

STUDY OF SEAL OIL IN REDUCING THE  
NEPHROTOXICITY OF CYCLOSPORINE A

CENTRE FOR NEWFOUNDLAND STUDIES

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WEI YANG







**STUDY OF SEAL OIL IN REDUCING THE NEPHROTOXICITY OF  
CYCLOSPORINE A**

By

©Wei Yang

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## Abstract

Nephrotoxicity is a common and serious side effect associated with cyclosporine A (CsA). Fish oil, rich in  $\omega$ -3 polyunsaturated fatty acids (PUFAs), has been reported to be beneficial in alleviating the toxicities induced by CsA. Seal oil is another source of  $\omega$ -3 PUFAs. This project was designed to investigate the potential of using seal oil to reduce the nephrotoxicity of CsA.

The cytotoxicity of CsA alone and in combination with seal oil emulsion, docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), main PUFAs in seal oil, was determined using the MTT assay and lactate dehydrogenase (LDH) leakage in LLC-PK1 cell line. The effects of seal oil on the *in vivo* nephrotoxicity of CsA were investigated on two different experimental protocols: In the first experiment, CsA in 20% of seal oil or corn oil emulsion was administered to SD rats at 50 mg/kg/day *p.o* or 25 mg/kg/day *i.p.* for 28 days. In the second experiment, rats were kept on diets containing 7% sunflower oil, or mixture of 5.95% seal oil with 1.05% sunflower oil, four weeks prior to and during the four-week *i.v.* administration of normal saline (control) or CsA (15 mg/kg/day).

The results of the *in vitro* studies revealed that DHA reduced the cytotoxicity induced by CsA. *In vivo* studies demonstrated that the nephrotoxicity associated with CsA was attenuated by the concomitantly supplemented seal oil. The possible mechanism of beneficial effects of seal oil may be exerted via modulating biosynthesis of prostanoids, leading to a higher ratio of prostaglandins to thromboxanes production, with a net favorable vasodilatory effect.

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## Abbreviation

$\omega$  / n: omega

AA: arachidonic acid

ALA: alpha-linolenic acid

ATCC: American Type Culture Collection

BUN: blood urea nitrogen

CaN: calcineurin

Clcr: creatinine clearance

cm: centimeter

COX: cyclooxygenase

Cyp: cyclophilin

D: Dalton(s)

DGLA: dihomo- $\gamma$ -linolenic acid

DHA: Docosahexanoic acid

DMSO: dimethylsulfoxide

DPA: docosapentaenoic acid

FCS: fetal calf serum

EIA: enzymeimmunoassay

EPA: eicosapentaenoic acid

eV: electron-volt

FBS: fetal bovine serum

FDA: Food and Drug Administration

g: gram(s) or g-force (standard unit of centrifugation)

GC: gas chromatography

GFR: glomerular filtration rate

h: hour(s)

HCCS: Health Care Corporation of St. John's

HE: hematoxylin and eosin

HETE: hydroxyeicosatetraenoic acids

HPETE: hydroperoxyeicosatetraenoic acids  
IC<sub>50</sub>: concentration of drugs causing 50% inhibition of cell growth  
IFN- $\gamma$ : interferon- $\gamma$   
IL-2: interleukine-2  
k: kilo  
L: liter(s)  
LA: linoleic acid  
LC-MS: liquid chromatography- mass spectrometry  
LDH: lactate dehydrogenase  
LOX: lipoxygenase  
LT: leukotrienes  
M: mol/L  
M199: Medium 199  
MAF: macrophage activating factor  
MCF: macrophage chemotactic factor  
MHC: major histocompatibility complex  
min: minute(s)  
m: milli-  
MLR: mixed lymphocyte reaction  
MRM: multiple reaction monitoring  
MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tertrazolubromide  
NAG: urinary N-Acetyl-1- $\beta$ -D-glucosaminidase  
NK cells: natural killer cells  
nm: nanometer  
NS: normal saline  
PBS: phosphate-buffered saline  
PG: prostaglandins  
P-gp: P-glycoprotein  
RIA: radioimmunoassay

ROS: reactive oxygen species  
RPF: renal plasma flow  
rpm: rounds per minute  
SBP: systolic blood pressure  
SD: Sprague-Dawley  
sec: second(s)  
TAG: triacylglyceride  
TBARS: thiobarbituric acid reactive substances  
TNF: tumor necrosis factor  
TX: thromboxanes  
V<sub>E</sub>: Vitamin E  
w: week(s)

# Chapter 1. Introduction

The focus of this work was to study whether seal oil, rich in long chain omega-3 ( $\omega/n-3$ ) polyunsaturated fatty acids (PUFAs), could render protection against cyclosporine A (CsA) induced nephrotoxicity. A general overview about CsA, the characteristics of CsA-induced nephrotoxicity and the beneficial health effects of