serial dilution. The final DMSO concentration in all preparations was less than 0.5% (v/v), which was previously determined to be non-toxic to cells (Butt, 2004). To induce apoptosis, 100 nM paclitaxel solution was prepared by diluting the 100 μM paclitaxel with DMEM prior to experiments.

The 10% (w/v) seal oil emulsion containing 0.02% vitamin E, 1.2% lecithin and 2.5% glycerol was prepared using high pressure lipid extrusion method. The mean particle size of seal oil emulsion was determined by a Beckman Coulter Delsa-44 particle size analyzer (Miami, FL, USA) and was found to be in the range of 300±30 nm in diameter.

Prior to each experiment, the 10% seal oil emulsion prepared above and 20% soybean oil derived from Intralipid® were diluted with DMEM to 0.004%, 0.008%, 0.016% and 0.032%

3.2.3 Assessment of cytotoxicity by MTT assay

The MTT assay was used for the examination of cytotoxicity. The method is based on the conversion of the yellow tetrazolium salt, MTT, to a coloured formazan product by mitochondrial dehydrogenase enzymes present only in the living and metabolically active cells (Sladowski *et al.*, 1993). The absorbance read through a spectrometer reflects the number of living cells.

3.2.3.1 Cytotoxicity of seal oil in MCF-7 and MDA-MB-231 cells

Cells were seeded at $0.5x10^5$/100 μL/well in 96-well tissue culture plates and were incubated

at 37 °C in a 5% CO_2 humidified incubator for 24 h. The medium was then removed and cells were treated with medium containing 0.004%, 0.008%, 0.016% and 0.032% seal oil for 24, 48 and 72 h, respectively. In parallel, cells treated with fresh medium under the same conditions were used as control. After the desired incubation period, medium was removed and cells were washed twice with PBS. Cells were then incubated with 100 µL of MTT in PBS (0.5 mg/mL) at 37 °C for 4 h. Then, the supernatant was removed by aspiration and 100 µL of DMSO was added to each well to dissolve the formazan crystals formed. The plates were then agitated on a shaker at room temperature for 20 min to ensure that all crystals had been dissolved, and read in a Bio-Rad Model 550 microplate reader at a measurement wavelength of 570 nm with a reference wavelength of 630 nm.

Data analysis was carried out using the Bio-Rad Microplate Manager® 4.0 program. The percentage of cell survival was calculated as: (mean absorbance of the treated well/ mean absorbance of the control well) x 100%. The experiments were repeated at least three times and the results were expressed as mean ± standard deviation (SD).

3.2.3.2 Cytotoxicity of paclitaxel in the absence and presence of seal oil in MCF-7 and MDA-MB-231 cells

Cells were seeded at $0.5x10^5$/100 µL/well in 96-well plates and were incubated at 37 °C in a 5% CO_2 humidified incubator for 24 hrs. The medium was then removed and cells were treated with medium containing varying concentrations of paclitaxel (0.39 μM, 1.56 μM, 6.25 μM, 25 μM, 100 μM and 400 μM) in the absence or presence of 0.016% seal oil for 24 h, respectively. Cells treated with fresh medium without drug under the same conditions were used as control. After 24 h,

medium was removed and cells were washed twice with PBS. Cells were then incubated with 100 μL of MTT solution (0.5 mg/mL) at 37 °C for 4 h, following which the supernatant was aspirated and 100 μL of DMSO was added to each well to dissolve the formazan crystals formed. Plates were then placed on a shaker and agitated at room temperature for 20 min to ensure that all crystals had been dissolved, and then read in a plate reader at a measurement wavelength of 570 nm with a reference wavelength of 630 nm.

The percentage of cell survival was obtained as previously described. A curve of the percentage of cell viability against the logarithmic concentrations of paclitaxel was obtained using SigmaPlot® 8.0 program. The paclitaxel concentration resulting in 50% of cell survival obtained from the curve was defined as the IC_{50} of paclitaxel. The experiments were repeated at least three times and the results were expressed as mean $IC_{50} \pm SD$ μM.

3.2.3.3 Cytotoxicity of soybean oil in MCF-7 and MDA-MB-231 cells

Cells were seeded at $0.5x10^5$/100 μL/well in 96-well tissue culture plates and were incubated at 37 °C in a 5% CO_2 humidified incubator for 24 hrs. The medium was then removed and cells were treated with medium containing 0.004%, 0.008%, 0.016% and 0.032% soybean oil for 24, 48 and 72 h, respectively. Cells treated with fresh medium under the same conditions were used as control. After the desired incubation periods, cells were washed with PBS twice followed by incubation with 100 μL of MTT solution (0.5 mg/mL) for 4 h at 37 °C. Then, the supernatant was aspirated and 100 μL of DMSO was added to each well to dissolve the formazan crystals generated. The plates were then agitated on a shaker at room temperature for 20 min and read in a plate reader using a measurement wavelength of 570 nm with a reference wavelength of 630 nm.

The percentage of cell survival was defined as previously described. The experiments were repeated at least three times and the results were expressed as mean ± SD.

3.2.4 Examination of cell morphology using Hoechst staining

Apoptotic cells are characterized by the appearance of condensed nuclear chromatin and fragmented nuclei. Hoechst 33342 is a fluorescent dye used to stain DNA structures in living cells. Normal nuclei stained with the Hoechst dye are in dimly blue, whereas apoptotic nuclei are demonstrated as condensed, smaller, and very intensely bright blue (Mgbonyebi *et al.*, 1999).

In this study, cells were seeded at $1x10^5$/400 μL/well in 8-well Lab-Tek chamber slides. Following incubation overnight at 37 °C in a 5% CO_2 humidified incubator, cells were treated with medium containing 0.016% seal oil, 100 nM paclitaxel, 100 nM paclitaxel plus 0.016% seal oil for 24 h, respectively. Cells were also treated with 0.016% soybean oil for 24, 48, 72 h, respectively. Cells treated with fresh medium under the same conditions were used as control.

After incubation for desired periods, medium was removed and cells were washed with PBS twice. Then cells were fixed with ice-cold methanol at room temperature for 10 min. After being washed with PBS twice, fixed cells were stained with 100 μL of Hoechst dye in PBS (0.5 μg/mL) at room temperature for 10 min in dark. The slides were then immersed in PBS three times with 10 min each time. The fixed cells were mounted with VectaShield® mounting reagent and covered by slide cover glasses. The slides were viewed and photographed at 40X magnification using an Olympus BX 50 fluorescence microscope at an excitation wavelength of 360-730 nm and a detection wavelength of 420 nm. Experiments were repeated at least three times. A minimum of 500-1000 cells was counted in randomly selected areas and the results were expressed as the mean

percentage of apoptotic cells over the total number of cells counted ± SD of three separate experiments.

3.2.5 Determination of DNA strand breaks using terminal deoxynucleotidyltransferase-mediated bio-dUTP nick-end labelling (TUNEL) assay

Cleavage of genomic DNA during apoptosis may yield double-stranded, low molecular weight DNA fragments (mono- and oligonucleosomes) as well as single strand breaks of high molecular weight DNA. These DNA strand breaks can be identified by labelling free 3'- OH termini with labelled nucleotides by a terminal deoxynucleotidyl transfererase (TdT) which catalyzes nucleotide polymerization. The red labelled nucleotides incorporated in nucleotide polymers can then be detected by fluorescence microscopy.

In this study, cells were seeded at $1x10^5$/400 μL/well in 8-well Lab-Tek chamber slides. Following incubation overnight, cells were treated with 0.016% seal oil, 100 nM paclitaxel or 100 nM paclitaxel plus 0.016% seal oil, respectively, for 24 h. Cells treated with fresh medium under the same conditions were used as control.

After incubation for 24 h, the medium was removed. The cells were then washed with PBS twice and fixed using acetone-methanol (1:1) at -20 ^{o}C for 10 min in dark. The fixed cells were washed with PBS twice, and 75 μL of labelled solution which consisted of TdT labelled nucleotide (Enzyme solution: label solution, 1: 9, *In Situ* Cell Death Detection Kit, Roche) was then added into each well. Slides were then incubated in a 5% CO_2 humidified atmosphere at 37 ^{o}C for 1 h in dark. After rinsed with PBS twice, slides were viewed and photographed by a fluorescence microscope at an excitation wavelength of 530-550 nm and a detection wavelength of 590 nm.

Five hundred to 1000 cells were counted in randomly selected areas and the results were expressed as the mean percentage of apoptotic cells over the total number of cells counted ± SD of three separate experiments.

3.2.6 Assessment of membrane alteration using Annexin-V-Fluorescein (Annexin-V-FLUOS) staining

This analysis was performed using Annexin-V-fluorescein isothiocyanate conjugate (Annexin-V-FITC, green dye) and propidium iodide (PI, orange dye) to differentiate apoptotic cells from necrotic and intact cells.

In the early stage of apoptosis, changes occur at the cell membrane. One of the alterations is the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer. Annexin-V is a Ca^{2+} dependent phospholipid binding protein with high affinity for PS. This protein can hence be used as a probe for PS exposure upon the outer leaflet of the cell membrane when labelled with fluorescence (e.g. FITC). PI is a DNA dye, which can penetrate into late apoptotic and necrotic cells that have lost their membrane integrity. However, both Annexin-V-FITC and PI do not bind to normal cells. Therefore, simultaneous application of these two staining dyes can discriminate apoptotic cells characterized with high Annexin-V-FITC and low PI staining from necrotic cells shown with high staining of Annexin-V-FITC and PI as well as normal cells shown with low Annexin-V-FITC and PI staining.

Cells were seeded at $1x10^5$ cells/400 μL/well in 8-well Lab-Tek chamber slides. One day after seeding, cells were treated with 0.016% seal oil for 24 h, after which medium was removed, and cells were washed with PBS and fixed with ice-cold methanol at room temperature for 10 min.

Following being washed with PBS twice, fixed cells were stained with 100 μL/well Annexin-V-FLUOS labelling solution (Annexin-V-FITC: PI: HEPES buffer, 1: 1: 50, Annexin-V-FLUOS Staining Kit, Roche) and incubated at room temperature for 10 min in dark. The slides were then covered with slide cover glasses, viewed and photographed under a fluorescence microscope at an excitation wavelength of 470-490 nm and a detection wavelength of 515 nm. Photographs obtained were representative of three experiments.

3.2.7 Western blotting analysis of p53 and Bcl-2 protein expression

3.2.7.1 Extraction of protein from MCF-7 and MDA-MB-231 cells

When cells in 6-well plates grew to 80-90% confluence, they were respectively treated with 0.016% seal oil, 100 nM paclitaxel, 100 nM paclitaxel plus 0.016% seal oil, 0.016% soybean oil or 0.016% soybean oil plus 100 nM paclitaxel. After incubation for 24 h, medium was removed and cells were washed with ice-cold PBS twice. Then, 250 μL of ice-cold lysis buffer (50 mM tris-HCl pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1% igepal CA-630, 0.1% SDS, 0.5% sodium deoxycholate, 1% PMSF ethanol solution and 0.5% aprotinin solution) was added to each well. The cells were scraped off and the lysate was transferred into ice chilled 1.5 mL centrifuge tubes. After being placed on ice for 30 min, the lysate was centrifuged at 12,000 g at 4 °C for 10 min. The supernatant was then transferred into another tube and stored at -80 °C until further analysis.

3.2.7.2 Protein quantification

Prior to the determination of protein concentration in the extracts, 20 μL of reagent S (surfactant) from the DC protein assay kit was added to 1 mL of reagent A (alkaline copper tartrate solution) to eliminate the interference from the detergents in the lysis buffer. BSA stock solution

(1.44 μg/μL in pH 7.4 PBS) was diluted with lysis buffer to obtain BSA solutions at 0.09 μg/μL, 0.18 μg/μL, 0.36 μg/μL and 0.72 μg/μL.

Five μL of the above BSA solutions, lysate samples or lysis buffer (blank) were respectively added to each well of the 96-well plates, followed by adding 25 μL of reagent A and 200 μL of reagent B (folin reagent). The plates were placed at room temperature for 15 min, and then read on a Bio-Rad Model 550 microplate reader at a measurement wavelength of 630 nm. The protein concentration in the extracts was obtained from the standard curve constructed with the standard BSA solutions using the Bio-Rad Microplate Manager® 4.0 software program.

3.2.7.3 Western blotting assay

Before the protein extracts were resolved in a discontinuous polyacrylamide gel consisted of a resolving gel (lower) and a stacking gel (upper), the minigel apparatus (Mini-Protean® II apparatus Bio-Rad.) was assembled according to the instruction provided. To prepare 10% resolving gel for p53 protein assay, 3.3 mL of 30% acrylamide mix, 2.5 mL of 1.5 M Tris (pH 8.8), 0.1 mL of 10% SDS and 4.0 mL of distilled water (dH_2O) were mixed; while 15% resolving gel used for Bcl-2 protein was prepared by mixing 5.0 mL of 30% acrylamide mix, 2.5 mL of 1.5 M Tris (pH 8.8), 0.1 mL of 10% SDS and 2.3 mL of dH_2O. The mixture was degassed under vacuum for 15 min, following which 0.1 mL of 10% ammonia persulphate and 4 μL of TEMED were added, and the mixture was gently shaken. The mixture was then immediately added to the gap of the apparatus. Sufficient space was left for the stacking gel, and the acrylamide solution was covered with dH_2O.

After polymerizing for 30 min, the overlay water was poured off and the top of the gel was

washed with dH_2O. Five percent stacking gel solution with 3.4 mL of dH_2O, 0.83 mL of 30% acrylamide mix, 0.63 mL of 10% SDS, 50 μL of 10% ammonium persulfate and 5 μL of TEMED was added onto the top of the polymerized resolving gel. Immediately, a clean Teflon comb was inserted into the stacking gel solution. The gel was then placed at room temperature for 30 min for polymerization.

Protein samples (10 μg for p53 assay and 20 μg for Bcl-2 assay) were mixed at 1:1 (v/v) with 2X SDS gel-loading buffer prepared with 200mM Tris, 4% SDS, 0.2% bromophenol blue and 20% glycerol, and placed in boiling water for 3 min to denature the proteins. The samples were then loaded into stacking gel wells and the electrophoresis was conducted at 200 volts until the bromophenol blue reached the bottom of the resolving gel.

Following electrophoresis, gels were equilibrated in transfer buffer prepared with 25 mM Tris, 192 mM glycine and 20% methanol for 30 min, and the buffer was changed twice during equilibration. During the equilibration period, the nitrocellulose membranes (Hybond ™ ECL™ Amersham Biosciences) were soaked in transfer buffer for 15-30 min. Once the equilibration was completed, the transfer sandwich consisted of the gel and the membrane in the middle of fibre pads and 12 pieces of filter papers was assembled into the transfer cassette and placed in a buffer tank containing ice-cold transfer buffer.

Protein transfer was performed in the ice-cold transfer buffer at 100 volts for 2 h. To ensure transfer was successful, the membranes were stained with Ponceau S staining solution (0.5% Ponceau S and 1% glacial acetic acid) for a few seconds to obtain visible pink protein bands, and then washed off by dH_2O.

Following transfer, the membranes were washed with dH_2O twice, and immersed into 5% non-fat milk blocking buffer solution prepared with tris-base and Tween-20 (TBST, 0.1% Tween-20, 0.8% NaCl and 0.24% Tris, HCl adjusted to pH 7.6) and shaken at room temperature for 1 h to block the non-specific binding sites of the proteins.

Then, after rinsed with TBST three times for 15 min each time, membranes were incubated in diluted primary antibody in 5% non-fat milk TBST blocking buffer (1:1000 for anti-p53, 1:500 for anti-Bcl-2 antibody) with shaking at room temperature overnight.

After rinsed with TBST twice for 15 min each time, membranes were incubated in the diluted secondary antibody in 5% non-fat milk TBST blocking buffer (1:1500) for 1 h.

The membranes were then washed with TBST twice again and incubated with enhanced chemilumiscence ECL™ detection solution (Amersham Biosciences) for a few seconds. Excess detection reagent was then drained off, and the membranes were exposed to Hyperfilm™. Finally, films were developed to reveal the protein bands.

Considering that the observed changes in protein expression could be the result of different amounts of protein loaded, β-actin protein was employed as the control protein. To re-probe β-actin with anti-β-actin mAb in the same membranes, the primary signal on the membranes was striped off with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris/HCl pH 6.8) by occasional agitating at 50 °C for 10 min, and then the membranes were washed with TBST buffer twice or 10 min each time. Subsequently, the membranes were immersed into 7% non-fat milk TBST blocking buffer, and shaken at room temperature for 2 h to block the non-specific binding sites again. Finally, the membranes were incubated with diluted primary anti-β-actin mAb

(1:5000) in 5% non-fat milk TBST blocking buffer overnight and subsequently secondary antibody for 1 h. The β-actin protein was then detected as described previously.

The optical density (O.D.) of the protein bands was measured and quantified using Eagle Eye II® Still Video System (Stratagene Co.). The relative O.D. value was calculated as:

$$\frac{\text{O.D. of protein with treatment} \times \text{O.D. of β-actin without treatment (control)}}{\text{O.D. of β-actin with treatment} \times \text{O.D. of protein without treatment (control)}}$$

Protein expression levels were then expressed as the ratio of p53 or Bcl-2 of the samples over control (without drug treatments). The photographs were representative of at least four separate experiments. The data were expressed as mean ± SD.

3.2.8 Determination of lipid peroxide products by TBARS assay

As a result of oxidative stress, PUFAs produce lipid peroxide products such as aldehyde, which can react with thiobarbituric acid (TBA) to form a 2:1 pink adduct (Figure 3.1) that can be measured by a spectrophotometer. In this assay, malondialdehyde (MDA) was used as a standard to react with TBA and the amount of TBARS in samples was expressed in terms of MDA equivalents.

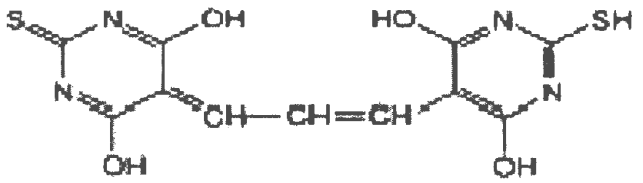


Figure 3.1 **Structure of MDA adduct with TBA** (Adapted from TBARS Assay Kit, ZeptoMetrix)

Cells were seeded in 25 cm^2 flasks. When cells grew to 80-90% confluence, they were treated with 0.016% seal oil and 100 nM paclitaxel plus 0.016% seal oil for 24 h, respectively. Cells treated with fresh medium were used as control. After incubation for 24 h, cells were dissociated and lipid binding proteins were extracted as described in Section 3.2.7.1. The amount of protein was quantified as described in Section 3.2.7.2.

MDA standards (2.5 nmol/mL, 5.0 nmol/mL, 7.5 nmol/mL and 10 nmol/mL) were prepared by diluting the MDA stock solution (100 nmol/mL) with the dilution buffer provided in the kit. One hundred μL of samples and standards were respectively added to test tubes, followed by adding 100 μL of SDS solution and 2.5 mL of TBA/Buffer reagent. The test tubes were then incubated at 95 °C for 1 h. After incubation, the temperature of samples was allowed to return to room temperature before the tubes were centrifuged at 3000 RPM for 15 min. The supernatant was collected and read by a Beckman DU-70 spectrophotometer at a wavelength of 532 nm. Results were obtained from the MDA standard curve and expressed as the amount of MDA in per mg protein. Experiments were repeated at least three times and the values were represented as the mean nmol of MDA equivalents in per mg protein ± SD.

3.2.9 Determination of lipid composition in MCF-7 and MDA-MB-231 cells by gas chromatographic (GC) assay

3.2.9.1 Extraction of lipids from MCF-7 and MDA-MB-231 cells

MCF-7 and MDA-MB-231 cells were seeded in 25 cm^2 flasks. When cells grew to 80-90% confluence, they were respectively treated with 0.016% seal oil or 100 nM paclitaxel plus 0.016% seal oil for 24 h. Cells treated with fresh medium under the same conditions were used as control. After incubation for 24 h, cells were washed with PBS twice and dissociated by trypsin-EDTA solution, followed by centrifuging at 3000 RPM for 5 min. Supernatant was then removed, and the cell pellets were suspended in 0.5 mL of methanol and sonicated for 15 min. Then, 0.5 mL of chloroform was added and the mixture was sonicated for another 15 min. After that, 0.5 mL of 0.88% KCl was added into the suspension and the suspension was placed at room temperature for 2 h to allow the organic layer to separate from the aqueous layer. The organic layer (lower part) was then transferred to clean tubes and washed with 1 mL of methanol: dH_2O (1:1). The mixture was centrifuged at 3000 RPM for 5 min and the aqueous portion was removed.

3.2.9.2 Preparation of fatty acid methyl esters

The organic layer obtained above was transferred to the transmethylation vials and was subjected to evaporation under N_2. One mL of transmethylation reagent (94% methanol and 6% HCl) and a few crystals of hydroquinone were added into the vials. The vials were then placed into protective cylinders and put in pre-heated (65 ^{o}C) oven for 2 h.

After the transmethylation was completed and the temperature was returned to room temperature, 2 mL of hexane was added, and the vials were vortex gently. The mixture was left for

a few seconds to allow separation, following which the hexane layer (upper part) was transferred to new test tubes. The remaining portion was washed with 1 mL of hexane twice and the hexane portions were combined. One mL of dH_2O was then added into the hexane portion to dissolve any water-soluble impurities. The hexane part (upper layer) was then collected in new test tubes. Again, the water part was washed with 1 mL of hexane twice and all hexane portions were combined. The collected hexane was kept at -20 °C for at least 2 h to remove frozen water, and then was subjected to evaporation under N_2. The dry lipid film was stored at -80 °C until further analysis.

3.2.9.3 GC assay

Prior to GC assay, the dry lipid film was dissolved in 200 μL of CS_2 and the solution was transferred to the GC test vials. Then, the solution was dried under N_2 and 20 μL of CS_2 was added to dissolve each sample. The test vials were sealed tightly with caps and placed into the auto-sampler of a Hewlett Packard 5890 Series II gas chromatograph equipped with Omegawax 320 fused silica capillary column (30 m x 0.32 mm ID, 0.2 μm film thickness). The oven, inlet and flame ionization detector temperature of the GC instrument were respectively set at 200 °C, 240 °C and 260 °C. Helium-carrier gas was running at a flow rate of 1 mL/min. The injection volume was 10 μL and the running time of each sample was 60 min. The split ratio was 100:1. Before running samples, fatty acid methyl ester standards PUFA-2 and PUFA-3 were tested by GC for peak identification in the chromatograms. Identification of the peaks of samples was made by the comparison of retention time with known standards composed of different fatty acids. Percentage of each fatty acid over total lipid was provided by the chromatograms. Data from at least three separate experiments was expressed as mean ± SD.

3.3 Statistic analysis

Significant differences between values obtained in a population of cells treated under different conditions were determined by the Student's *t*-test analysis with the SigmaPlot® 8.0 software program. Differences with a value of *: $P< 0.05$ were considered to be statistically significant; those with a value of **: $P< 0.01$ were considered statistically very significant.

Chapter 4 Results

4.1 Cytotoxicity of seal oil in MCF-7 and MDA-MB-231 cells

4.1.1. Cytotoxicity of seal oil in MCF-7 cells

MCF-7 cells were treated with different concentrations of seal oil ranging from 0.004% to 0.032% for 24, 48 and 72 h, respectively. Cytotoxicity was determined using MTT assay. As shown in Figure 4.1, the cytotoxicity was concentration dependent, and seal oil showed significant toxicity to MCF-7 cells at the concentrations of 0.016% and 0.032%. It was found that the viability of cells treated with 0.016% and 0.032% seal oil for 24 h was decreased to 83.9±3.7% ($P<0.01$) and 81.1±3.3% ($P<0.01$), respectively, while the cell viability was 98.4±1.2% and 97.5±2.5% when cells were exposed to 0.004% and 0.008% seal oil under the same conditions. The cell viability was found to be 96.4±3.3%, 92.7±4.9%, 87.8±4.0% ($P<0.05$) and 82.8±4.8% ($P<0.05$), respectively, when cells were exposed to 0.004%, 0.008%, 0.016% and 0.032% seal oil for 48 h. After 72 h, the cell viability was found to be 94.3±4.9% and 89.9±4.2% when cells were exposed to 0.004% and 0.008% seal oil, respectively, and 82.7±3.4% ($P<0.05$) and 72.9±4.6% ($P<0.01$) when cells were exposed to 0.016% and 0.032% seal oil, respectively. The results suggested that although the concentration of seal oil was shown to affect the cell viability, the incubation time did not appear to make any significant difference.

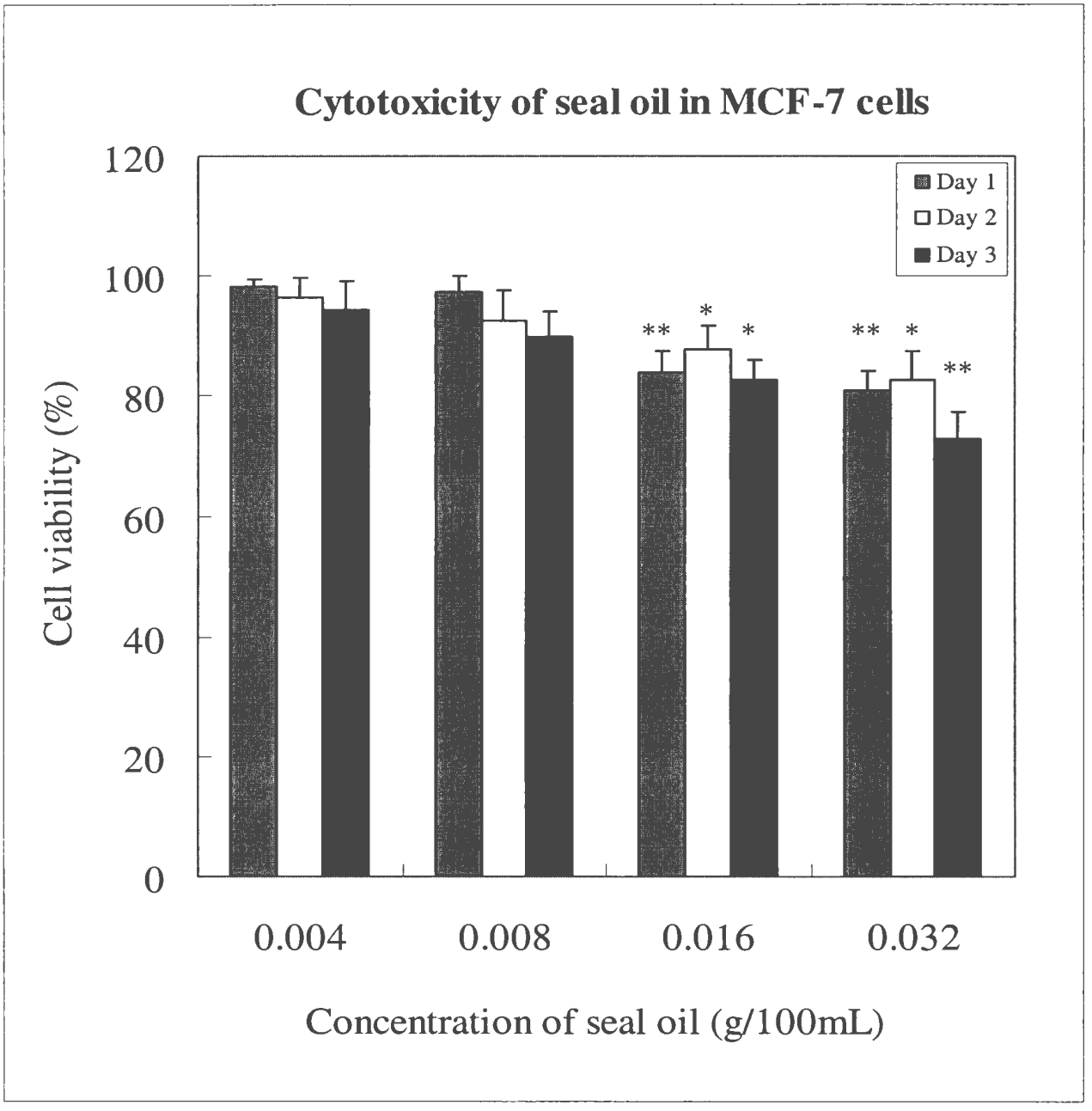


Figure 4.1 The cytotoxicity of seal oil in MCF-7 cells determined by MTT assay.

Cells were treated for 24, 48 and 72 h, respectively. **: statistical significance at $P<0.01$; *: statistical significance at $P<0.05$ compared with the results of the cells treated with 0.004% seal oil. Results are expressed as mean ± SD from three separate experiments.

4.1.2 Cytotoxicity of seal oil in MDA-MB-231 cells

MDA-MB-231 cells were also treated with different concentrations of seal oil ranging from 0.004% to 0.032% for 24, 48 and 72 h, respectively. As shown in Figure 4.2, seal oil was toxic to MDA-MB-231 cells at all concentrations and time intervals tested. Following treatment for 24 h, the viability of cells treated with 0.016% and 0.032% seal oil was decreased to 82.7±3.1% ($P<0.05$) and 78.7±3.7% ($P<0.05$) from 94.3±4.4% when treated with 0.004% and 89.9±4.0% when treated with 0.008% seal oil, respectively. In comparison with cells treated with 0.004% and 0.008% seal oil for 48 h, the cell viability was significantly decreased to 73.5±0.8% ($P<0.01$) when cells were treated with 0.016% seal oil and to 72.9±3.1% ($P<0.05$) when treated with 0.032% seal oil. When cells were treated for 72 h, 0.016% seal oil caused the cell viability to decrease to 70.1±6.1%, and 0.032% seal oil caused the viability to decrease further to 69.4±5.3%, while the viability was decreased to 72.1±4.1% and 78.4±4.6% by 0.008% and 0.004% seal oil, respectively.

In MDA-MB-231 cell line, the cytotoxicity of seal oil was found to have increased as the concentration of seal oil was increased when cells were treated for 24 and 48 h. However, when the cells were treated for 72 h, no significant difference of cytotoxicity was found as the concentration of seal oil was increased.

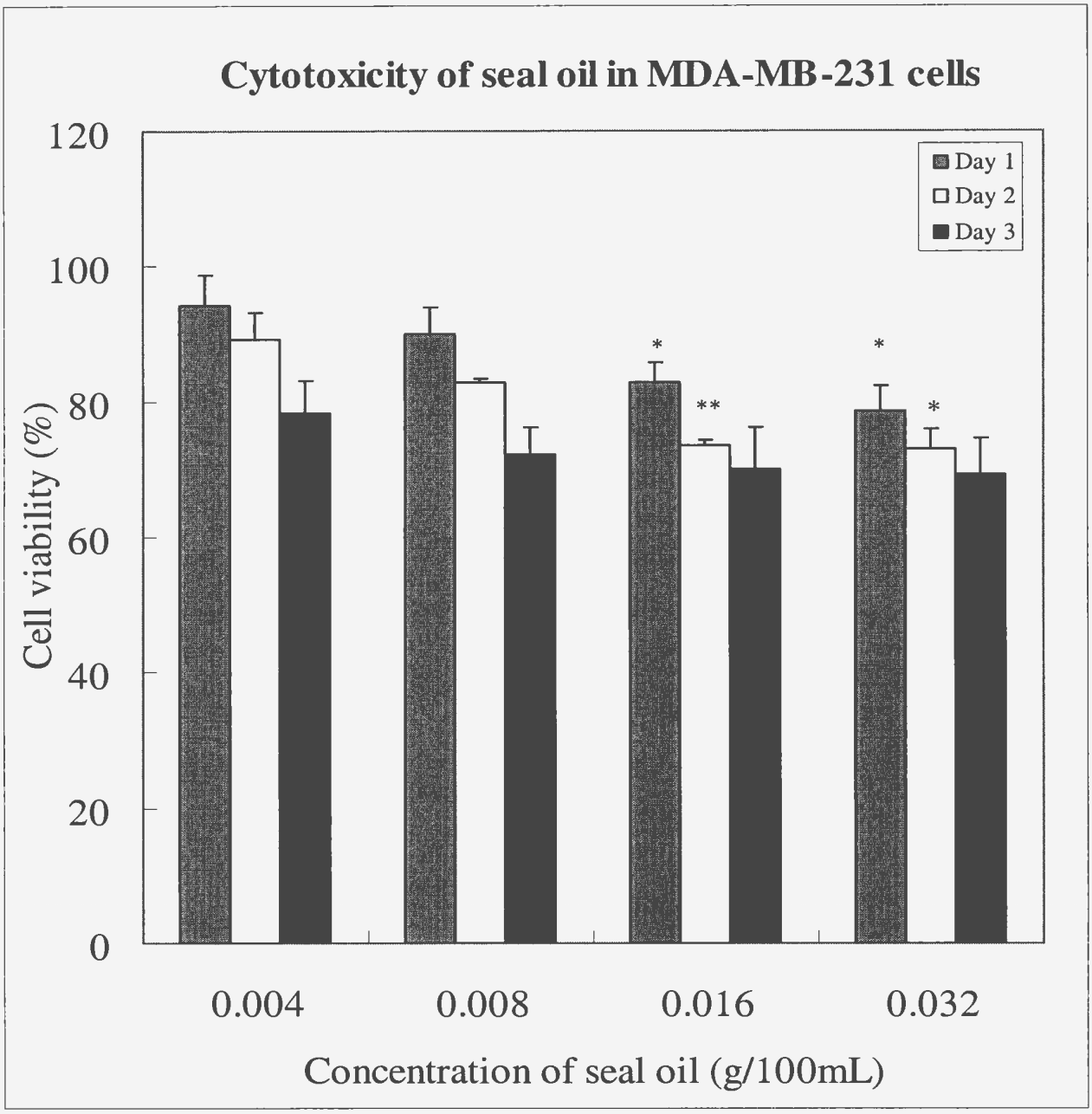


Figure 4.2 The cytotoxicity of seal oil in MDA-MB-231 cells determined by MTT assay.

Cells were treated for 24, 48 and 72 h, respectively. **: statistical significance at $P<0.01$; *: statistical significance at $P<0.05$ compared with the results of the cells treated with 0.004% seal oil. Results are expressed as mean ± SD from three separate experiments.

4.2 Cytotoxicity of paclitaxel in the absence and presence of seal oil in MCF-7 and MDA-MB-231 cells

Both MCF-7 and MDA-MB-231 cell lines were treated with varying concentrations of paclitaxel ranging from 0.39 μM to 400 μM alone and in combination with 0.016% seal oil for 24 h. In order to investigate the effect of seal oil on paclitaxel-induced cytotoxiity, MTT assay was employed and results are shown in Figures 4.3 and 4.4. It was found that the cytotoxicity induced by paclitaxel was concentration-dependent in both cell lines. The IC_{50} values of paclitaxel in MCF-7 and MDA-MB-231 cells were found to be 15.2 ±5.9 μM and 57.0 ±8.6 μM, respectively. It was found that 0.016% seal oil enhanced the cytotoxicity of paclitaxel in both cell lines and the IC_{50} values were significantly decreased to 4.6 ±2.4 μM ($P<0.05$) in MCF-7 cells and 24.1±2.8 μM ($P<0.01$) in MDA-MB-231 cells, respectively (Table 4.1). The increased cytotoxicity was found to be more remarkable at lower concentrations of paclitaxel. In addition, the lower IC_{50} value of paclitaxel in MCF-7 cells than that in MDA-MB-231 cells suggests that MCF-7 cell line is more sensitive to paclitaxel than MDA-MB-231 cell line.

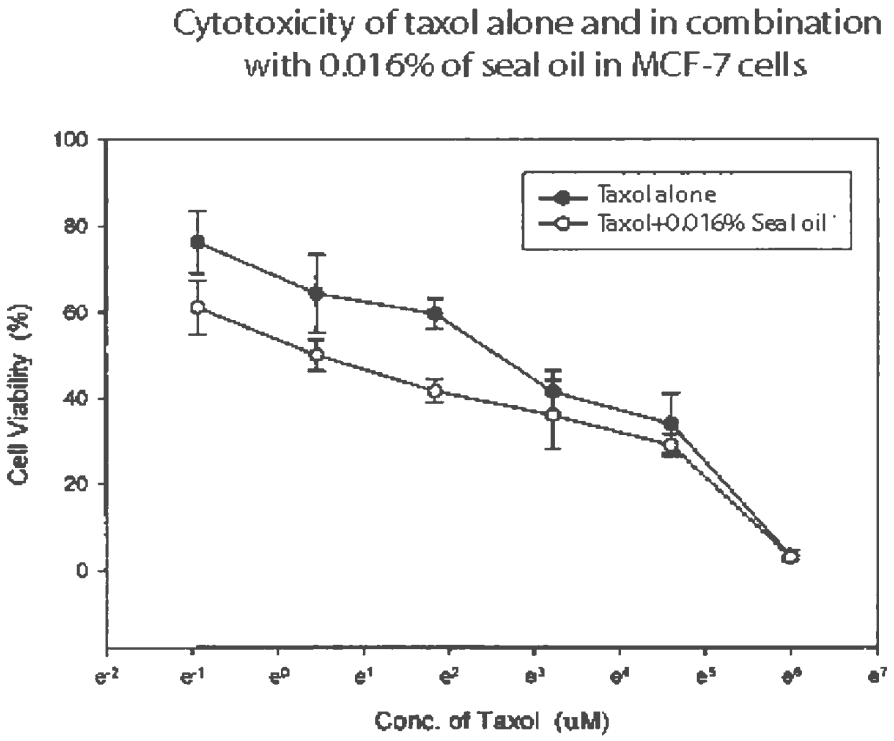


Figure 4.3 Cell viability of MCF-7 cells treated with varying concentrations of paclitaxel alone (-●-) and in combination with 0.016% seal oil (-o-) for 24 h determined by MTT assay.

Results are expressed as mean ± SD from three separate experiments.

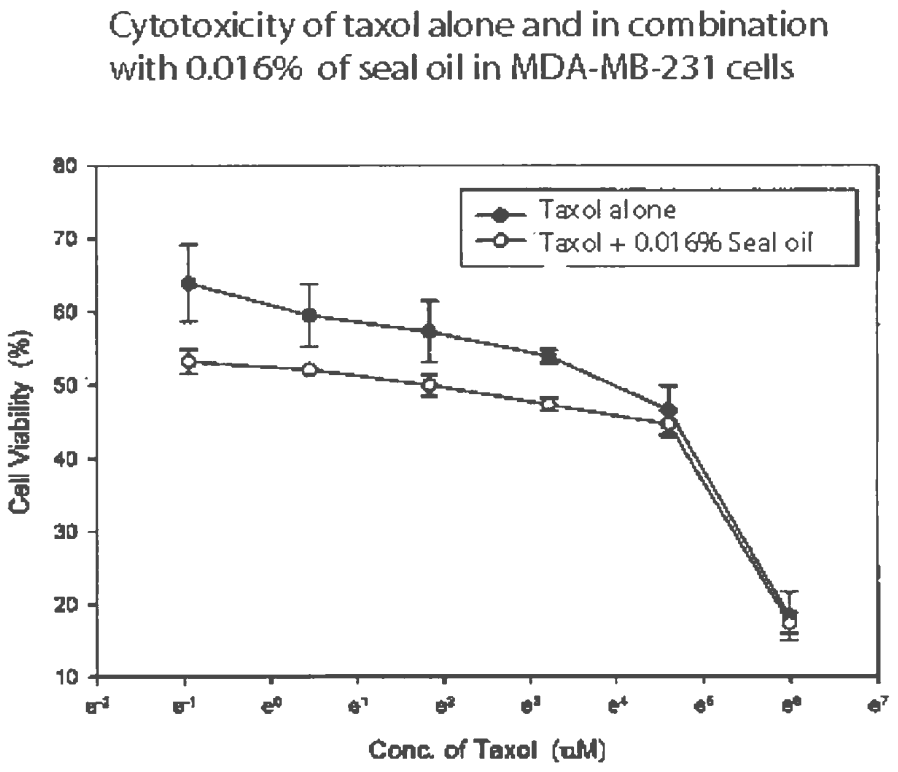


Figure 4.4 Cell viability of MDA-MB-231 cells treated with varying concentrations of paclitaxel alone (-•-) and in combination with 0.016% seal oil (-o-) for 24 h determined by MTT assay.

Results are expressed as mean ± SD from three separate experiments.

Table 4.1 IC_{50} values of paclitaxel in the absence and presence of 0.016% seal oil in MCF-7 and MDA-MB-231 cell lines.

	IC_{50} (μM)	
Cell line	Paclitaxel	Paclitaxel + 0.016% seal oil
MCF-7	15.2 ±5.9	4.6 ±2.4 *
MDA-MB-231	57.0 ±8.6	24.1 ±2.8 **

Results are expressed as mean ± SD from four separate experiments. Statistical significance was obtained by *t*-test as compared with paclitaxel alone treatment. *: statistical significance at $P<0.05$; **: statistical significance at $P<0.01$.

4.3 Examination of cell morphology using Hoechst staining

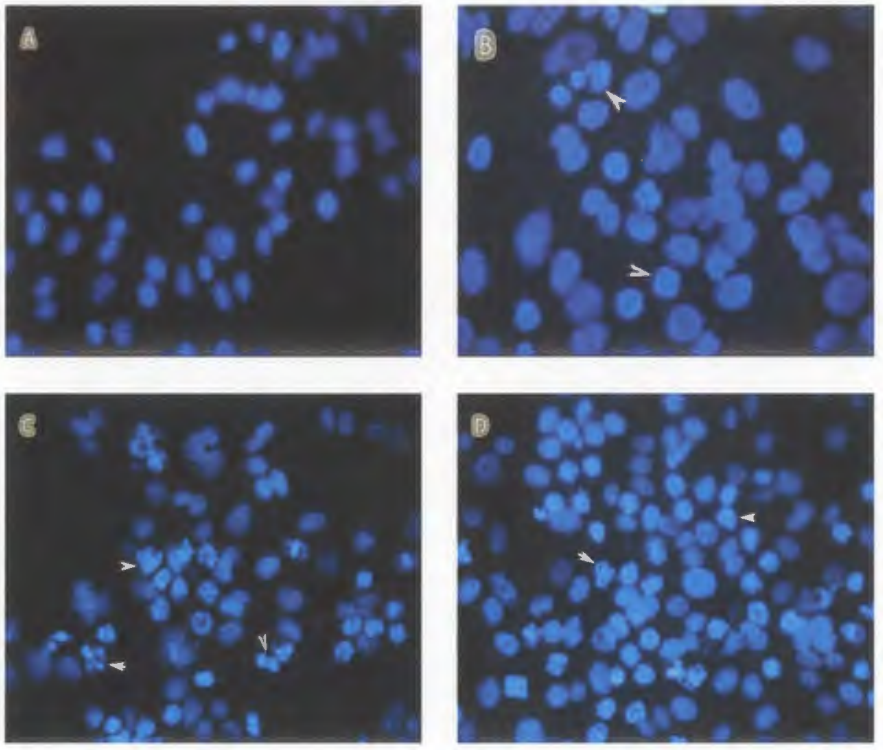
To investigate the apoptotic manifestation, cells were exposed to 0.016% seal oil, 100 nM paclitaxel alone or in combination with 0.016% seal oil for 24 h. Hoechst staining was used to assess chromatin aggregation, one of the characteristic morphological changes associated with apoptosis. The number of apoptotic cells in randomly selected areas was counted. A minimum of 500-1000 cells was examined for each experiment and the results were expressed as the mean percentage of apoptotic cells over the total number of cells counted. Results are shown in Figures 4.5 and 4.6, and Table 4.2.

Table 4.2 The percentage of apoptotic cells with morphological changes determined by Hoechst staining.

Cell line	Control	Treated with 0.016% seal oil	Treated with 100 nM paclitaxel	Treated with 100 nM paclitaxel+0.016% seal oil
MCF-7	0	6.9±0.7	12.3±1.3	17.5±1.9*
MDA-MB-231	0	6.7±1.2	8.8±1.5	13.3±2.4*

Results are expressed as mean ± SD from three separate experiments *: statistical significance at $P<0.05$ was obtained by *t*-test as compared with paclitaxel treatment.

In both cell lines, chromatin aggregation, cytoplasmic and nuclear condensation characterized as intensively bright blue colour was observed after cells were treated with paclitaxel alone and in combination with 0.016% seal oil. The treatment with paclitaxel in the presence of seal oil was found to have induced more apoptotic cells than paclitaxel alone. It was also found that seal oil alone led cells to undergo early stage of apoptosis as evidenced by condensed chromatin. These results suggested that the increased amount of apoptotic cells observed with paclitaxel in combination with seal oil was likely due to the fact that seal oil itself could induce apoptosis.



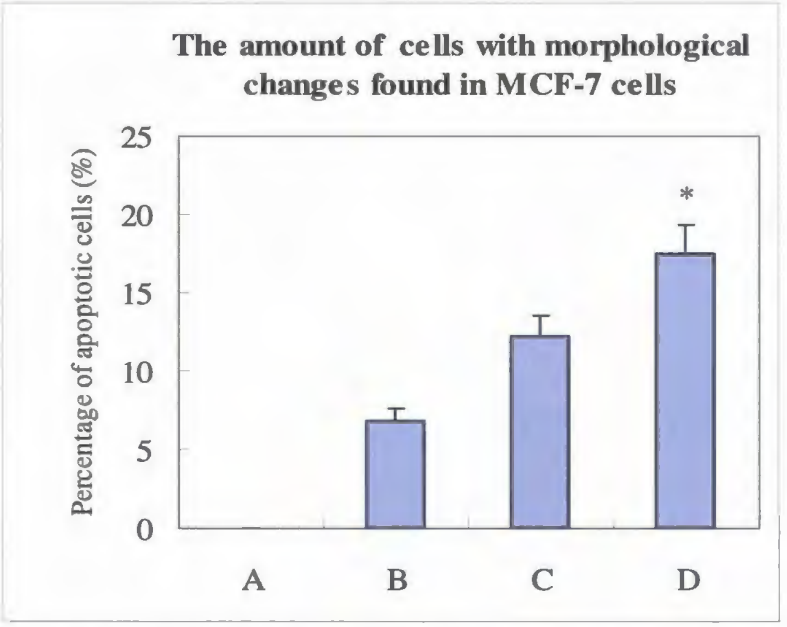
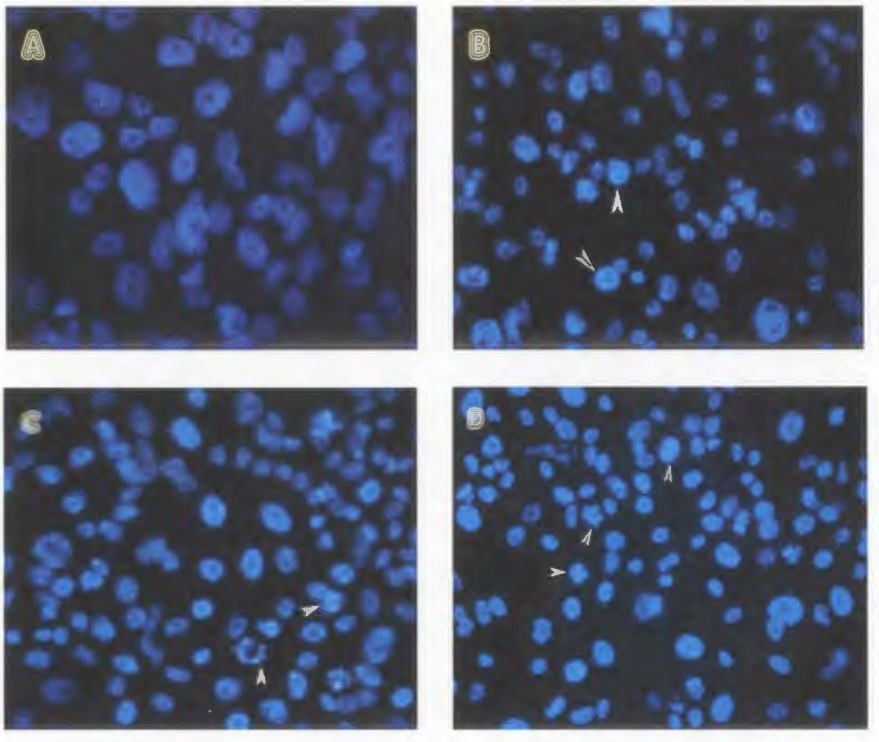


Figure 4.5 Morphological changes in MCF-7 cells determined by Hoechst staining (40X magnification). A: control (cells without treatments); B: cells treated with 0.016% of seal oil; C: cells treated with 100 nM of paclitaxel alone, and D: cells treated with 100 nM of paclitaxel in combination with 0.016% of seal oil for 24 hrs, respectively. Photographs and data are representative of three separate experiments.*: statistical significance at $P<0.05$ compared with paclitaxel alone treatment.



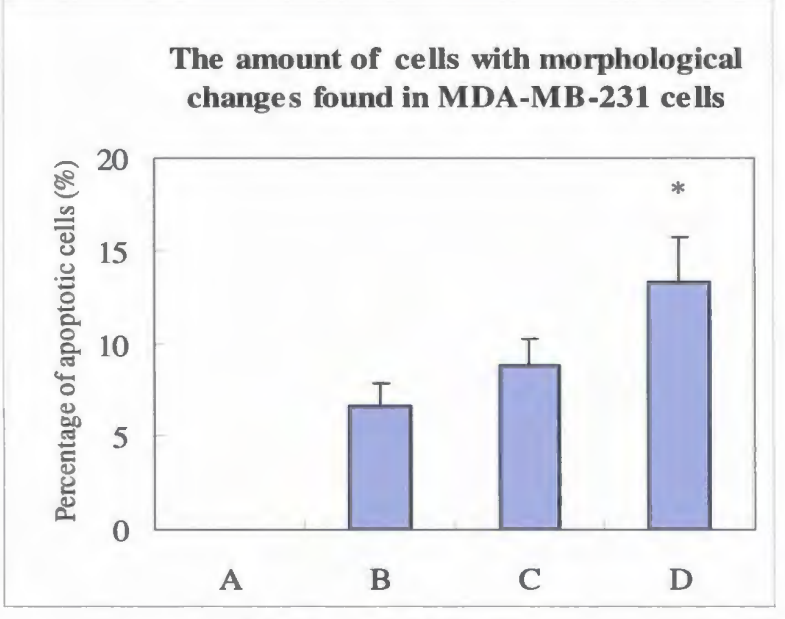


Figure 4.6 Morphological changes in MDA-MB-231 cells determined by Hoechst staining (40X magnification). A: control (cells without treatments); B: cells treated with 0.016% of seal oil; C: cells treated with 100 nM of paclitaxel alone, and D: cells treated with 100 nM of paclitaxel in combination with 0.016% of seal oil for 24 hrs, respectively. Photographs and data are representative of three separate experiments.*: statistical significance at $P<0.05$ compared with paclitaxel alone treatment.

4.4 Determination of DNA strand breaks using TUNEL assay

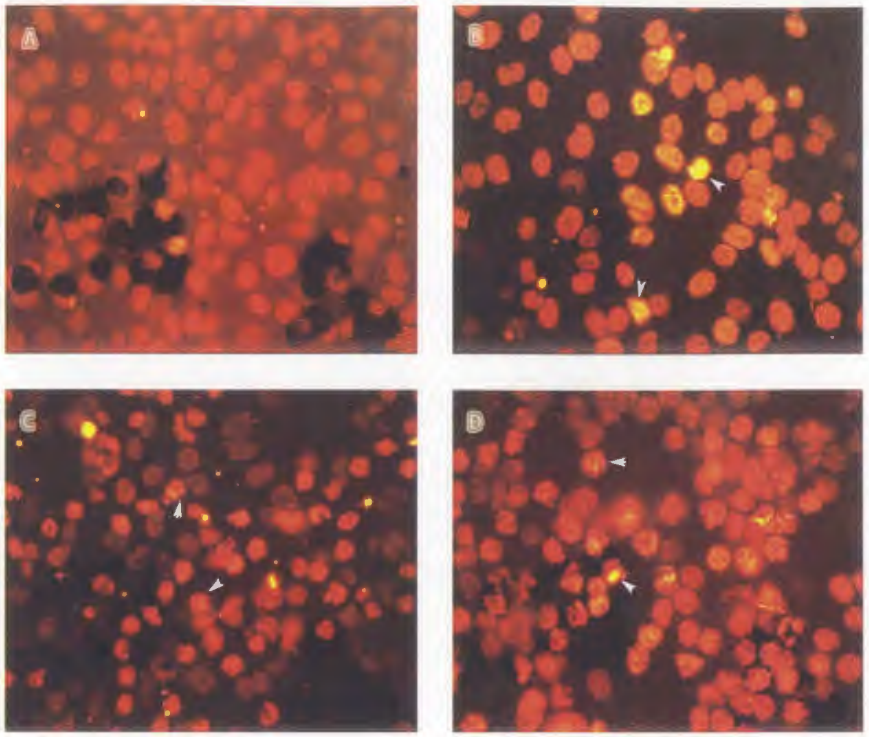
TUNEL was employed to investigate the DNA strand breaks in apoptotic cells. A minimum of 500-1000 cells in randomly selected areas was examined for each experiment and the results were expressed as the mean percentage of apoptotic cells over the total number of cells counted. Results are shown in Figures 4.7 and 4.8, and Table 4.3.

Table 4.3 The percentage of apoptotic cells with DNA strand breaks determined by TUNEL.

Cell line	Control	Treated with 0.016% seal oil	Treated with 100 nM paclitaxel	Treated with 100 nM paclitaxel+0.016% seal oil
MCF-7	0	4.0±0.5	17.3±1.4	22.1±2.3*
MDA-MB-231	0	8.4±1.6	17.7±1.5	25.8±2.6*

Results are expressed as mean ± SD from three separate experiments. *: statistical significance at $P<0.05$ was obtained by *t*-test as compared with paclitaxel alone treatment.

Figures 4.7 and 4.8 show cells with DNA fragmentation (bright orange) observed when cells were exposed to 100 nM paclitaxel alone and 100 nM paclitaxel in combination with 0.016% seal oil for 24 h. In comparison with paclitaxel alone treatment, the treatment of paclitaxel in combination with 0.016% seal oil induced more apoptotic cells as evidenced by the incidence of cells showing condensed DNA fragments. Seal oil alone also led some cells to undergo early apoptosis with aggregation of DNA, which was consistent with the results shown by Hoechst staining.



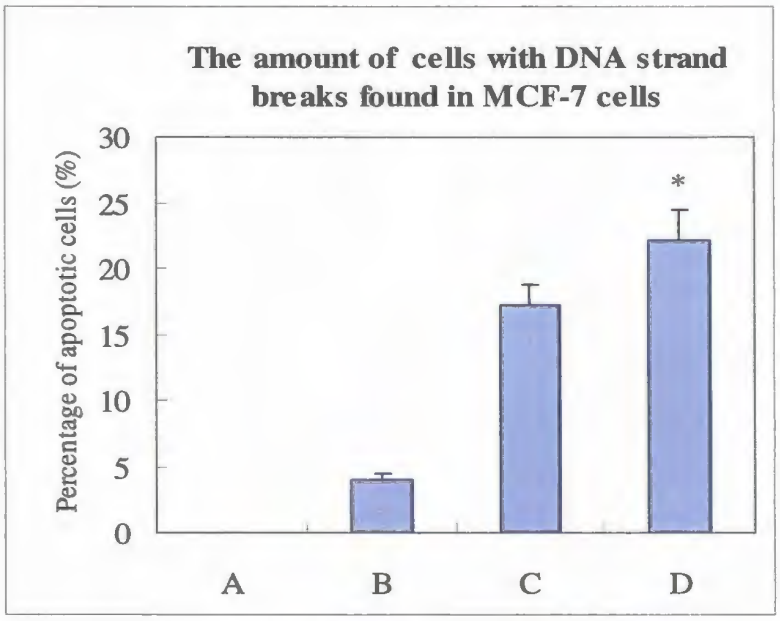
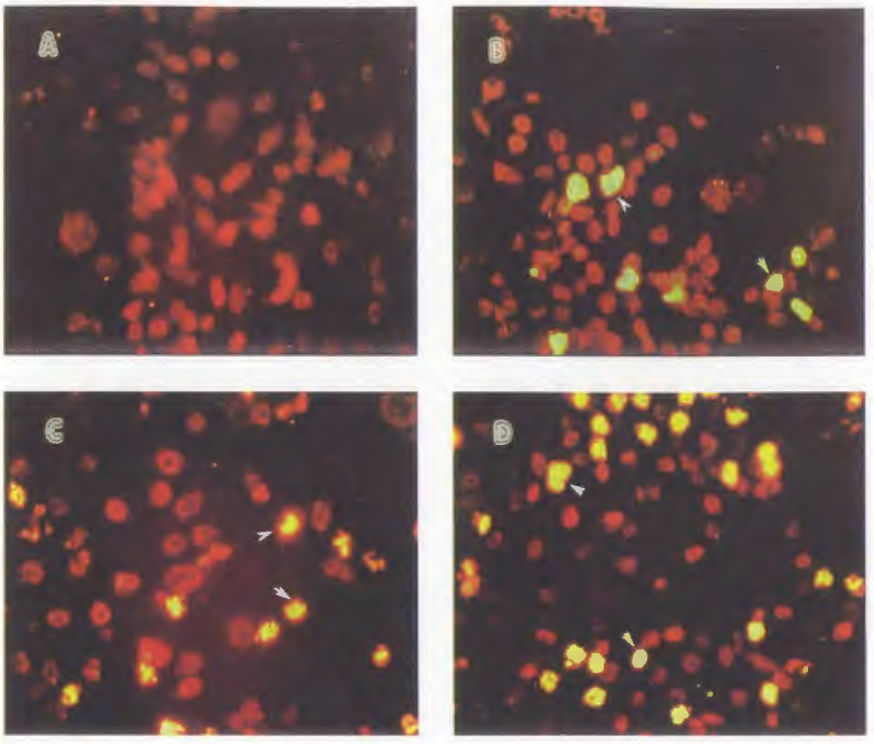


Figure 4.7 DNA fragmentations observed in MCF-7 cells determined using TUNEL procedure (40X magnification). A: control (cells without treatments); B: cells treated with 0.016% of seal oil; C: cells treated with 100 nM of paclitaxel alone, and D: cells treated with 100 nM of paclitaxel in combination with 0.016% of seal oil for 24 hrs, respectively. Photographs and data are representative of three separate experiments.*: statistical significance at $P<0.05$ compared with paclitaxel alone treatment.



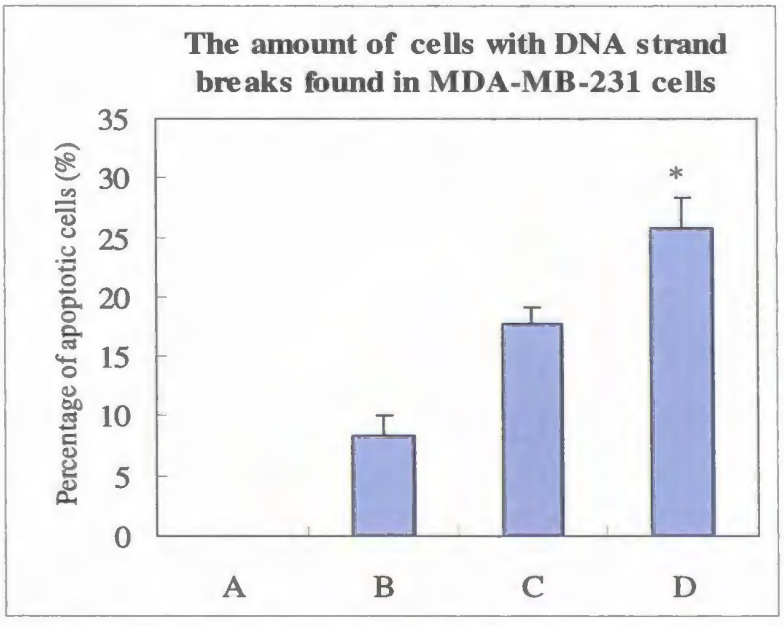


Figure 4.8 DNA fragmentations observed in MDA-MB-231 cells determined using TUNEL procedure (40X magnification). A: control (cells without treatments); B: cells treated with 0.016% of seal oil; C: cells treated with 100 nM of paclitaxel alone, and D: cells treated with 100 nM of paclitaxel in combination with 0.016% of seal oil for 24 hrs, respectively. Photographs and data are representative of three separate experiments.*: statistical significance at $P<0.05$ compared with paclitaxel alone treatment.

4.5 Assessment of membrane alteration using Annexin-V-FLUOS staining

MCF-7 and MDA-MB-231 cells treated with 0.016% seal oil for 24 h were stained with Annexin-V-FITC and PI to measure membrane changes. As shown in Figure 4.9, early stage of apoptosis with PS translocation to the outer layer of the cell membrane as evidenced by the green membrane was observed. Some cells were found to bind to both fluorescent dyes showing green membrane and orange condensed cytoplasm, which revealed that these cells were undergoing late stage of apoptosis with the loss of integrity of membrane and fragmented nuclei.

When cells were treated with 100 nM paclitaxel in the absence and presence of 0.016% seal oil, most apoptotic cells were observed showing late stage of apoptotic characteristics with fragmented nuclei and loss of integrity of membrane. No distinct difference was observed between cells treated with paclitaxel alone and paclitaxel plus seal oil by Annexin-V-FLUOS staining. Since seal oil-induced apoptosis only appeared to be in its early stage as evidenced by PS translocation in the cell membrane according to the results of Annexin-V-FLUOS staining, it indicated that the ability of seal oil to induce apoptosis was much weaker than that of paclitaxel.

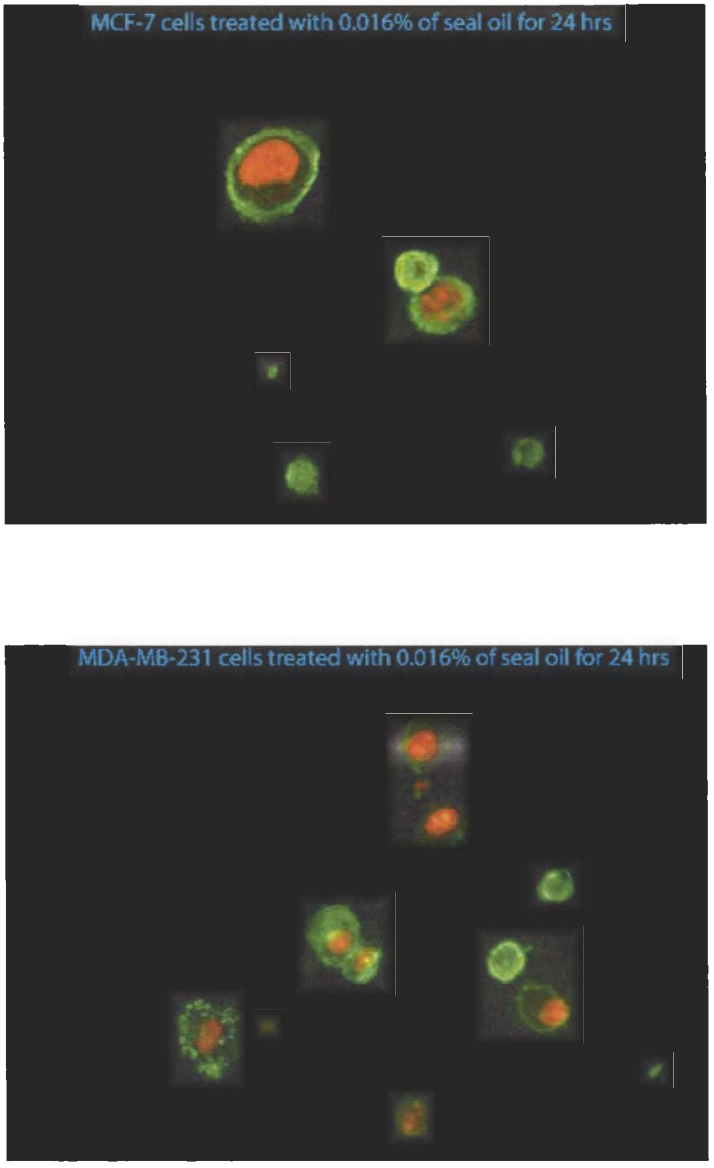


Figure 4.9 Membrane alterations of apoptotic MCF-7 and MDA-MB-231 cells determined by Annexin-V-FLUOS staining (40X magnification). Photographs are representative of three separate experiments.

4.6 Western blot analysis of p53 and Bcl-2 protein expression

4.6.1 Expression of p53 and Bcl-2 proteins in MCF-7 cells

The expression of p53 and Bcl-2 proteins was examined by Western blot assay. Figure 4.10 and Table 4.4 show that the expression of p53 protein in MCF-7 cells was increased by 29% when cells were exposed to 100 nM paclitaxel for 24 h, compared with the control ($P<0.05$). However, the expression of p53 was decreased slightly by 0.016% seal oil to 90% of the control. The inclusion of 0.016% seal oil to paclitaxel reduced the expression of p53 to 82% of the control.

As shown in Figure 4.11 and Table 4.4, the expression of Bcl-2 protein in MCF-7 cells treated with 100 nM paclitaxel or 0.016% seal oil was down-regulated by 40% and 27%, compared with the control ($P<0.05$), respectively. The expression of Bcl-2 protein was further decreased by 100 nM paclitaxel in combination with 0.016% seal oil to 35% of the control ($P<0.01$).

Table 4.4 The expression of p53 and Bcl-2 proteins in MCF-7 cells determined by Western blot and expressed as O.D. values. The values represent mean ± SD from four separate experiments. Statistical significance was obtained by *t*-test as compared with control. *: statistical significance at $P<0.05$; **: statistical significance at $P<0.01$.

O.D. values	Control	Treated with 0.016% seal oil	Treated with 100 nM paclitaxel	Treated with 100 nM paclitaxel+0.016% seal oil
p53	1	0.90±0.10	1.29±0.17*	0.82±0.09
Bcl-2	1	0.73±0.14*	0.60±0.09*	0.35±0.08**

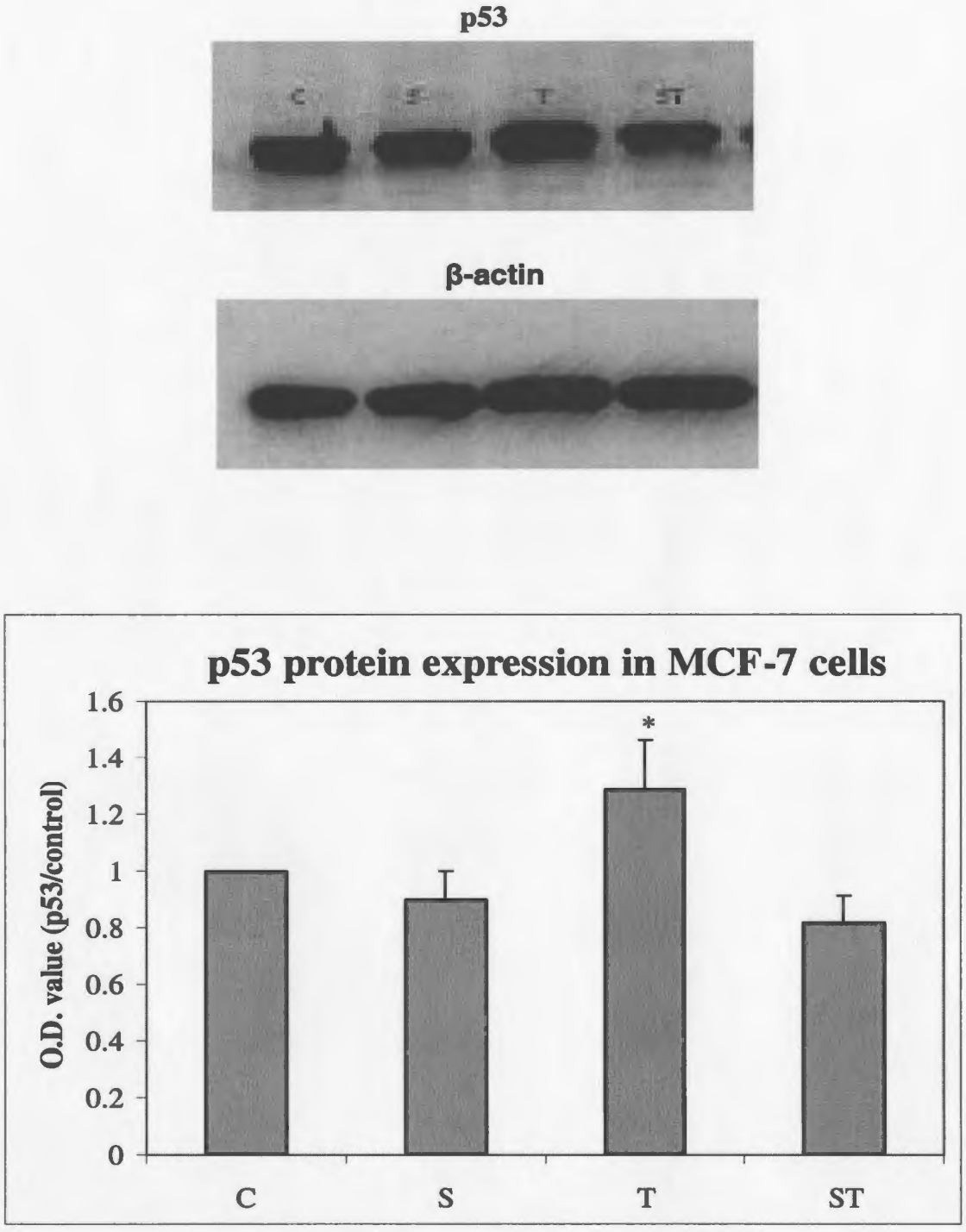
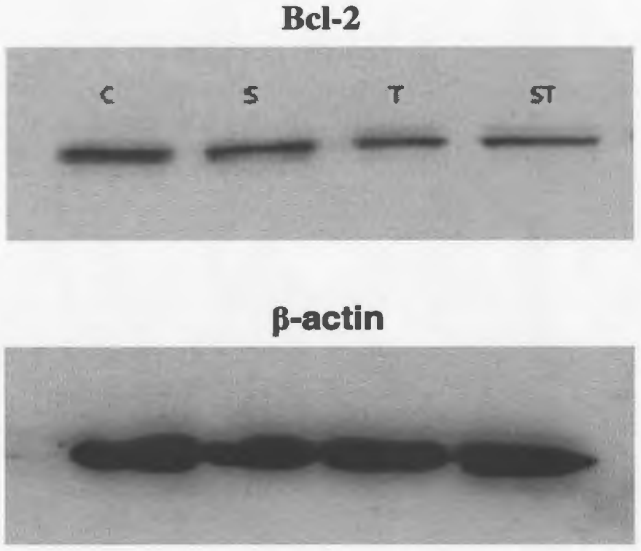


Figure 4.10 The expression of p53 protein in MCF-7 cells determined by Western blot.

C: control (cells without treatments); S: cells treated with 0.016% seal oil; T: cells treated with 100 nM paclitaxel alone, and ST: cells treated with 100 nM paclitaxel in combination with 0.016% seal oil for 24 h, respectively. *: statistical significance at $P<0.05$ compared with control. The O.D. values are expressed as mean ± SD from four separate experiments. Photographs are representative of four separate experiments.



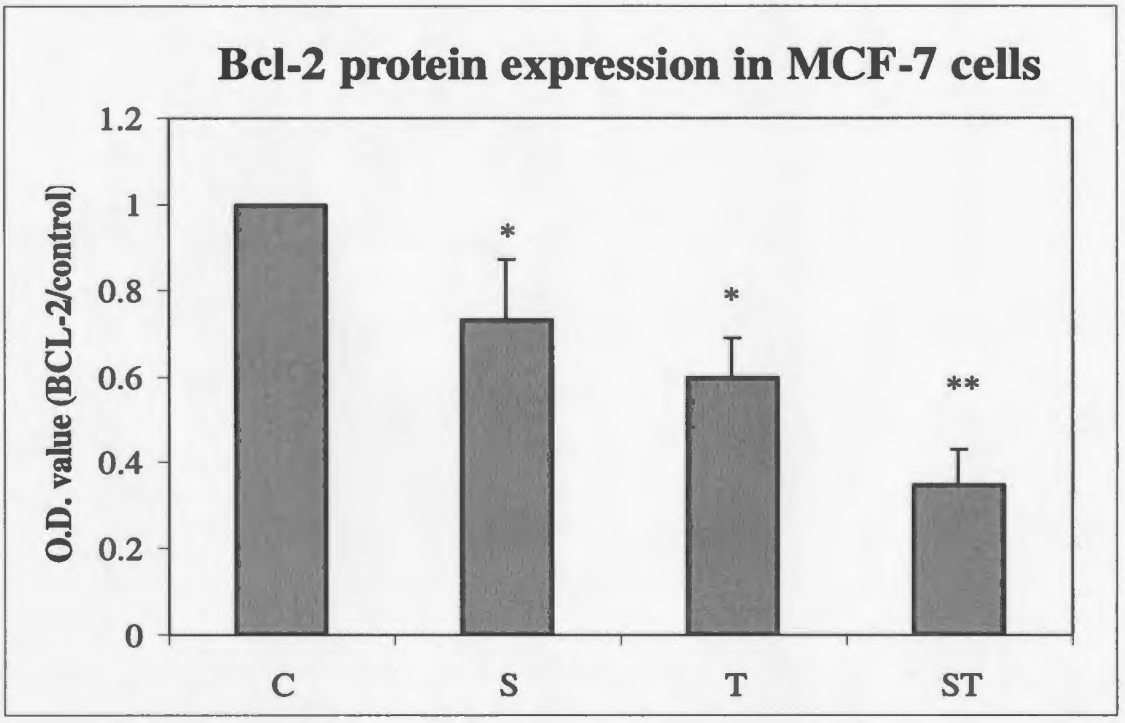


Figure 4.11 The expression of Bcl-2 protein in MCF-7 cells determined by Western blot.

C: control (cells without treatments); S: cells treated with 0.016% seal oil; T: cells treated with 100 nM paclitaxel alone, and ST: cells treated with 100 nM paclitaxel in combination with 0.016% seal oil for 24 h, respectively. *: statistical significance at $P<0.05$; **: statistical significance at $P<0.01$ compared with control. The O.D. values are expressed as mean ± SD from four separate experiments. Photographs are representative of four separate experiments.

These results suggest that the apoptosis induced by paclitaxel in MCF-7 cells was related to the up-regulation of p53 protein and down-regulation of Bcl-2 protein expression. Seal oil-induced apoptosis was likely associated with the inhibition of Bcl-2 protein expression and independent of p53 protein. The enhanced apoptosis by paclitaxel in combination with seal oil was probably related to the enhanced down-regulation of Bcl-2 protein expression.

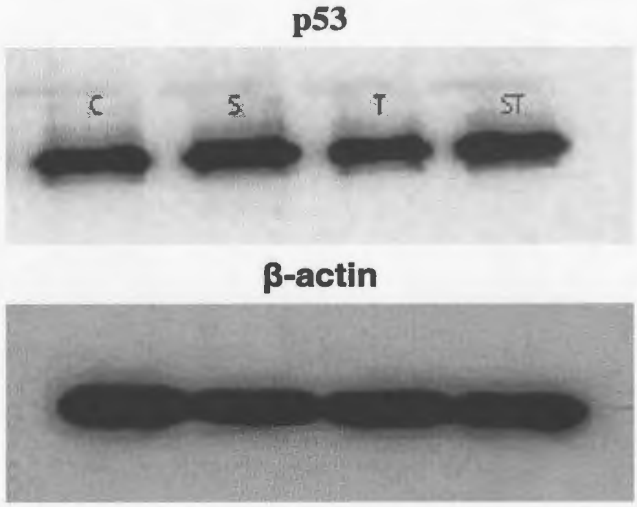
4.6.2 Expression of p53 and Bcl-2 proteins in MDA-MB-231 cells

As shown in Figures 4.12 and 4.13, and Table 4.5, both p53 and Bcl-2 proteins in MDA-MB-231 cells were found to be down-regulated by 100 nM paclitaxel, which were approximately 60% and 40% of the control, respectively ($P<0.01$). The results suggested that paclitaxel-induced apoptosis in MDA-MB-231 cells was associated with the down-regulation of Bcl-2 protein expression, rather than through the up-regulation of p53 protein expression as the p53 protein in MDA-MB-231 cells was reported to be mutant and non-functional (Toillon *et al.*, 2002).

Table 4.5 The expression of p53 and Bcl-2 proteins in MDA-MB-231 cells determined by Western blot and expressed as O.D. values. The values represent mean ± SD from four separate experiments. Statistical significance was obtained by *t*-test as compared with control. **: statistical significance at $P<0.01$.

O.D. values	Control	Treated with 0.016% seal oil	Treated with 100 nM paclitaxel	Treated with 100 nM paclitaxel+0.016% seal oil
p53	1	2.01±0.14**	0.58±0.10**	0.73±0.08**
Bcl-2	1	0.71±0.05**	0.39±0.10**	0.35±0.07**

The expression of p53 protein was found to be up-regulated by approximately 2-fold by 0.016% seal oil in comparison with the control ($P<0.01$). However, compared with the control, the expression of p53 protein was reduced by about 25% when 0.016% seal oil was combined with 100 nM paclitaxel ($P<0.01$). The expression of Bcl-2 protein was found to be inhibited by seal oil to approximately 70% of the control ($P<0.01$). The inhibition of Bcl-2 expression was enhanced further by paclitaxel in combination with seal oil and was only 35% of the control ($P<0.01$). According to these results, it can be concluded that apoptosis induced by seal oil in MDA-MB-231 cell line was likely related to the down-regulation of Bcl-2 protein expression, and seal oil enhanced paclitaxel-induced apoptosis by down-regulating Bcl-2 protein expression further.



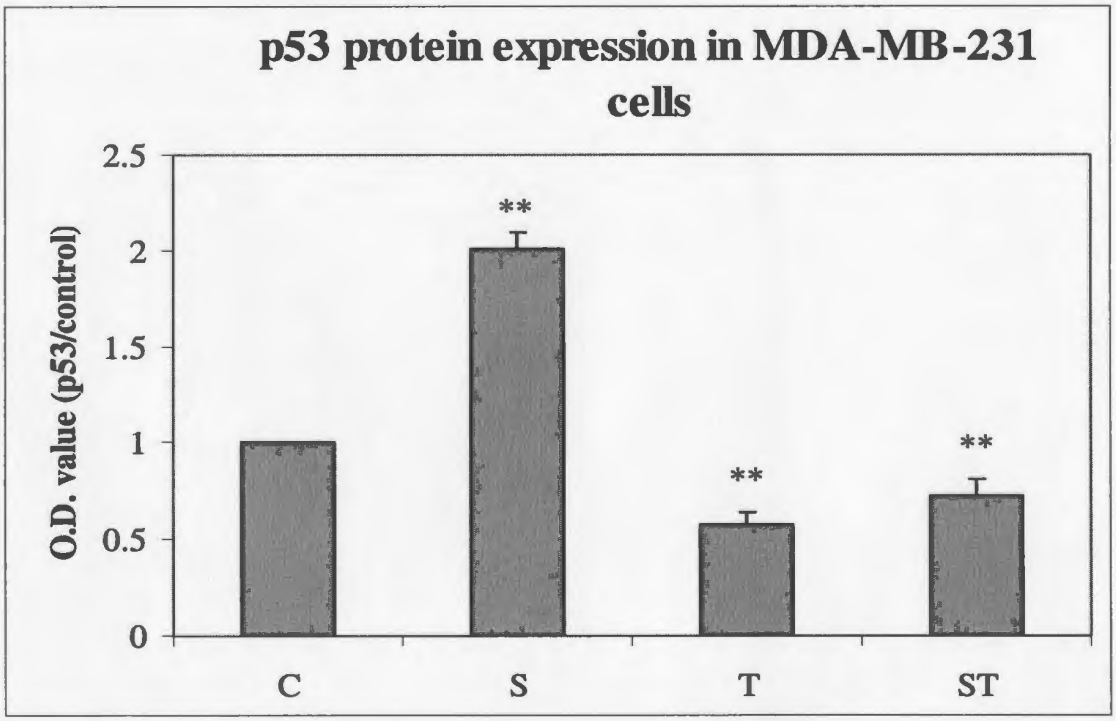
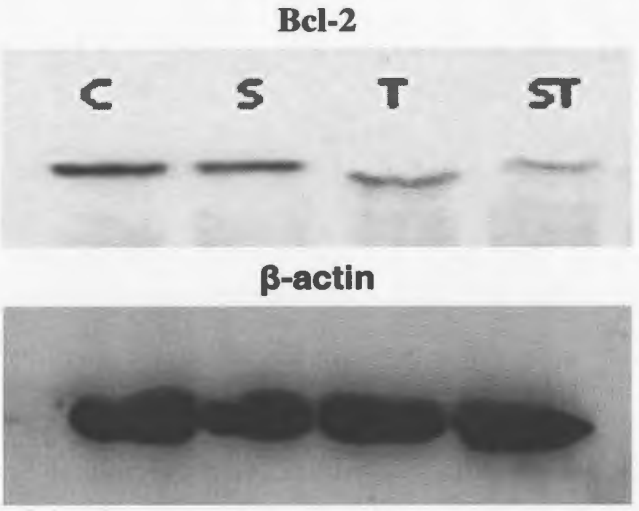


Figure 4.12 The expression of p53 protein in MDA-MB-231 cells determined by Western blot.

C: control (cells without treatments); S: cells treated with 0.016% seal oil; T: cells treated with 100 nM paclitaxel, and ST: cells treated with 100 nM paclitaxel in combination with 0.016% seal oil for 24 h, respectively. **: statistical significance at $P<0.01$ compared with control. The O.D. values are expressed as mean ± SD from four separate experiments. Photographs are representative of four separate experiments.



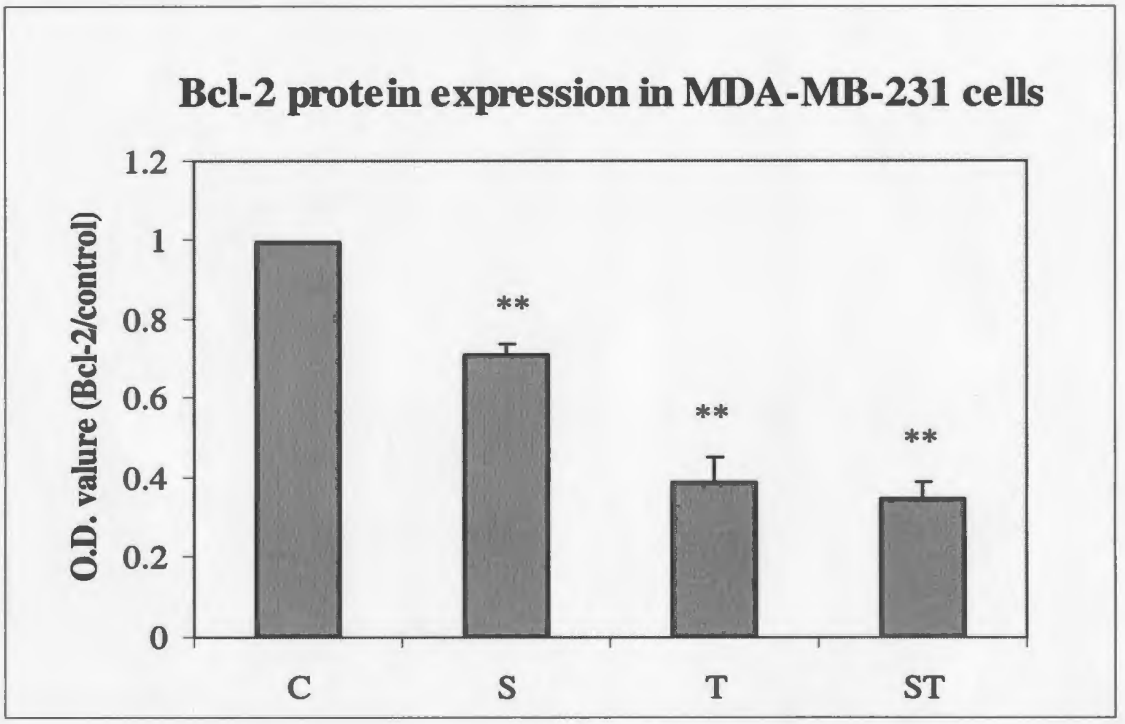


Figure 4.13 The expression of Bcl-2 protein in MDA-MB-231 cells determined by Western blot.

C: control (cells without treatments); S: cells treated with 0.016% seal oil; T: cells treated with 100 nM paclitaxel, and ST: cells treated with 100 nM paclitaxel in combination with 0.016% seal oil for 24 h, respectively. **: statistical significance at $P<0.01$ compared with control. The O.D. values are expressed as mean ± SD from four separate experiments. Photographs are representative of four separate experiments.

4.7 Determination of lipid peroxide products by TBARS assay

Both MCF-7 and MDA-MB-231 cell lines were treated with 0.016% seal oil or 100 nM paclitaxel in combination with 0.016% seal oil for 24 h, respectively. The lipid peroxide products were then measured by TBARS assay and quantified in terms of MDA equivalents, one of the end products of lipid peroxidation. The results are shown in Figures 4.14 and 4.15, and Table 4.6.

Table 4.6 MDA equivalents measured in MCF-7 and MDA-MB-231 cells.

Cell line	MCF-7	MDA-MB-231
C	0.292±0.094	0.941±0.072
S	0.620±0.091 *	1.494±0.244 *
ST	0.632±0.073 *	1.474±0.272 *

C: control, S: cells exposed to 0.016% seal oil and ST: cells exposed to 100 nM paclitaxel plus 0.016% seal oil. Data are expressed as mean ± SD nmol MDA/mg protein from three separate experiments. Statistical significance was obtained by *t*-test as compared to the control, *: statistical significance at $P<0.05$.

The results show that the amounts of MDA in cells treated with seal oil were significantly higher than that of the control in both cell lines, with 2-fold in MCF-7 cells ($P<0.05$) and 1.5-fold in MDA-MB-231 cells ($P<0.05$), respectively. However, the amounts of MDA in cells treated with seal oil and paclitaxel in combination with seal oil were found to be similar, suggesting that paclitaxel had little effect on the production of lipid peroxide products. Since seal oil was found to

induce apoptosis and to enhance paclitaxel-induced cytotoxicity, it can therefore be concluded that the production of lipid peroxide products from seal oil might be responsible for the cytotoxicity and apoptosis caused by seal oil.

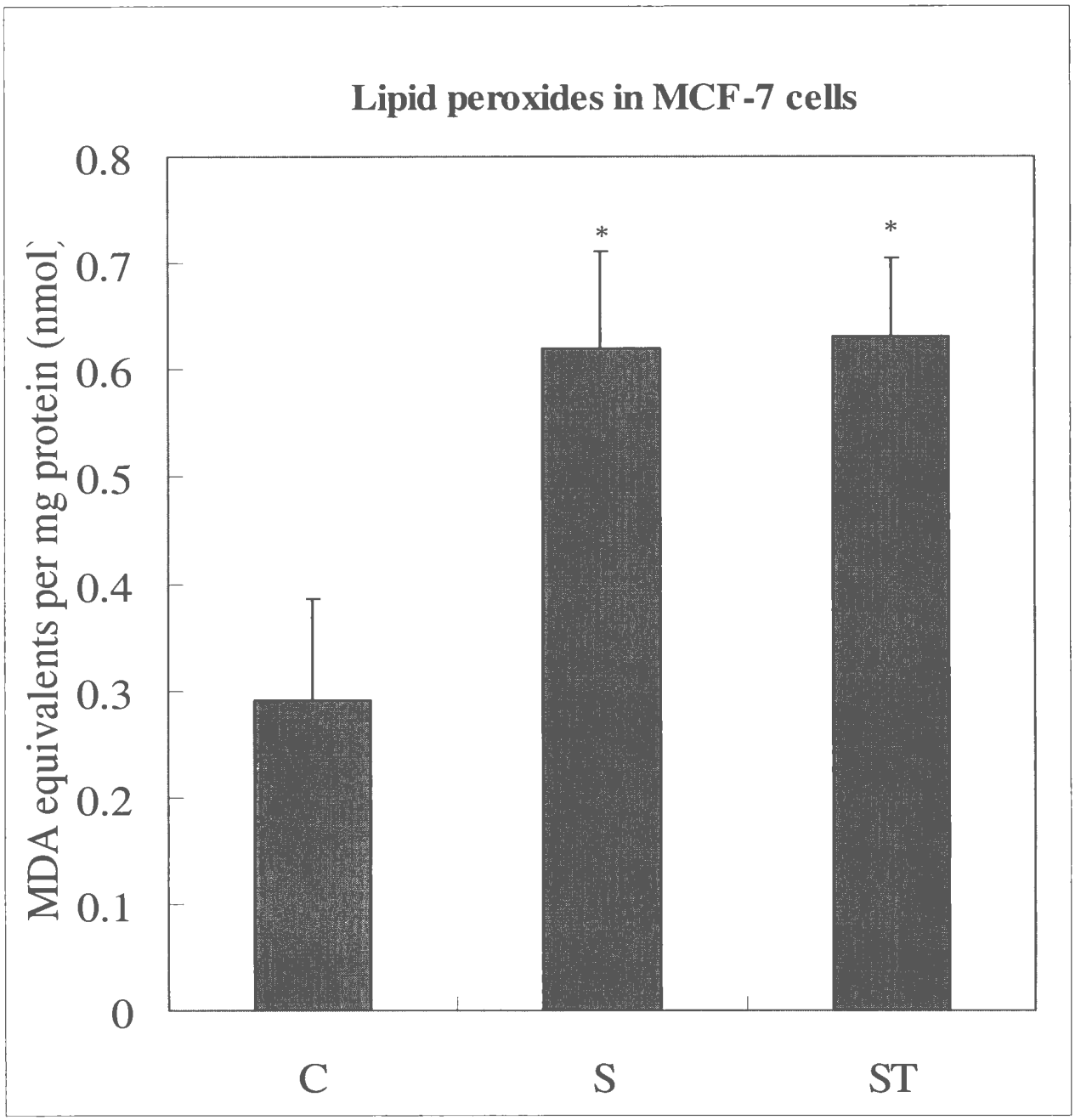


Figure 4.14 Lipid peroxide products in MCF-7 cells determined by TBARS assay.

C: control (cells without treatments); S: cells treated with 0.016% seal oil, and ST: cells treated with 100 nM paclitaxel in combination with 0.016% seal oil for 24 h, respectively. *: statistical significance at $P<0.05$ compared with control. Results are expressed as mean ± SD from three separate experiments.

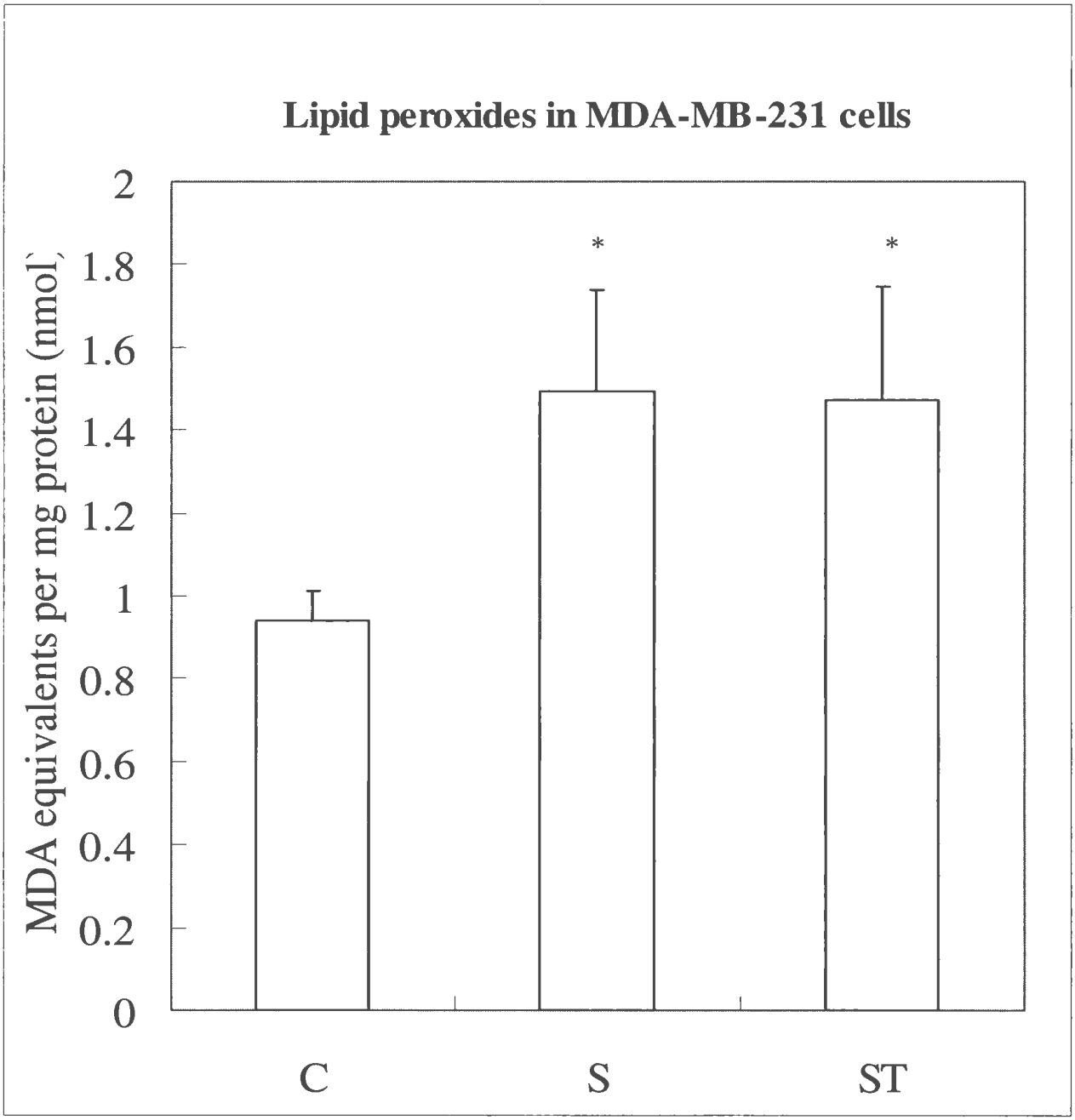


Figure 4.15 Lipid peroxide products in MDA-MB-231 cells determined by TBARS assay.

C: control (cells without treatments); S: cells treated with 0.016% seal oil, and ST: cells treated with 100 nM paclitaxel in combination with 0.016% seal oil for 24 h, respectively. *: statistical significance at $P<0.05$ compared with control. Results are expressed as mean ± SD from three separate experiments.

4.8 Lipid composition in MCF-7 and MDA-MB-231 cells determined by GC assay

4.8.1 Lipid composition in MCF-7 cells

The fatty acid composition of total cellular lipids was determined by GC. The fatty acids extracted with methanol: chloroform 1:1 (v/v) were methyl esterified using methanol: HCl (96:4 v/v) reagent. The fatty acid methyl esters were subsequently separated through GC and identified by comparison of the retention times with those of the known standards.

As shown in Table 4.7 and Figure 4.16, saturated fatty acids, e.g., palmitic acid, (16:0) and stearic acid, (18:0), were found to be predominant in MCF-7 cells. After cells were treated with 0.016% seal oil for 24 h, γ-LA (18:3ω-6), AA (20:4ω-6), α-LA (18:3 ω-3), EPA (20:5ω-3) and DHA (22:6ω-3) were detected at 0.36 ± 0.03%, 0.31 ± 0.10%, 0.14 ± 0.04%, 0.28 ± 0.06% and 0.19 ± 0.08% of total lipids, respectively. Following the treatment with 100 nM paclitaxel in combination with 0.016% seal oil for 24 h, the amounts of γ-LA, AA, α-LA, EPA and DHA were found to be 0.34 ± 0.08%, 0.54 ± 0.04%, 0.15 ± 0.01%, 0.22 ± 0.06% and 0.35 ± 0.05% of total lipids, respectively. The total amount of ω-3 PUFAs was changed from 0 in the control to 0.61 ± 0.18% following seal oil treatment and to 0.92 ± 0.13% following paclitaxel in combination with seal oil treatment, respectively ($P<0.05$). The total amount of ω-6 PUFAs was also increased from 0.46 ± 0.09% in the control to 1.00 ± 0.26% in the seal oil group and 1.32 ± 0.23% in the paclitaxel in combination with seal oil group, respectively ($P<0.05$) (Figure 4.16).

Table 4.7 Fatty acid compositions expressed as % of total lipids in MCF-7 cells.

Fatty acids	Control	Treated with 0.016% seal oil	Treated with 100 nM paclitaxel +0.016% seal oil
16:0 (palmitic acid)	9.10 ± 1.68	10.12 ± 1.29	10.33 ± 1.59
18:0 (stearic acid)	13.91 ± 0.10	17.95 ± 4.81	18.52 ± 4.31
18:2 ω-6 (LA)	0.46 ± 0.09	0.33 ± 0.13	0.44 ± 0.11
18:3 ω-6 (γ-LA)	ND	0.36 ± 0.03	0.34 ± 0.08
20:4 ω-6 (AA)	ND	0.31 ± 0.10	0.54 ± 0.04
18:3 ω-3 (α-LA)	ND	0.14 ± 0.04	0.15 ± 0.01
20:5 ω-3 (EPA)	ND	0.28 ± 0.06	0.22 ± 0.06
22:5 ω-3 (DPA#)	ND	ND	0.20 ± 0.01
22:6 ω-3 (DHA)	ND	0.19 ± 0.08	0.35 ± 0.05
Σ ω-6	0.46 ± 0.09	1.00 ± 0.26 *	1.32 ± 0.23 *
Σ ω-3	ND	0.61 ± 0.18 *	0.92 ± 0.13 *

#: DPA: docosapentaenoic acid

ND: not detected

Cells were treated without any drug treatment (control), treated with 0.016% seal oil and 100 nM of paclitaxel plus 0.016% seal oil for 24 h, respectively. Data are expressed as mean ± SD from four separate experiments. *: statistical significance at $P<0.05$ compared with control.

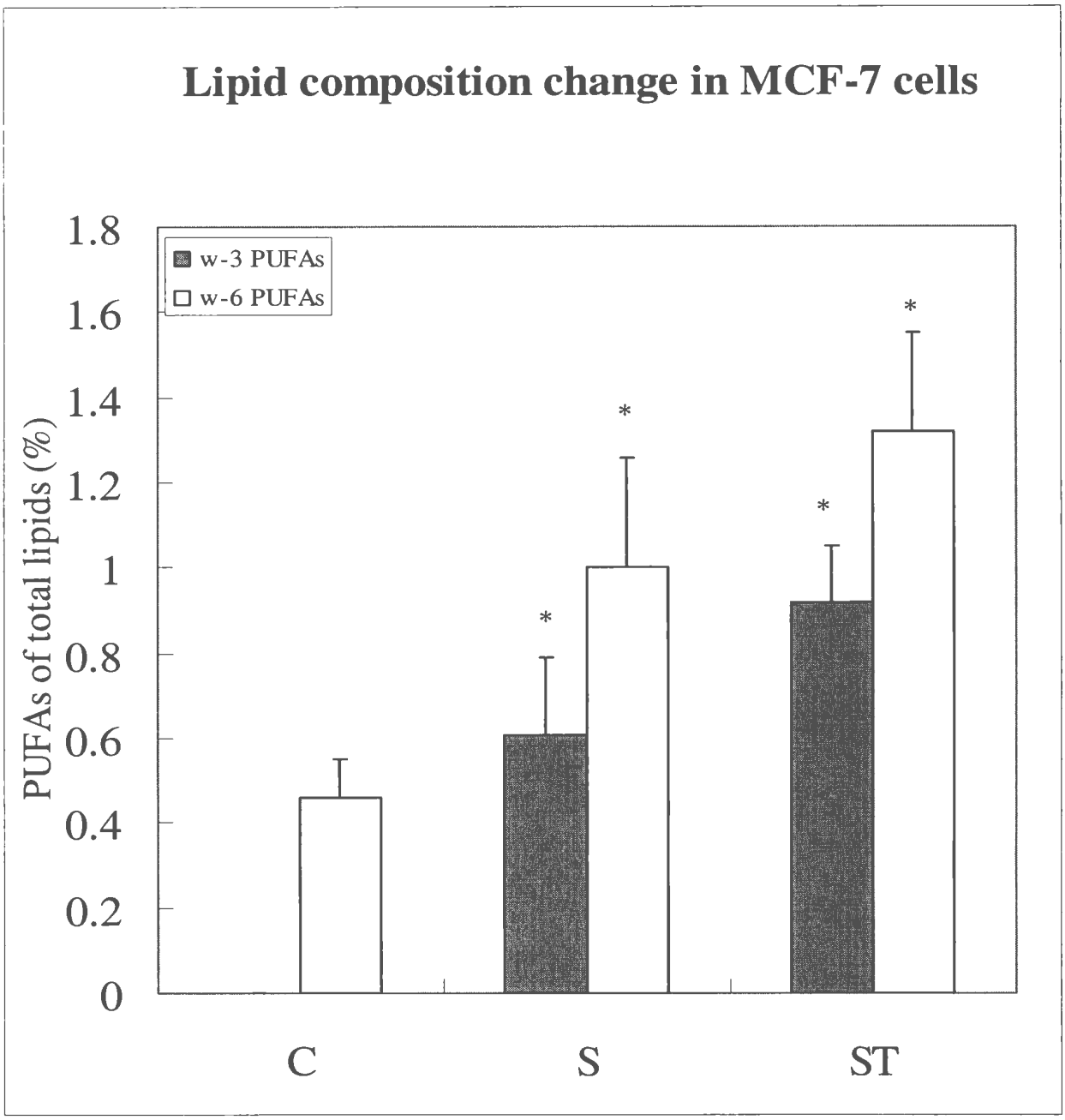


Figure 4.16 The ω-3 and ω-6 fatty acids in MCF-7 cells determined by GC.

C: control (cells without treatments); S: cells treated with 0.016% seal oil, and ST: cells treated with 100 nM paclitaxel in combination with 0.016% seal oil for 24 h, respectively. *: statistical significance at $P<0.05$ compared with control. Results are expressed as mean ± SD from four separate experiments.

4.8.2 Lipid composition in MDA-MB-231 cells

Similarly, as shown in Table 4.8 and Figure 4.17, lipids initially found in MDA-MB-231 cells were saturated fatty acids. Following the treatment with 0.016% seal oil for 24 h, ω-3 PUFAs including α-LA (18:3ω-3), EPA (20:5ω-3), DPA (22:5ω-3), DHA (22:6ω-3) and ω-6 PUFA, γ-LA (18:3ω-6), were detected at 0.37 ± 0.05%, 0.16 ± 0.02%, 0.27 ± 0.01%, 0.40 ± 0.01% and 0.47 ± 0.12% of total lipids, respectively. The respective PUFAs were found to be 0.42 ± 0.09%, 0.22 ± 0.09%, 0.44 ± 0.14%, 0.30 ± 0.01% and 0.47 ± 0.01% following incubation with 100 nM paclitaxel in combination with 0.016% seal oil. The total amount of ω-3 PUFAs was changed from 0 in the control to 1.20 ± 0.09% following the treatment of seal oil and 1.38 ± 0.33% following the treatment of paclitaxel in combination with seal oil, respectively ($P<0.05$). The total amount of ω-6 PUFAs was also changed from 0 in the control to 0.47 ± 0.12% following the treatment of seal oil and 0.47 ± 0.01% following the treatment of paclitaxel in combination with seal oil, respectively ($P<0.05$) (Figure 4.17).

Table 4.8 Fatty acid compositions expressed as % of total lipids in MDA-MB-231 cells.

Fatty acids	Control	Treated with 0.016% seal oil	Treated with 100 nM paclitaxel + 0.016% seal oil
16:0 (palmitic acid)	9.17 ± 0.85	8.64 ± 2.46	8.48 ± 1.69
18:0 (stearic acid)	16.47 ± 1.59	15.95 ± 4.19	15.70 ± 3.69
18:2 ω-6 (LA)	ND	ND	ND
18:3 ω-6 (γ-LA)	ND	0.47 ± 0.12	0.47 ± 0.01
20:4 ω-6 (AA)	ND	ND	ND
18:3 ω-3 (α-LA)	ND	0.37 ± 0.05	0.42 ± 0.09
20:5 ω-3 (EPA)	ND	0.16 ± 0.02	0.22 ± 0.09
22:5 ω-3 (DPA)	ND	0.27 ± 0.01	0.44 ± 0.14
22:6 ω-3 (DHA)	ND	0.40 ± 0.01	0.30 ± 0.01
Σ ω-6	ND	0.47 ± 0.12 *	0.47 ± 0.01 *
Σ ω-3	ND	1.20 ± 0.09 *	1.38 ± 0.33 *

ND: not detected

Cells were treated without any treatment (control), treated with 0.016% seal oil and 0.016% seal oil plus 100 nM paclitaxel for 24 h, respectively. Data are expressed as mean ± SD from four separate experiments. *: statistical significance at $P<0.05$ compared with control.

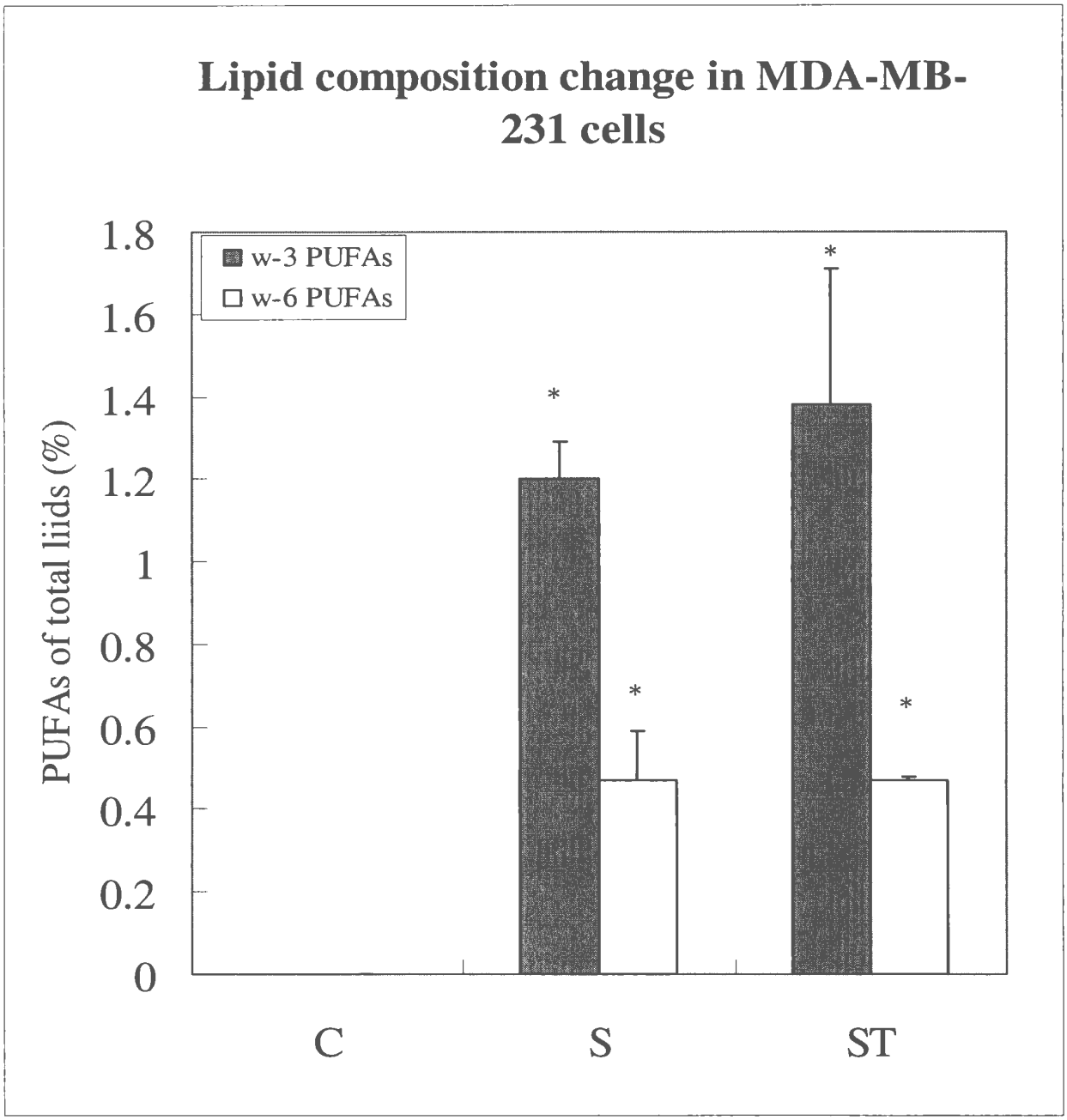


Figure 4.17 The ω-3 and ω-6 fatty acids in MDA-MB-231 cells determined by GC.

C: control (cells without treatments); S: cells treated with 0.016% seal oil, and ST: cells treated with 100 nM paclitaxel in combination with 0.016% seal oil for 24 h, respectively. *: statistical significance at $P<0.05$ compared with control. Results are expressed as mean ± SD from four separate experiments.

In conclusion, following the treatment of 0.016% seal oil for 24 h, the composition of lipids in the two cell lines tested, MCF-7 and MDA-MB-231, was found to have changed. In MCF-7 cell line, the total ω-3 PUFAs increased from 0 in the control to 0.6-1.0% of total lipids, and the total ω-6 PUFAs increased from 0.5% in the control to 1.0-1.3% of total lipids. In MDA-MB-231 cell line, neither ω-3 nor ω-6 PUFAs was detected in the control; however, following the treatments of seal oil, ω-3 and ω-6 PUFAs were detected at 1.2 – 1.4% and 0.5% of total lipids, respectively. Since no significant difference was found in the lipid composition of the cells treated with seal oil and paclitaxel in combination with seal oil, it is therefore believed that paclitaxel had little effect on the change of the lipid composition in the cell membrane. Because PUFAs cannot be bio-synthesized *de novo* in mammalian cells, the increase of PUFAs was thought to be derived from the seal oil the cells were exposed to. The increase of PUFAs in the cells might be responsible for the elevated production of intracellular lipid peroxides, which was observed in the TBARS assay.

4.9 Effect of soybean oil on cell growth, cell morphology, and expression of p53 and Bcl-2 proteins in MCF-7 and MDA-MB-231 cells

Intralipid® is a currently available fat emulsion on the market. It is used in clinic as a dietary supplement by intravenous infusion for patients who are unable to obtain enough fat in their diet due to certain illnesses or recent surgery, to provide energy and nutritional substances needed for normal functions.

The main ingredient in Intralipid® is the purified soybean oil, which contains approximately

15% saturated fatty acids (palmitic acid, 16:0 and stearic acid, 18:0), 24% monounsaturated fatty acid (oleatic acid, 18:1ω-9) and 61% PUFAs including more than 50% linoleic acid (18:2ω-6) and about 7% α-linolenic acid (18:3ω-3) (USDA, 1979). Unlike seal oil emulsion, Intralipid® has a higher ratio of ω-6/ω-3 and lacks long chain PUFAs such as DHA and EPA.

To compare the effect of soybean oil on breast cancer cells with that of seal oil, MTT assay, Hoechst staining and Western blot assay were performed.

4.9.1 Effect of soybean oil on MCF-7 and MDA-MB-231 cell growth

4.9.1.1 Effect of soybean oil on MCF-7 cell growth

MCF-7 cells were treated with different concentrations of soybean oil varying from 0.004% to 0.032% for 24, 48 and 72 h, respectively. As shown in Figure 4.18, after the cells were treated with 0.004%, 0.008%, 0.016% and 0.032% soybean oil for 24 h, the cell viability was decreased to from 83.1±7.7% to 75.5±2.4% of the control. The cell viability was decreased further when cells were treated for 48 and 72 h to approximately 60% of the control ($P<0.01$ and $P<0.05$), compared with that for 24 h.

In comparison with the cytotoxicity induced by seal oil in MCF-7 cells, soybean oil showed greater toxicity to MCF-7 cells under the same conditions. (Figures 4.19-21)

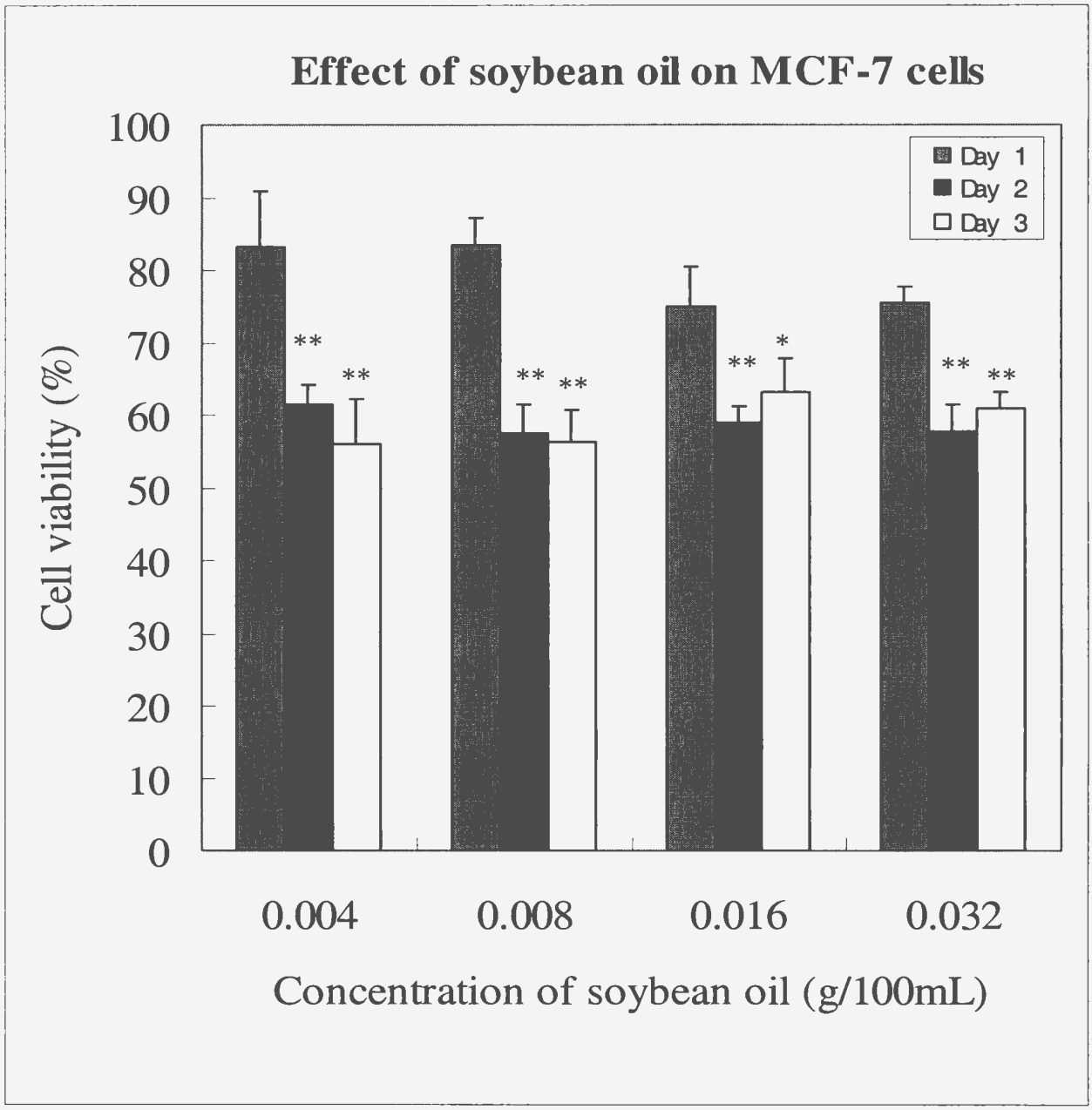


Figure 4.18 The effect of soybean oil on MCF-7 cells treated for 24, 48 and 72 h at different concentrations.

**: statistical significance at P<0.01; *: statistical significance at P<0.05 compared with data of 24 h. Results are expressed as mean ± SD from three separate experiments.

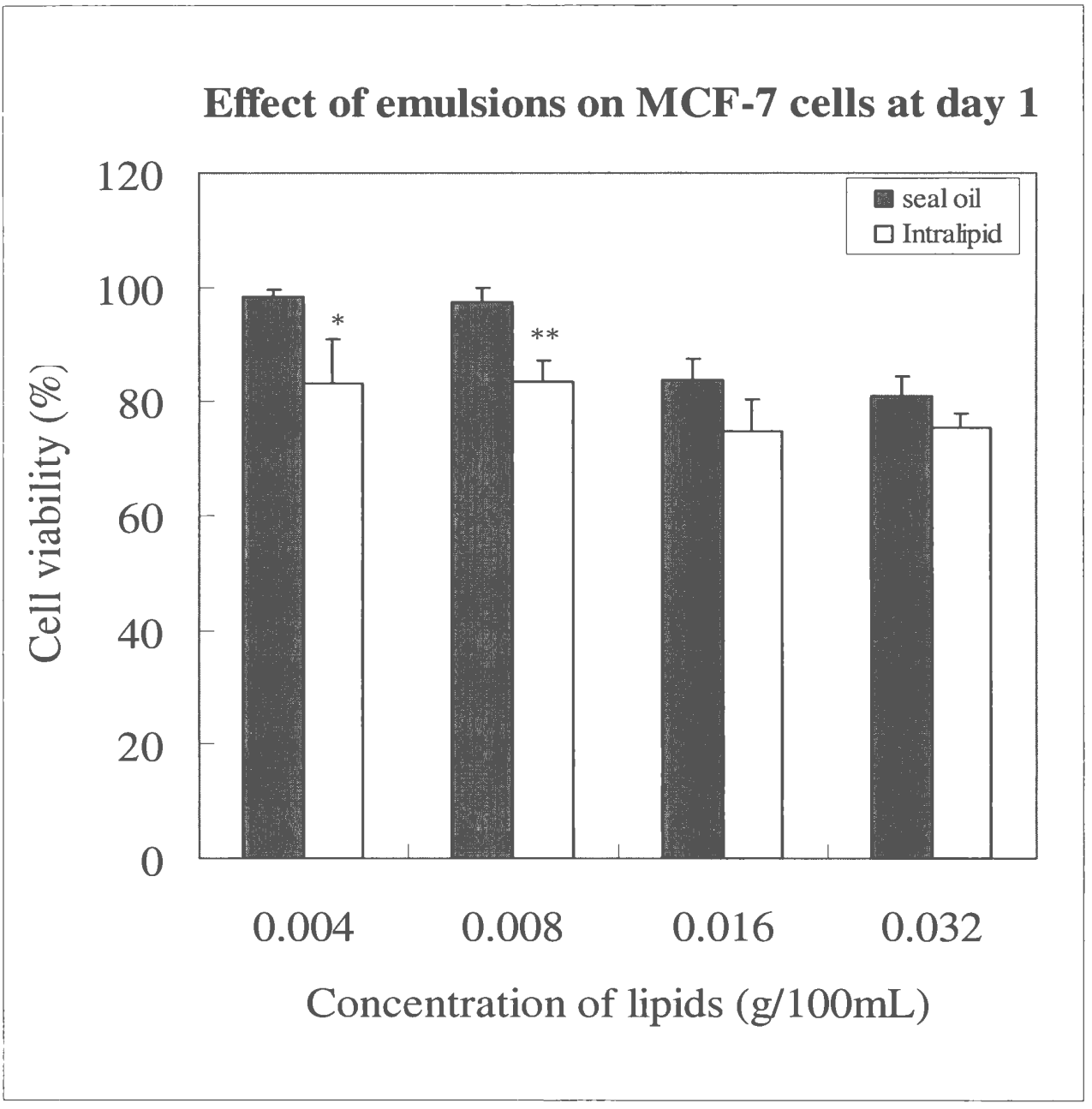


Figure 4.19 The effect of seal oil and soybean oil on MCF-7 cells treated for 24 h at different concentrations.

**: statistical significance at $P<0.01$; *: statistical significance at $P<0.05$ compared with seal oil. Results are expressed as mean ± SD from three separate experiments.

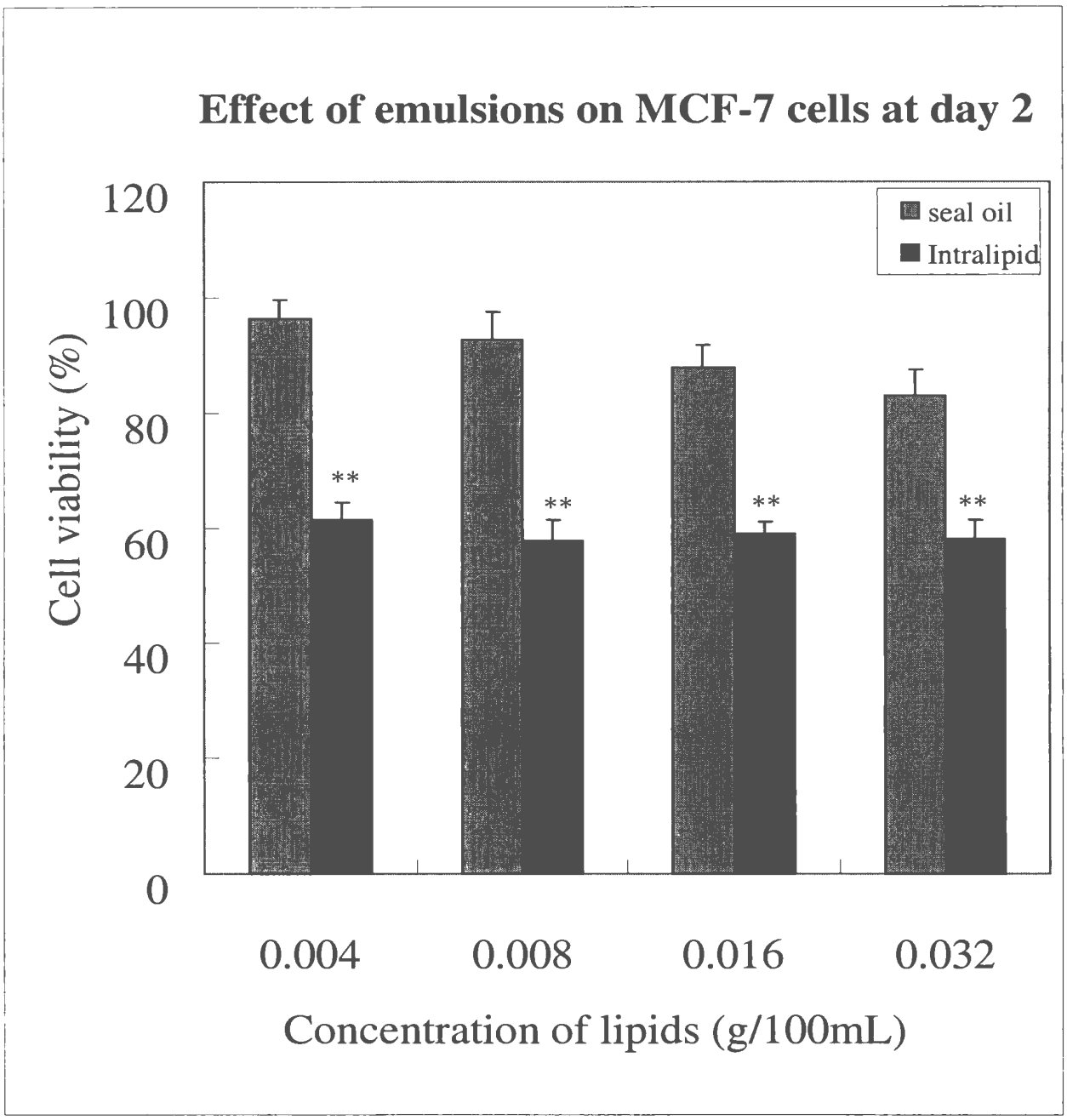


Figure 4.20 The effect of seal oil and soybean oil on MCF-7 cells treated for 48 h at different concentrations.

**: statistical significance at $P<0.01$ compared with seal oil. Results are expressed as mean ± SD from three separate experiments.

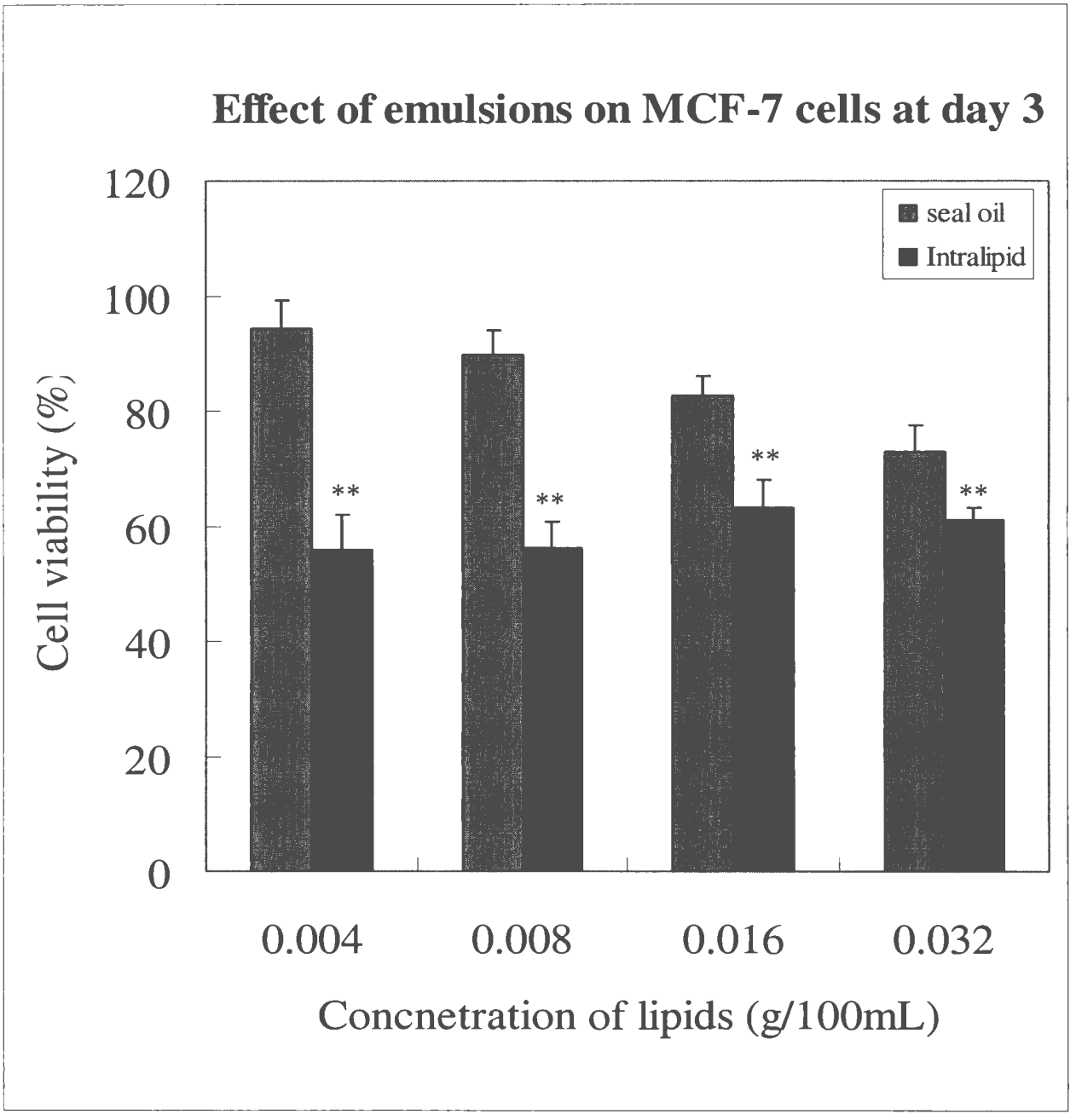


Figure 4.21 The effect of seal oil and soybean oil on MCF-7 cells treated for 72 h at different concentrations.

**: statistical significance at $P<0.01$ compared with seal oil. Results are expressed as mean ± SD from three separate experiments.

4.9.1.2 Effect of soybean oil on MDA-MB-231 cell growth

MDA-MB-231 cells were treated with soybean oil as well. Figure 4.22 shows that when cells were treated with soybean oil varying from 0.004% to 0.032% for 24 h, the cell viability was found to be decreased to from 92.5±3.9% to 80.9±2.6% of the control. However, the cell viability was improved when cells were treated for 48 h, to more than 95% of the control ($P<0.01$). When cells were treated for 72 h, the cell viability was further increased to 115% - 140% of the control ($P<0.01$and $P<0.05$). The results suggest that instead of killing cells, soybean oil promoted MDA-MB-231 cell growth following incubation for 48 and 72 h.

Compared with the cytotoxicity induced by seal oil in MDA-MB-231 cells, the cell viability was decreased only following the treatment of soybean oil for 24 h, and was increased following the treatment of soybean oil for 48 and 72 h. (Figures 4.23-25)

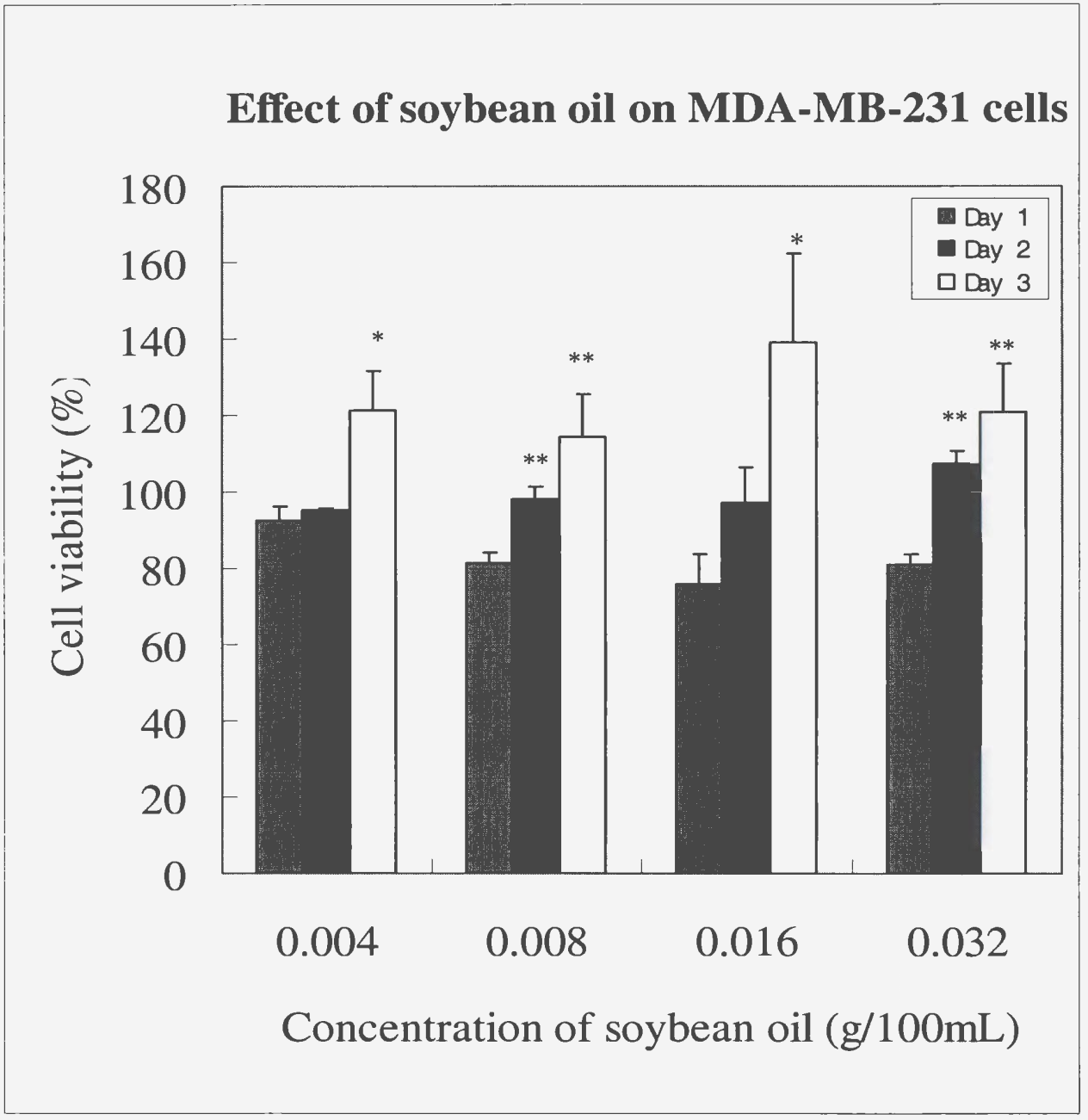


Figure 4.22 The effect of soybean oil on MDA-MB-231 cells treated for 24, 48 and 72 h at different concentrations.

**: statistical significance at P<0.01; *: statistical significance at P<0.05 compared with data of 24 h. Results are expressed as mean ± SD from three separate experiments.

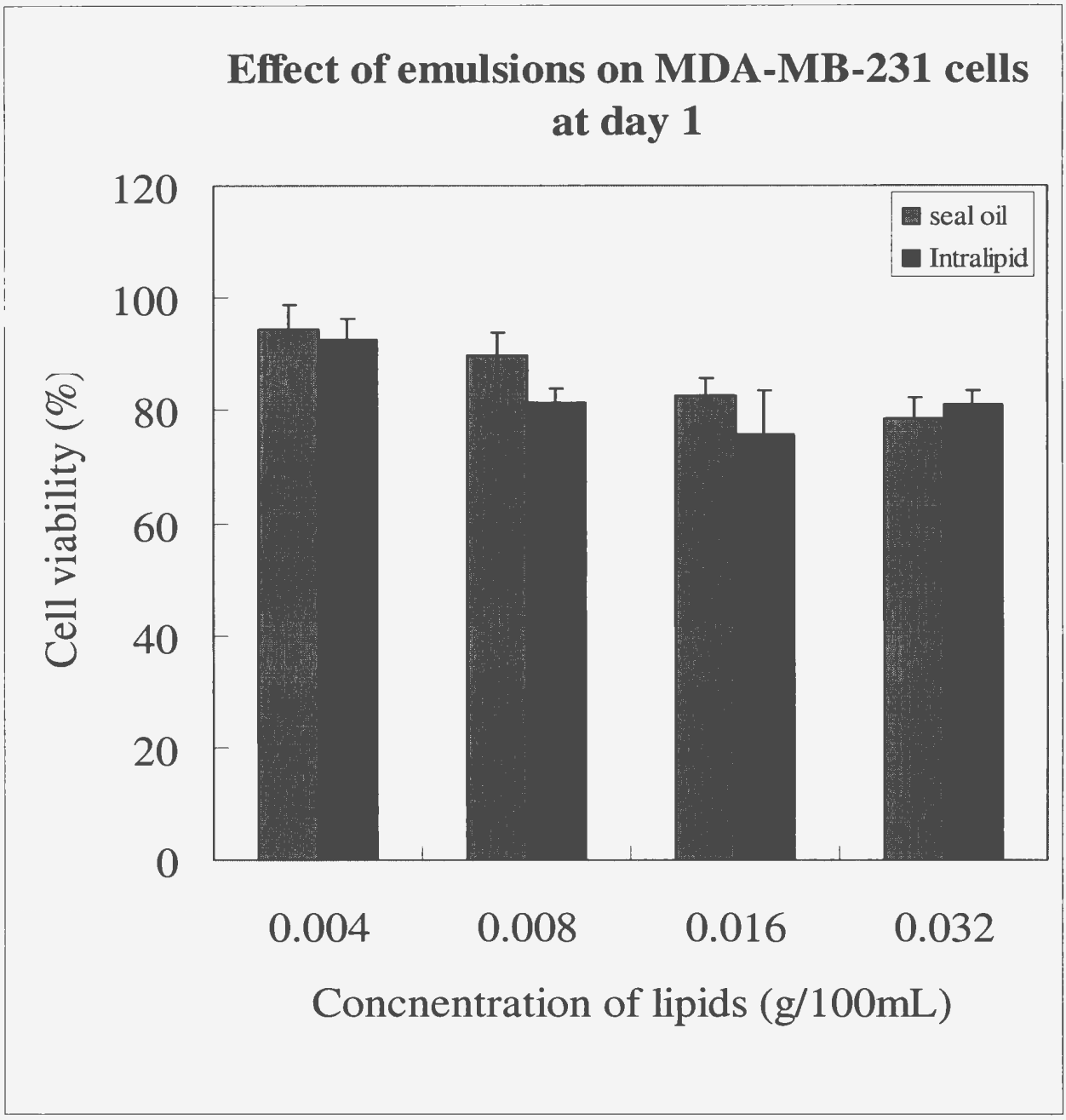


Figure 4.23 The effect of seal oil and soybean oil on MDA-MB-231 cells treated for 24 h at different concentrations.

Results are expressed as mean ± SD from three separate experiments.

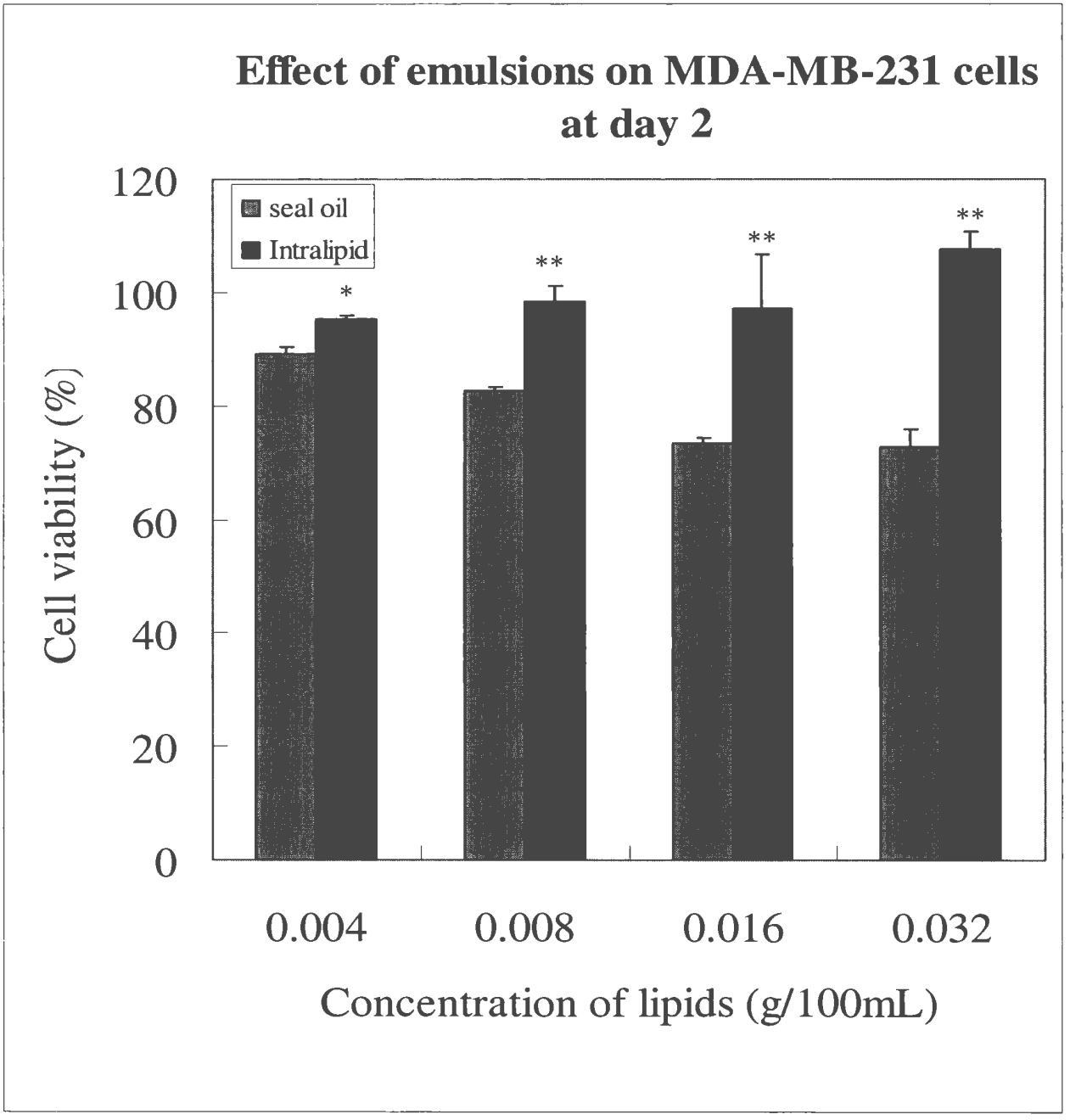


Figure 4.24 The effect of seal oil and soybean oil on MDA-MB-231 cells treated for 48 h at different concentrations.

**: statistical significance at $P<0.01$; *: statistical significance at $P<0.05$ compared with seal oil. Results are expressed as mean ± SD from three separate experiments.

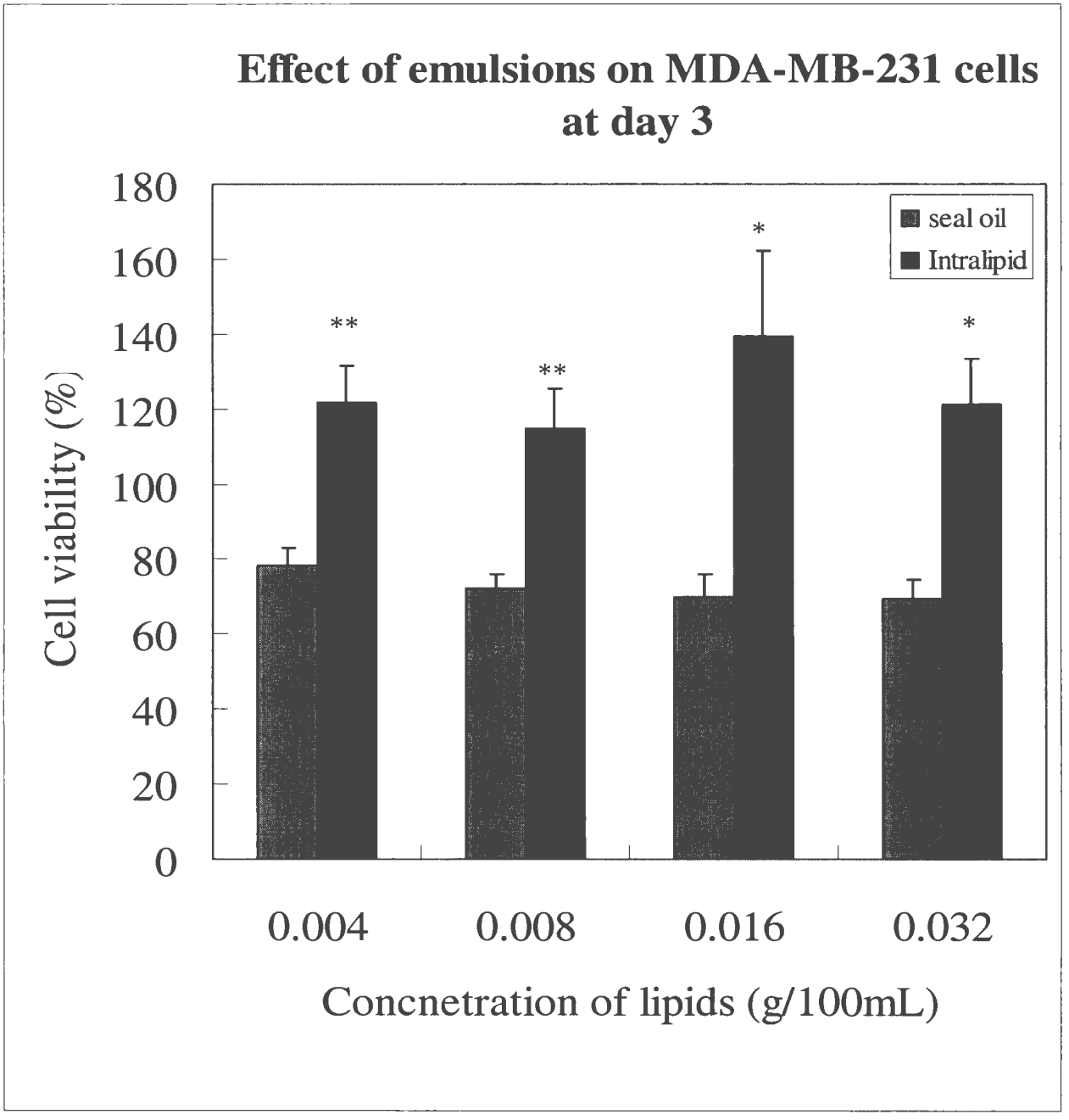


Figure 4.25 The effect of seal oil and soybean oil on MDA-MB-231 cells treated for 72 h at different concentrations.

**: statistical significance at P<0.01; *: statistical significance at P<0.05 compared with seal oil. Results are expressed as mean ± SD from three separate experiments.

4.9.2 Effect of soybean oil on cell morphology in MCF-7 and MDA-MB-231 cells

Morphological changes of the cells respectively treated with 0.016% soybean oil for 24, 48 and 72 h were assessed by Hoechst staining as described previously.

4.9.2.1 Morphological changes in MCF-7 cells

As shown in Figure 4.26, no cells were found undergoing apoptosis when they were treated with soybean oil for 24 h. However, a few apoptotic cells were observed when cells were treated for 48 and 72 h. The results were not consistent with the cell viability results determined by MTT assay where soybean oil showed toxicity at all time intervals, suggesting that apoptosis was not the only mechanism of soybean oil-induced cell death in MCF-7 cells.

4.9.2.2 Morphological changes in MDA-MB-231 cells

As shown in Figure 4.27, no MDA-MB-231 cells were found to present aggregated chromatin and condensed cytoplasm and nuclei following the treatment of soybean oil at all the time intervals. It suggests that soybean oil was unable to induce apoptosis in MDA-MB-231 cells.

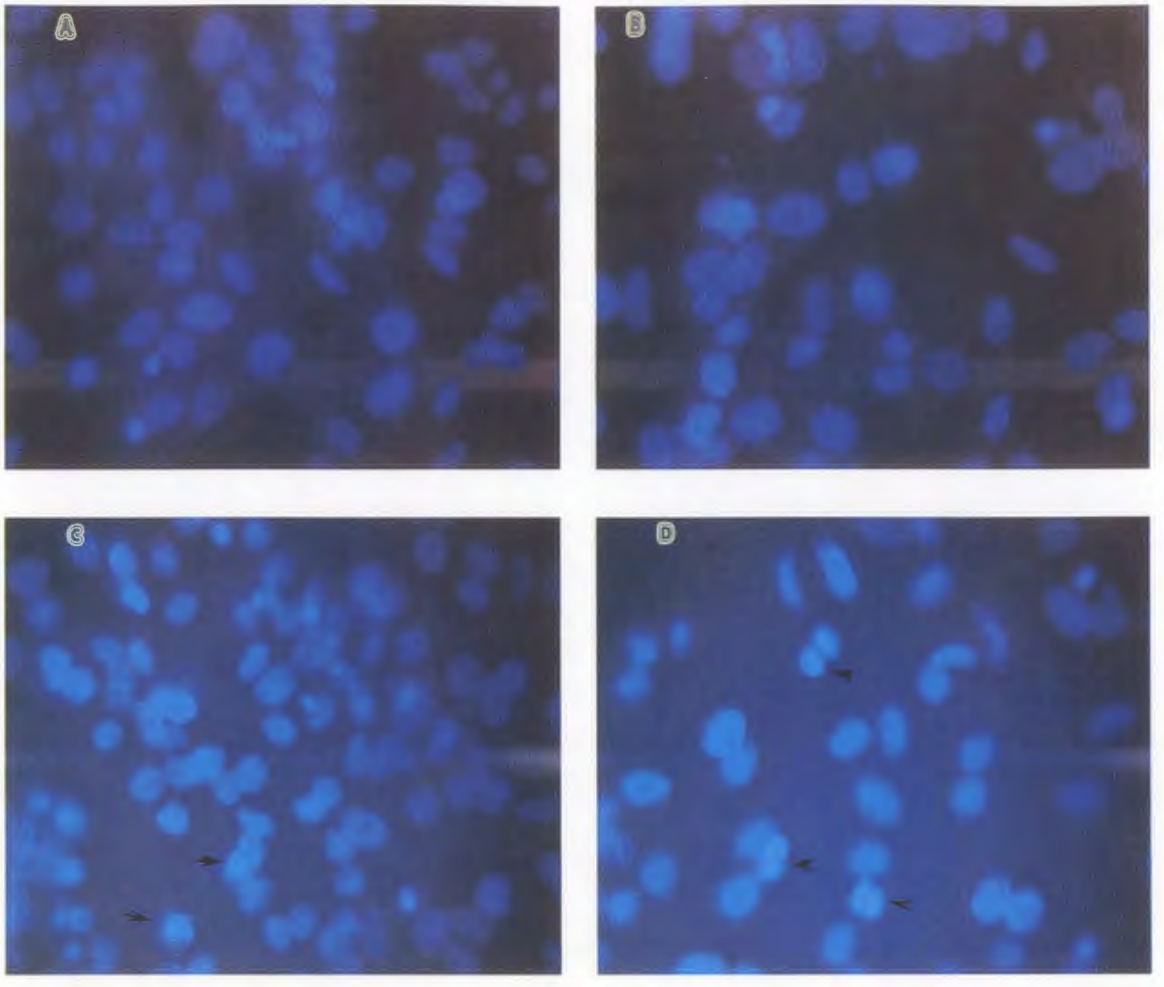


Figure 4.26 Morphological changes in MCF-7 cells treated with soybean oil determined by Hoechst staining (40X magnification). A: control (cells without treatments); B: cells treated for 24 hrs; C: cells treated for 48 hrs, and D: cells treated for 72 hrs. Photographs are representative of three separate experiments.

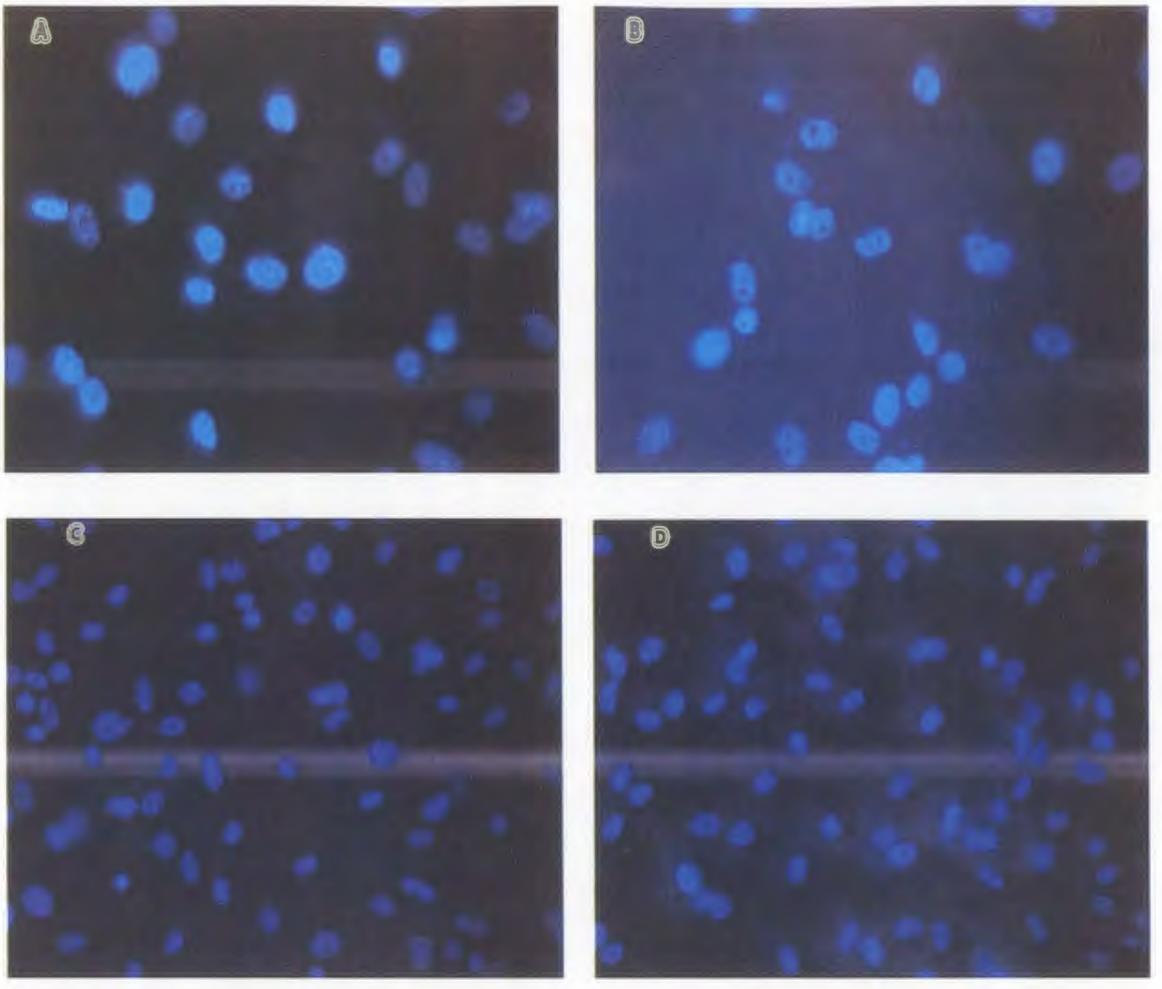


Figure 4.27 Morphological changes in MDA-MB-231 cells treated with soybean oil determined by Hoechst staining (40X magnification). A: control (cells without treatments); B: cells treated for 24 hrs; C: cells treated for 48 hrs, and D: cells treated for 72 hrs. Photographs are representative of three separate experiments.

4.9.3 Effect of soybean oil on the expression of p53 and Bcl-2 proteins in MCF-7 and MDA-MB-231 cells

Western blotting assay was employed to detect the effect of soybean oil on the expression of the apoptosis markers, p53 and Bcl-2 proteins. Cells were treated with 0.016% soybean oil in the absence and presence of 100 nM of paclitaxel for 24 h. The proteins were extracted and quantified, and the electrophoresis was performed as previously described. The expression of p53 and Bcl-2 proteins was expressed as O.D. values and was compared with that of the control.

4.9.3.1 Expression of p53 and Bcl-2 proteins in MCF-7 cells

As shown in Figure 4.28 and Table 4.9, soybean oil alone had little effect on the expression of p53 protein in MCF-7 cells. Although the expression of p53 protein in cells treated with soybean oil in combination with paclitaxel was 1.42-fold of the control ($P<0.05$), it showed no significant difference with the result from the cells treated with paclitaxel alone, which was 1.29-fold of the control. (See Table 4.4)

As shown in Figure 4.29 and Table 4.9, Bcl-2 protein expression was found to be up-regulated by 40% by soybean oil ($P<0.05$). When cells were treated with soybean oil in combination with paclitaxel, the Bcl-2 protein expression was increased by 30% ($P<0.05$), although paclitaxel alone inhibited the expression by 40%. (See Table 4.4)

These results indicated that soybean oil was unable to trigger the apoptotic protein, p53, and promote the expression of the anti-apoptotic protein, Bcl-2, to induce apoptosis in MCF-7 cells.

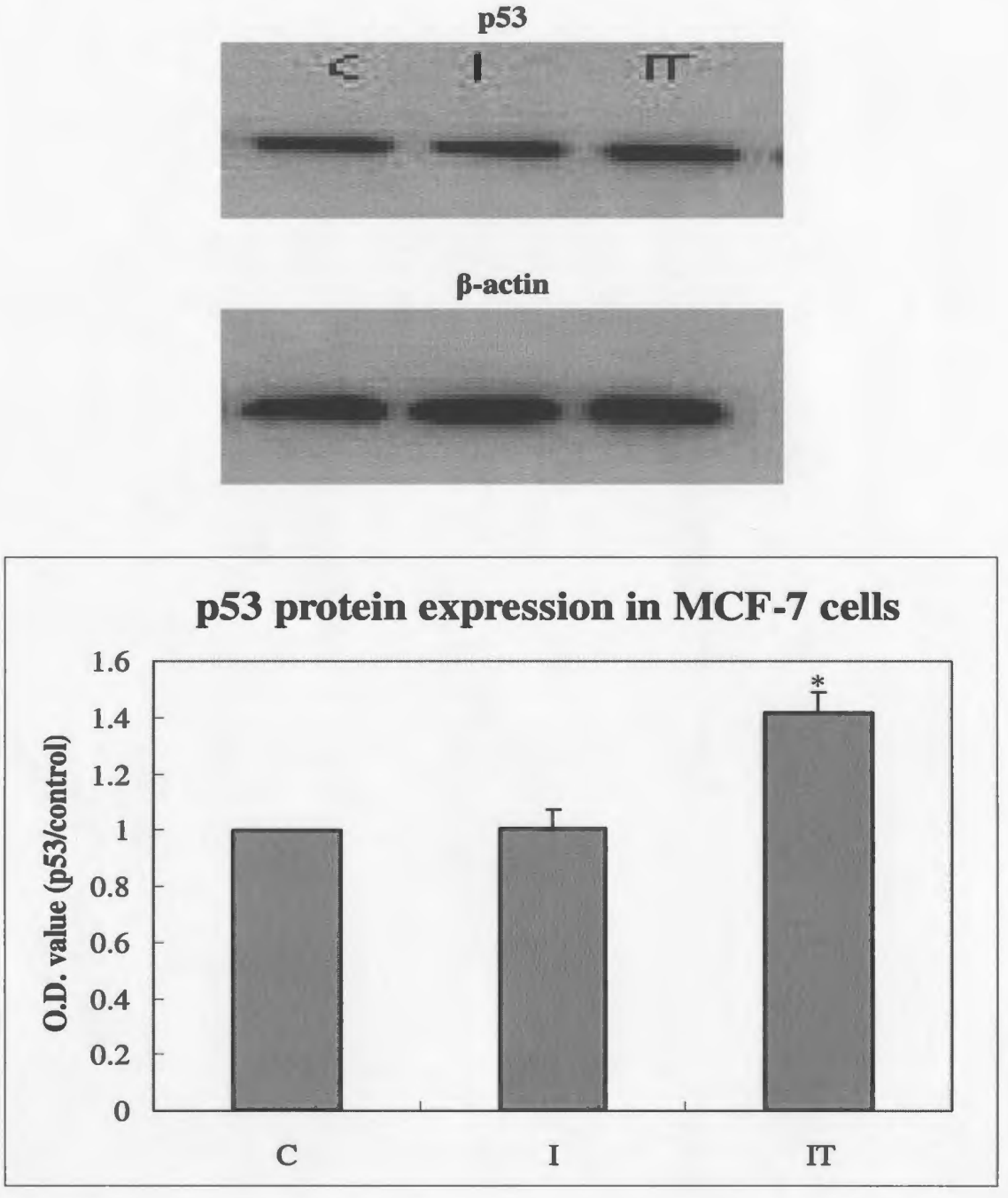


Figure 4.28 The expression of p53 protein in MCF-7 cells determined by Western blot.

C: control (cells without treatments); I: cells treated with 0.016% soybean oil, and IT: cells treated with 0.016% soybean oil in combination with 100 nM paclitaxel for 24 h, respectively. *: statistical significance at $P<0.05$ compared with control. The O.D. values are expressed as mean ± SD from four separate experiments. Photographs are representative of four separate experiments.

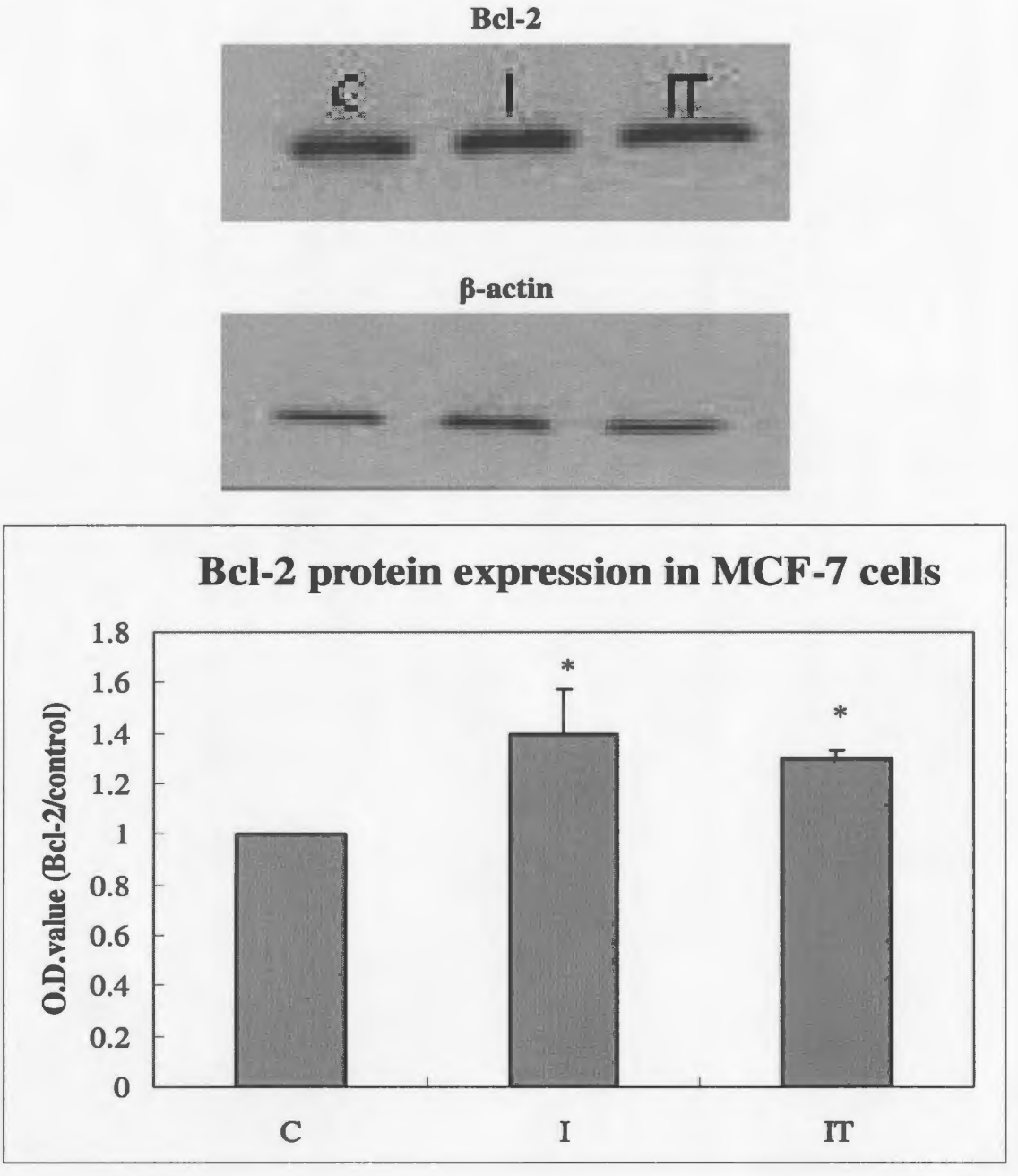


Figure 4.29 The expression of Bcl-2 protein in MCF-7 cells determined by Western blot.

C: control (cells without treatments); I: cells treated with 0.016% soybean oil, and IT: cells treated with 0.016% soybean oil in combination with 100 nM paclitaxel for 24 h, respectively. *: statistical significance at $P<0.05$ compared with control. The O.D. values are expressed as mean ± SD from four separate experiments. Photographs are representative of four separate experiments.

Table 4.9 The expression of p53 and Bcl-2 proteins in MCF-7 cells determined by Western blot and expressed as O.D. values.

O.D. values	Control	Treated with 0.016% soybean oil	Treated with 0.016% soybean oil + 100 nM paclitaxel
p53	1	1.01±0.06	1.42±0.07*
Bcl-2	1	1.40±0.17*	1.30±0.03*

The results are expressed as mean ± SD from four separate experiments. Statistical significance was performed by the Student *t*-test as compared with control.*: statistical significance at $P<0.05$.

4.9.3.2 Expression of p53 and Bcl-2 proteins in MDA-MB-231 cells

Figure 4.30 and Table 4.10 show that soybean oil down-regulated the expression of p53 protein ($P<0.05$) in MDA-MB-231 cells and such down-regulation was found to have been further enhanced by 100 nM of paclitaxel from 47% to 42% of the control ($P<0.01$). As shown in Figure 4.31 and Table 4.10, it was also found that soybean oil alone had little effect on the Bcl-2 protein expression in MDA-MB-231 cells. The expression of Bcl-2 protein in MDA-MB-231 cells following the treatment of soybean oil in combination with paclitaxel was found to have reduced to 51% of the control ($P<0.01$); however, this result was not significantly different from that of the cells treated with paclitaxel alone. (See Table 4.5)

Table 4.10 The expression of p53 and Bcl-2 proteins in MDA-MB-231 cells determined by Western blot and expressed as O.D. values.

O.D. values	Control	Treated with 0.016% soybean oil	Treated with 0.016% soybean oil + 100 nM paclitaxel
p53	1	0.47±0.22*	0.42±0.04**
Bcl-2	1	0.93±0.14	0.51±0.06**

The results are expressed as mean ± SD from four separate experiments. Statistical significance was performed by the Student *t*-test as compared with control.*: statistical significance at $P<0.05$; **: statistical significance at $P<0.01$.

According to these results, it can be concluded that the effect of soybean oil on cell growth appeared to be cell dependent and soybean oil was found cytotoxic to MCF-7 cells, but beneficial to MDA-MB-231 cells. Soybean oil did not result in the induction of apoptosis in neither of the two cell lines tested,likely due to the fact that it didn't show to trigger the expression of p53 protein or inhibit the expression of Bcl-2 protein.

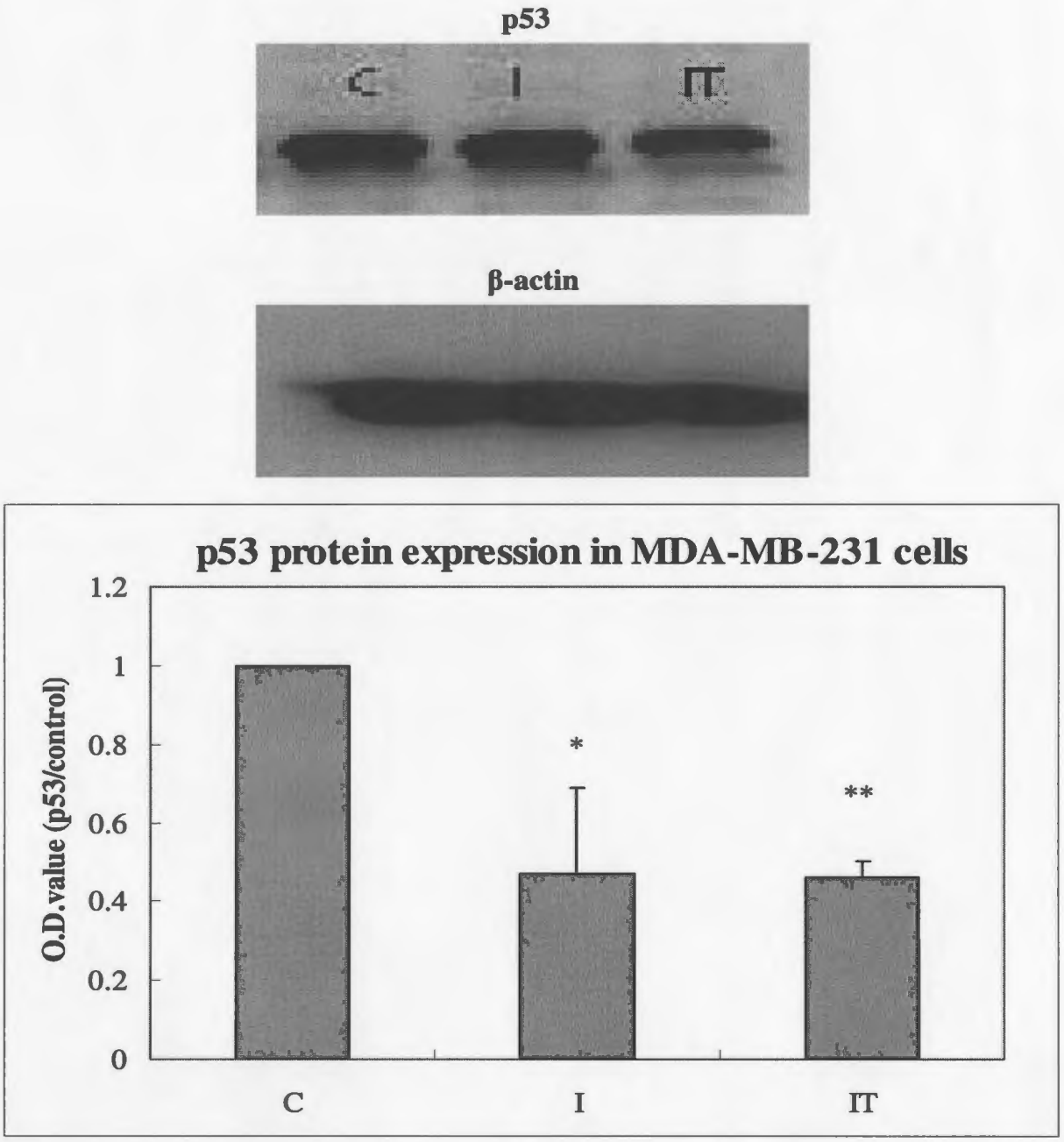


Figure 4.30 The expression of p53 protein in MDA-MB-231 cells determined by Western blot.

C: control (cells without treatments); I: cells treated with 0.016% soybean oil, and IT: cells treated with 0.016% soybean oil in combination with 100 nM paclitaxel for 24 h, respectively. **: statistical significance at $P<0.01$; *: statistical significance at $P<0.05$ compared with control. The O.D. values are expressed as mean ± SD from four separate experiments. Photographs are representative of four separate experiments.

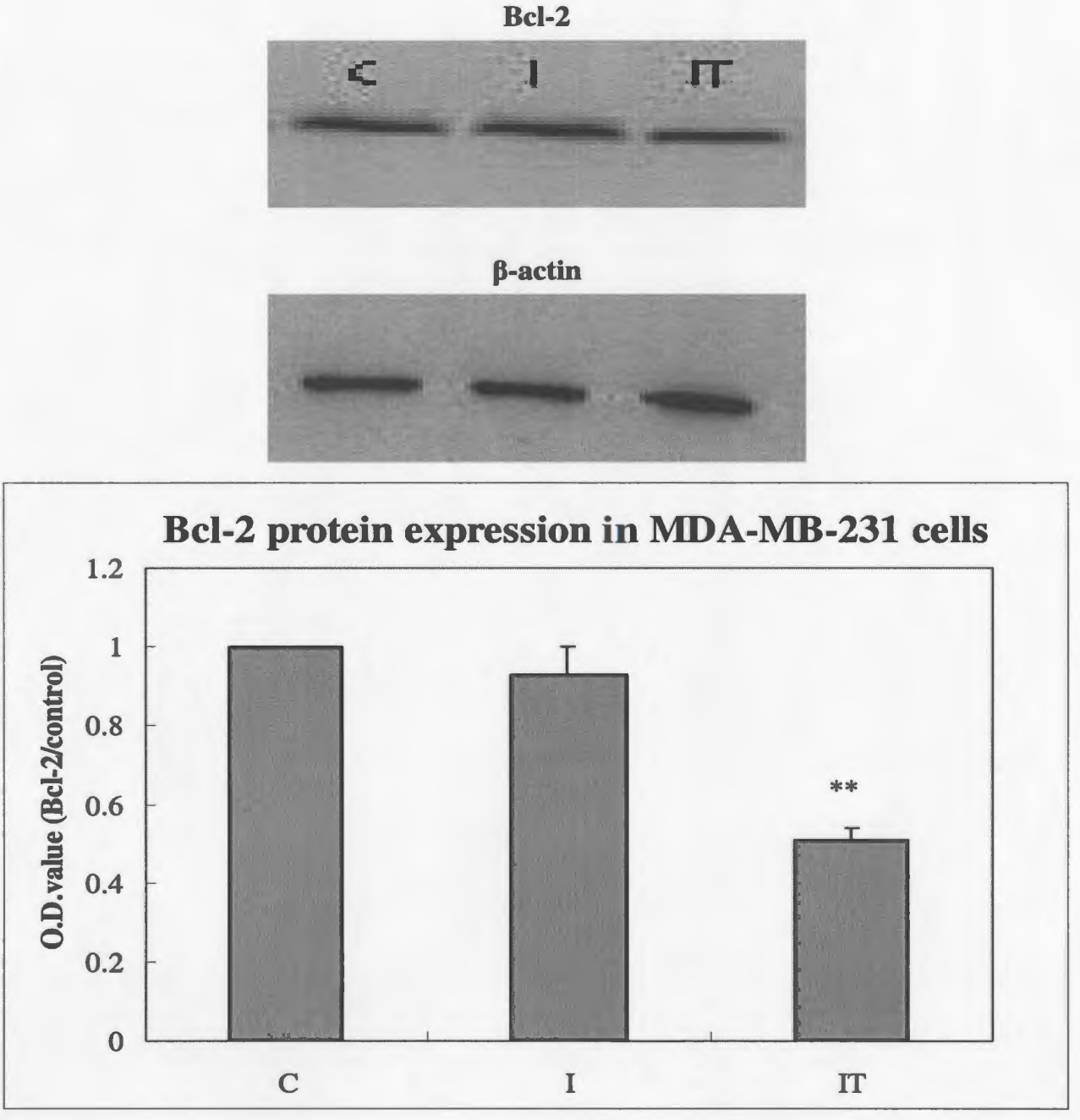


Figure 4.31 The expression of Bcl-2 protein in MDA-MB-231 cells determined by Western blot.

C: control (cells without treatments); I: cells treated with 0.016% soybean oil, and IT: cells treated with 0.016% soybean oil in combination with 100 nM paclitaxel for 24 h, respectively. **: statistical significance at $P<0.01$ compared with control. The O.D. values are expressed as mean ± SD from four separate experiments. Photographs are representative of four separate experiments.

Chapter 5 Conclusions

5.1 Seal oil induced cytotoxicity and apoptosis in MCF-7 and MDA-MB-231 cells

This study showed that seal oil ranging from 0.004% to 0.032% was toxic and resulted in cell death in both MCF-7 cells, an estrogen-dependent breast cancer cell line, and MDA-MB-231 cells, an estrogen-independent breast cancer cell line.

Apoptosis was shown to be responsible for the seal oil-induced cell death. Morphological changes in cells exposed to 0.016% seal oil for 24 h were observed and such changes are characteristic to apoptosis. In addition, DNA strand breaks and membrane alterations were also demonstrated. The seal oil-induced apoptosis appeared to be associated with the down-regulation of the expression of Bcl-2, an anti-apoptotic protein, in both cell lines. The production of lipid peroxides was also increased in cells treated with 0.016% seal oil for 24 h, which was accompanied by an increased level of PUFAs in the lipid composition in cells as determined by GC. These results suggested that the increased amount of intracellular PUFAs was likely responsible for lipid peroxide increasing, and in turn, might be responsible for the apoptosis induction.

5.2 Seal oil enhanced paclitaxel-induced cytotoxicity and apoptosis in MCF-7 and MDA-MB-231 cells

When combined with different concentrations of paclitaxel ranging from 0.39 μM to 400 μM, 0.016% seal oil was shown to enhance the paclitaxel-induced cytotoxicity upon incubation for 24 h in both MCF-7 and MDA-MB-231 cell lines. The IC_{50} values of paclitaxel were decreased

3.3-fold in MCF-7 cells and 2.4-fold in MDA-MB-231 cells, respectively.

Paclitaxel (100 nM) was shown to induce apoptosis in both cell lines upon incubation for 24 h. The apoptosis in MCF-7 cells was accompanied by the up-regulation of p53 protein expression and down-regulation of Bcl-2 protein expression; whereas, the down-regulation of both p53 and Bcl-2 proteins were demonstrated in MDA-MB-231 cell line. More cells were found undergoing apoptosis and the expression of Bcl-2 was further inhibited in both MCF-7 and MDA-MB-231 cell lines when cells were treated with 100 nM paclitaxel in combination with 0.016% seal oil. In addition, the levels of PUFAs in the intracellular lipid composition and lipid peroxide products were shown to be elevated significantly in the cells when seal oil was included. These results suggested that the enhanced apoptosis by 100 nM paclitaxel in combination with 0.016% seal oil in comparison with 100 nM paclitaxel alone was likely due to the increased inhibition of Bcl-2 protein expression and the increased intracellular lipid peroxide products.

5.3 Effect of soybean oil on MCF-7 and MDA-MB-231 cells

The effect of soybean oil on the growth of MCF-7 and MDA-MB-231 cells was found to be cell type-dependent. Soybean oil showed a greater cytotoxicity than seal oil in MCF-7 cells. However, it promoted the growth of MDA-MB-231 cells. Unlike seal oil, soybean oil did not induce apoptosis neither of the two cell lines based on the assessment of cell morphology. Western blot assay showed that soybean oil had little effect on the expression of p53 protein but increased the expression of Bcl-2 protein in MCF-7 cells. In MDA-MB-231 cells, the expression of mutant p53 protein was decreased by soybean oil but little change was observed with the expression of

Bcl-2 protein.

In conclusion, this study suggested that seal oil rich in ω-3 PUFAs may be beneficial in treating breast cancer. Seal oil may be given as a dietary supplement or an adjunct therapy with chemotherapy to cancer patients. In addition, seal oil emulsion may have the potential to be used for the formulation of paclitaxel or other chemotherapeutic drugs if it indeed is proven to be beneficial for treating cancer conditions because it may serve as a good solvent for hydrophobic drugs.

Chapter 6 Discussion

6.1 Experiment design and optimization

6.1.1 Cell culture and MTT assay

The purpose of this study was to investigate the activity of harp seal oil and its effect on paclitaxel-induced cytotoxicity and apoptosis in breast cancer cell lines. Because lipophilic seal oil is unable to be dispersed uniformly in the aqueous cell culture medium, seal oil stock emulsion containing 10% (w/v) of purified seal oil prepared by lipid extrusion method using high pressure homogenizer was employed in the experiments. In order to examine the cytotoxicity of seal oil, different concentrations of seal oil in cell culture medium ranging from 0.004% to 0.032% (w/v) were prepared by diluting seal oil stock emulsion with DMEM. MTT assay was used to measure the cell viability. When cells were treated with 0.004% seal oil for 24 h, it was found that the cell viability only slightly decreased to 98.4±0.9% in MCF-7 cell line and 94.3±3.7% in MDA-MB-231 cell line. Thus, concentrations below 0.004% were not tested. When the concentration of seal oil exceeded 0.032% in the cell culture medium, a visual flocculent and turbid mass was found in the medium, which was not desirable for cell growth. Therefore, concentrations higher than 0.032% were not studied either.

The MTT assay is based on the conversion of the yellow MTT tetrazolium salt to a purple formazan product by the living cells, which is reflected through the absorbance read by spectrometry. To quantify the living cells properly, the absorbance should be linear with the amount of living cells. It was found that there was a linear relation between the number of cells

seeded at 0.2 – 0.5 x 10^5 cells/100 μL/well in 96-well plates and the absorbance read at 570 nm with a reference wavelength of 630 nm. Hence, 0.5 x 10^5 cells/100 μL/well were seeded in 96-well plates for the MTT assay.

6.1.2 Cytotoxicity of DMSO

Paclitaxel is a highly hydrophobic compound. In this study, DMSO was used as a solvent to dissolve paclitaxel. It has been previously determined (Butt, 2004) that 0.5% (v/v) of DMSO in cell culture medium causes little toxicity to the cells, while it is sufficient to keep paclitaxel dissolved in DMEM. Hence, prior to each experiment, paclitaxel stock solutions (100 mM or 100 μM in DMSO stored at -20 ^{o}C) were diluted with DMEM and the concentration of DMSO was kept below 0.5% in all working solutions. In order to avoid any potential interaction between DMSO and seal oil in studies where a combination of seal oil and paclitaxel was used, paclitaxel in DMSO solution and seal oil were mixed by DMEM just prior to the experiments.

6.1.3 Apoptosis

Cytotoxic agents often induce only a fraction of cells to become apoptotic (Saunders *et al.*, 1997). It was demonstrated in this study that the amount of apoptotic cells determined by Hoechst staining and TUNEL was less than the mortality detected by MTT assay. The induction of apoptosis caused by cytotoxic agents is usually concentration and time dependent. It has been reported that the apoptotic responses to paclitaxel are often observed at 10 to 500 nM (McCloskey *et al.*, 1996, Saunders *et al.*, 1997 and Huang *et al.*, 1997), which are 100 to 1000-fold below those that are effective against breast cancer cells and bring about non-specific necrotic cell death (Saunders *et al.*, 1997).

In our lab, it has been previously determined that 100 nM of paclitaxel induced efficient apoptosis in MCF-7 and MDA-MB-231 cells upon incubation for 24 h (Butt, 2004). In this study, higher concentrations of paclitaxel including the IC_{50} concentrations and longer incubation periods i.e., 48 and 72 h were examined. However, under these conditions, the majority of cells underwent necrosis and the characteristics of apoptosis were not demonstrated. Therefore, in order to examine the apoptosis induced by paclitaxel, 100 nM paclitaxel and incubation of 24 h were chosen for subsequent studies. Based on the cytotoxicity results, seal oil at 0.016% showed significant toxicity to cells upon incubation for 24 h, resulting in approximately 20% of cell death. This concentration did not cause any visual flocculent phenomenon in the medium. Therefore, 0.016% seal oil was used to investigate the effect of seal oil on paclitaxel-induced apoptosis.

Apoptosis is a highly programmed process and apoptotic cells are associated with unique morphological and biochemical characteristics. Apoptosis starts with the alteration of cell membrane with no compromise in integrity, i.e., the translocation of PS from the inner side of the plasma membrane to the outer layer. And then chromatin aggregation at the nuclear membrane, condensation of the nucleus and shrinking of cytoplasm occur. Apoptosis ends with cell fragmentation into apoptotic bodies. Various methods developed based on these changes are used to evaluate the occurrence and/or progress of apoptosis. In this study, Hoechst staining that detects chromatin aggregation, TUNEL that assesses DNA fragmentation and Annexin-V-FLUOS assay that measures membrane alteration were performed to evaluate apoptotic changes occurred.

In addition to determining the membrane alteration in apoptotic cells upon fluorescence microscopy, Annexin-V-FLUOS assay can discriminate and quantify cells in difference stages

using flow cytometry. The simultaneous application of two staining dyes, Annexin-V-FITC and PI, can discriminate normal cells which would be Annexin-V-FITC negative and PI negative, from apoptotic cells which would appear Annexin-V-FITC positive and PI negative, and necrotic cells which would be Annexin-V-FITC positive and PI positive. Through a flow cytometer, the cells in the three stages can be clearly demarcated and quantified with different clusters. However, in this study, both MCF-7 and MDA-MB-231 cells are adherent cells. In order to apply the technology of flow cytometry properly, cells need to be dissociated from the surface of cell culture plates and suspended in test tubes. Trypsinization and any other sample processing methods may result in false positive staining due to the inevitable occurrence of cell death. Therefore, flow cytometry was not applied to quantify the apoptotic cells in these two types of adherent cell lines in this study.

6.2 Effects of seal oil and soybean oil on breast cancer cells

6.2.1 Apoptosis caused by seal oil

In this study, it was found that seal oil-induced apoptosis was accompanied by the down-regulation of Bcl-2 protein expression and increase of lipid peroxide generation. This suggests that there might be a relation between the lipid peroxidation and some organelles that are involved in seal oil-induced apoptosis such as mitochondria.

Mitochondria are considered as powerhouses for the vast majority of O_2 consumption and believed to be a major site of ROS production during respiration because electron transport throughout the mitochondrial respiratory chain is extraordinarily efficient. On the other hand,

mitochondrial dysfunction is one of the characteristics of apoptosis. Vladimir (Vladimir, 1996) suggested the opening of transition permeability pores of mitochondria upon apoptosis. It was reported that increased O_2 consumption in mitochondria might increase the amount of ROS production, while increased ROS levels would induce the opening of transition permeability pores of mitochondria. Maintaining the pore opening requires membrane potential ($\Delta\psi_m$). When ROS accumulates in mitochondria, long-term pore opening causes the membrane potential to collapse, preventing the uptake and retaining of precursors by the mitochondria for protein synthesis. In addition, long-term pore opening also causes imbalanced osmosis between the mitochondrial matrix and the intermembrane space because the pores are permeable for certain compounds with low molecular weights. As a result, the outer membrane would swell and burst. Consequently, proteins including pro-apoptotic factors such as cytochrome c and apoptosis-inducing factors (AIF) located in the intermembrane space would be released into the cytosol. When the pro-apoptotic factors reach a sufficient amount in the cytosol, apoptosis would be initiated and morphological changes would occur, leading to the elimination of ROS-producing cells by apoptosis (Vladimir, 1996). Thus, mitochondria are believed to be involved in ROS-induced apoptosis by releasing various classes of apoptotic molecules from the intermembrane space.

It has been reported that Bcl-2, Bax and Bcl-X_L proteins are able to form ion channels and pores with different conformations in the membrane (Fisher, 2001). In mitochondrial membrane, Bcl-2 protein interacts with the permeable pores and controls pore conformation. By forming pores, Bcl-2 protein can maintain the $\Delta\psi_m$ of mitochondrial membrane and prevent the membrane from disruption, impeding the release of apoptotic factors from mitochondria (Petit *et al.*, 1996).

Pro-apoptotic member Bax localizes largely in the cytosol, but following exposure of apoptotic stimuli, Bax inserts into the membrane of mitochondria to form homodimers and heterodimers. Translocated Bax might decrease Bcl-2 protein expression by forming heterodimers with Bcl-2 thereby abrogating Bcl-2 capability. In addition, Bax homodimers can form selective channels and result in increased plasma membrane permeability, inducing the loss of $\Delta\psi_m$, and allowing cytochrome c and AIF escape into cytoplasm to activate downstream caspases to execute apoptosis (Reed, 1998 and Hersey *et al.*, 2003).

In this study, seal oil-induced apoptosis accompanied by the down-regulation of Bcl-2 protein expression might be associated with the dysfunction of mitochondria, as seal oil rich in highly unsaturated PUFAs was an exogenous source of ROS in cells. The constant stimulation of seal oil-produced ROS might destroy the normal mitochondrial function, resulting in the release of the pro-apoptotic factors into cytoplasm. These factors are able to induce nuclear chromatin condensation and fragmentation, which were observed under fluorescence microscopy. Additionally, they might trigger some of the cascades to inhibit Bcl-2 protein expression. Under the apoptotic stimulation, the expression of Bcl-2 protein might be also inhibited by forming heterodimers with Bax protein which translocated from cytoplasm to the membrane of mitochondria.

6.2.2 Bcl-2 protein, lipid peroxidation and apoptosis

Bcl-2 protein has been suggested to possess antioxidant ability (Halder *et al.*, 1995 and Tyurina *et al.*, 1997). The majority of Bcl-2 protein localizes as a membrane protein to the intracellular sites where oxygen free radicals are generated including mitochondria, endoplasmic

reticulum and nuclear membrane (Hockenbery *et al.*, 1993). In mitochondria, for example, Bcl-2 protein has a patchy distribution linked to the contact sides between the outer and the inner membrane (Petit *et al.*, 1996), which is likely associated to its property as an antioxidant to inhibit apoptosis by suppressing the formation and effect of ROS.

Lipid peroxidation in mitochondria is initiated by hydrogen extraction of $OH^{\cdot}$ radical and other potential ROS. A conjugated diene resulted then acts with O_2 to form a peroxy radical $ROO^{\cdot}$, which extracts $H^{\cdot}$ from another fatty acid to establish an autocatalytic free radical chain reaction. The production of hydroxyl radicals results in hydrogen extraction, hydrogen addition, and electron transfer, which mediates the lipid peroxidation and oxidative damage to DNA and sulfhydryl modification of proteins (Lee *et al.*, 2004). Bcl-2 protein may decrease the generation or increase the scavenging of the ROS by functioning as a direct radical scavenging protein and an inhibitor of electron transfer in the mitochondria inner membrane (Kane *et al.*, 1993), thus decreasing the formation of ROS in order to prevent their damages.

The fact that Bcl-2 protein acts as an antioxidant and scavenging protein against oxidative stress that mediates cytotoxicity implies that there might be a balance among the level of Bcl-2 protein expression, exogenous PUFAs and the generation of lipid peroxide products. When cells were treated with seal oil, the redox balance might be affected by the exogenous PUFAs. With the increase of intracellular lipid peroxide produced by PUFAs, ROS would become predominant and the equilibrium might shift to the anti-oxidative side to suppress Bcl-2 activity and enhance lipid peroxidation, leading to the down-regulation of Bcl-2 protein expression and the occurrence of apoptosis.

In addition to changing the redox equilibrium, it is reported that peroxide products of some free PUFAs can also prevent Bcl-2 protein expression through binding Bcl-2 oncogenes or affecting other oncogenes such as suppressing *ras* and enhancing *fas* expression to block the trigger of Bcl-2 expression (Das, 1999).

Taken together, exogenous PUFAs can enhance lipid peroxidation and generate abnormal signalling species including free radicals and various oxidized fatty acid products, causing mitochondrial dysfunction and the release of pro-apoptotic factors. As a consequence, the signalling species suppress the expression of the anti-apoptotic Bcl-2 protein by inducing the formation of heterodimers with Bax protein, interrupting the redox equilibrium and affecting the oncogenes which are capable of triggering Bcl-2 gene expression.

Down-regulation of Bcl-2 protein is regarded as a strategy in the therapy of cancers because overexpression of Bcl-2 protein is a common problem and is also one of the mechanisms causing drug resistance. The fact that seal oil inhibited Bcl-2 protein expression indicates that it might be a promising agent in improving the response to chemotherapeutic drugs during cancer treatments.

6.2.3 Promising role of seal oil on chemotherapeutic and hormonal therapy in breast cancers

In this study, seal oil not only showed cytotoxicity in both MCF-7 and MDA-MB-231 cell lines, but also enhanced the cytotoxicity of paclitaxel in these cell lines, as evidenced by the greater toxicity of paclitaxel with lower IC_{50} values in combination with seal oil than paclitaxel alone. Since several receptors and transporters which are involved in the uptake of extracellular nutritional substances and drug molecular are located in the membrane, the accumulation of PUFAs derived from seal oil in cell membrane might alter the normal barrier function and the

physical and biochemical characteristics of the receptors and transporters located. It has been reported that in DU-145 prostate cell line, free PUFAs such as EPA, AA and OA were found to have a high affinity for androgen receptor (AR) and significantly decrease the binding of AR with androgen in DU-145 cells treated with PUFAs (Prinsloo *et al.*, 2002). In MCF-7 cells treated with DHA, it was found that the binding capacity and affinity for estrogen with the receptors was decreased by DHA and the decrease was in a DHA dose-dependent manner (Borras *et al.*, 1992). The change of biochemical properties of the receptors might lead to the alteration of intracellular signal transfer and cell survival. In addition, the accumulation of PUFAs in the membrane may also increase the membrane permeability so that higher intracellular concentration of drugs could be reached resulting in higher sensitivity and lower drug resistance of the cells.

ω-3 PUFAs were reported to be able to alleviate the resistance against endocrine therapy as well. Breast cancer with tamoxifen resistance has been found to have high activity of Akt, a downstream mediator in the protein kinase signalling pathway. Activation of Akt endows breast cancer with aggressiveness and resistance to the hormone- and chemotherapy-induced apoptosis. Cells with high Akt activity have also been reported to be resistant to cytotoxic agents such as paclitaxel and doxorubicin (deGraffenried *et al.*, 2003). ω-3 PUFAs have been proven to be potent and effective broad-spectrum protein kinase inhibitors. When employed to tamoxifen-resistant breast cancer cells with hyperactive Akt, EPA restored tamoxifen's activity against breast cancer cell growth, and significantly inhibited Akt kinase activity (deGraffenried *et al.*, 2003). Based on these reports and the results in this study, it is therefore supposed that seal oil rich in long chain ω-3 PUFAs such as DHA and EPA may be useful for the tamoxifen and paclitaxel-resistant breast

cancers to improve the therapeutic efficiency.

6.2.4 Effect of soybean oil on breast cancer cells

In this study, soybean oil, which contains high amounts of ω-6 PUFAs (mainly LA), exhibited different effects on the two cell lines. It was found to be cytotoxic to MCF-7 cells but beneficial to MDA-MB-231 cell growth. It has been reported that LA stimulated the proliferation of mammary epithelial cells *in vitro* in the presence of epidermal growth factor (EGF) (Bandyopadhyay *et al.*, 1987 and 1988). EGF is one of the growth factors to modulate the proliferative effects. Mammary epithelial cells with hormone-independent phenotype such as MDA-MB-231 cells have an increased expression of EGF-R and increased level of EGF replacing ER and estrogen (Clarke *et al.*, 1989). In this study, the effect of soybean oil on MDA-MB-231 cell growth might be ascribed to the higher level of EGF found in MDA-MB-231 cells in comparison to that in MCF-7 cells. In addition, unlike MCF-7 cells, MDA-MB-231 cells are known to possess efficient Δ6 and Δ4 desaturase activity and lipoxygenase activity (Rose, 1997); therefore, the high level of LA from soybean oil could have high tendency to undergo desaturation, elongation, to form AA and consequently subject to eicosanoid metabolism in MDA-MB-231 cells. Since AA and the eicosanoid metabolites are believed to stimulate tumour cells growth, MDA-MB-231 cell growth induced by soybean oil might be likely a result of the elevated AA metabolism.

6.3 Comparison of MCF-7 and MDA-MB-231 breast cancer cells

6.3.1 Sensitivity to paclitaxel

In this study, the MCF-7 breast cancer cell line was chosen to represent estrogen-dependent

cells with wild-type p53 protein, while the MDA-MB-231 cell line represents estrogen–independent cells with mutant p53 protein. MTT assay results indicated that the MCF-7 cell line was more sensitive to paclitaxel than the MDA-MB-231 cell line which had a lower IC_{50} value. One of the possible reasons could be that MCF-7 cells have a relatively low proliferation in the medium without additional estrogen supplement, and the treatment with paclitaxel further deteriorates the conditions for the cell growth, causing more MCF-7 cell death. This fact makes it understandable that the depletion of estrogen is a strategy of treating estrogen-dependent breast cancers, and in clinic, anti-hormone therapy such as using tamoxifen or androgen to antagonize estrogen with adjuvant chemotherapy has been applied as a treatment for patients with estrogen-dependent breast cancers (Mamounas *et al.*, 2001).

6.3.2 Effect of paclitaxel and seal oil on the expression of p53 protein

Wild-type p53 gene is a pro-apoptotic gene as well as DNA repair gene. It exerts most of its tumour suppressing activity through transcriptional activation of target genes. As a sequence-specific transcription factor, wild-type p53 gene binds to specific DNA as a tetramer and activates transcription of cell cycle arrest (Gartel *et al.*, 2002). It has been reported that about 50% of breast tumours experience p53 mutations (Bautista *et al.*, 1997 and Hartman *et al.*, 1997). Most p53 gene mutations are in the hydrophobic mid-region of the protein with missense points. For example, the majority of p53 mutations are with T:A or C:G transversions from G:C. These transversions produce missense proteins with altered or absent transcriptional regulation activities and high concentrations of p53 protein detectable by immunohistochemmistry (Hartman *et al.*, 1997) as compared with wild-type p53 protein having an extremely short

half-life and small amount present in the cell nucleus (Bautista *et al.*, 1997). Mutant p53 protein can also block wild-type p53 function by forming oligomers to abrogate the effect. The loss of p53 function eliminates the growth arrest in the G1 to S phase transition which is in response to certain DNA damage, and enhances the frequency of damaged gene amplification (Hartman *et al.*, 1997). Cells bearing defective p53 proteins bypass these checkpoints and progress through cell cycle with damaged DNA leading to the accumulation of unhealed chromosomal genome. Meanwhile, p53 mutations emerging during malignant progression in tumours leads to resistance to certain anticancer treatments, especially to those with radiotherapy and DNA-damaging agents such as doxorubicin (Bunz *et al.*, 1999 and Gartel *et al.*, 2002).

The Western blot study showed that the expression of p53 protein in MCF-7 cells was elevated by paclitaxel, suggesting that the wild-type p53 protein was involved in the paclitaxel-induced apoptosis in MCF-7 cell line. According to this result, it can be seen that paclitaxel plays roles on the entire MCF-7 cell cycle as it can not only induce necrosis by causing the polymerization of tubulin and sustained mitotic block in the late G2 or M phases of the cell cycle, but also induce apoptosis by triggering wild-type p53 expression to arrest cell cycle in the G1 to S phase.

The expression of mutant p53 protein in MDA-MB-231 cells was attenuated by paclitaxel. However, whether the decrease is associated with the paclitaxel-induced apoptosis is not clear.

The expression of the wild-type p53 protein was not increased by seal oil in MCF-7 cells although the ROS generated from PUFAs were expected to damage many subcellular structures including DNA, which could trigger the expression of p53 protein. On the other hand, seal oil was

found to increase the expression of mutant p53 protein in MDA-MB-231 cells. These results suggested that the seal oil-induced apoptosis in MCF-7 cells might be independent of p53 protein pathway.

6.3.3 Uptake of PUFAs from seal oil in the extracellular environment

The occurrence, development and proliferation of breast cancer not only need hormone stimuli but also other growth factors. Studies have demonstrated that both low- and high-density lipoproteins affect the proliferation of breast cancer cells in cell culture (Rotheneder *et al.*, 1989 and Stranzl *et al.*, 1997). The lipoproteins can bind to the glycoprotein receptor present in most types of cells in the cytosolic and nuclear fractions to form ligand-receptor complex. The complex stimulates protein synthesis and secretion for cell growth (Prinsloo *et al.*, 2002). Although MDA-MB-231 cell line is an estrogen receptor negative cell line, it has other factors attributed to the development and progression of malignancy. For example, it has a higher level of an otherwise regulated gene, low-density lipoprotein receptors (LDL-R) mRNA than that in MCF-7 cells (Rotheneder *et al.*, 1989 and Stranzl *et al.*, 1997). It also exhibits a higher affinity for low-density lipoproteins in comparison to MCF-7 cells (Rotheneder *et al.*, 1989). As a result, MDA-MB-231 cells can take up sufficient low-density lipoproteins, the carrier of cholesterol and other growth factors in plasma, to meet the need for significant proliferation (Rotheneder *et al.*, 1989).

PUFAs are precursors of steroidal hormones, and participate in the biosynthesis of some hormone derivatives, e.g., prostaglandin series. In the cell culture medium containing FBS, PUFAs

might be taken up by binding to proteins such as albumin and are transported through binding to the ERs and/or LDL-Rs. Thus, PUFAs have the tendency to compete with other necessary nutritional substances for cell growth, thereby blocking the receptors and transporters.

According to the results of GC assay, intracellular lipid composition change was different in these two cell lines. The increase of ω-3 PUFAs in MCF-7 cells was lower than that in MDA-MB-231 cells while the increase of ω-6 PUFAs in MCF-7 cells was relatively more than that in MDA-MB-231 cells. These differences indicate that the manner of PUFAs uptake and transport varies with different types of cell lines. In addition, from the cytotoxicity assay of seal oil, it can be seen that the two cell lines showed different sensitivities to seal oil, with MDA-MB-231 cell being more sensitive; suggesting that the cell sensitivity towards the PUFAs treatment was cell-type dependent and it might be attributed to the different receptors and transporters the cells possess.

6.3.4 Progression from estrogen-dependent to estrogen-independent growth in human breast cancers

The major problem in breast cancer therapy is that the treatments can cause changes that result in alteration of tumours in response to chemotherapeutical drugs and hormones. Many breast tumours with low metastatic potential initially express estrogen receptors and demonstrate high response to endocrine treatments, but ultimately lose estrogen-dependence and exhibit metastatic phenotype and resistance to endocrine treatments, revealing inevitable progression from estrogen-dependent to estrogen-independent growth (Clarke *et al.*, 1989). The potential mechanisms which could contribute to the progression of human breast cancer include growth factor production, increased sensitivity to growth factor and overexpression of oncogenes which

are associated with a more malignant phenotype. Dickson et al. (Dickson *et al.*, 1986) have presented that in an ovariectomized athymic nude mice model, some growth factor activities such as insulin-like growth factor I (IGF-I) activity and transforming growth factor α (TGF-α) like activity that binds to the epidermal growth factor receptor were completely stimulated by exogenous estrogen and sufficient to promote MCF-7 cells to form tumours. It is of note that these induced growth factors are partially capable of replacing estrogen, acting as estrogen-induced "second messengers" in estrogen responsive growth of human breast cancer, which might promote tumorigenesis (Dickson *et al.*, 1986). In addition, Reed et al. (Reed *et al.*, 1989 and Berkenstam *et al.*, 1989) reported that in MCF-7 cells, estrogen could reduce ER level and ER mRNA expression in a dose and time dependent manner, causing the loss of estrogen-dependence with long-term estrogen stimuli (Reed *et al.*, 1989 and Berkenstam *et al.*, 1989). Furthermore, without estrogen, estrogen-dependent cells have a potential to convert into malignant phenotype as well. Katzenellenbogen *et al.* (Katzenellenbogen *et al.*, 1987) reported that MCF-7 cells grown in absolute estrogen-free medium (5% charcoal-dextran-treated calf serum phenol red medium) maintained a reduced rate of cell proliferation in a one month treatment, whereas they showed a markedly increased rate of cell proliferation in during a 5-6 month treatment. This change in growth pattern might reflect altered regulation or sensitivity of growth factor in the cancer cells so that the cells could adapt to grow in the medium in the absence of estrogen. Cho *et al.* (Cho *et al.*, 1991) isolated an estrogen-independent MCF-7 subline from the parental estrogen-dependent MCF-7 cells after long term growth *in vitro* in the absence of estrogen. The authors demonstrated through this MCF-7 subline that the progression to estrogen-independent growth was

accompanied with a change in the regulation of some estrogen-induced genes such as pS2 gene, which is a useful marker under the direct control of estrogen in MCF-7 cells for estrogen responsiveness.

It is therefore suggested that estrogen-dependent cell line can change to estrogen-independent phenotype under certain conditions with long-term estrogen stimulation or absolute absence of estrogen due to the alteration of regulation of various growth factors. These observations aid to understand that estrogen-dependent breast cancer in patients may convert into malignant phenotype and the antiestrogen therapy in clinic causes resistance after long-term treatment. Currently, adjuvant therapy with hormonal therapy and systemic chemotherapy has been accepted as a strategy in breast cancer treatments, to prevent the local disease from progression to metastasis or to treat micrometastases before progression (Buzdar, 2001 and Mamounas *et al.*, 2001).

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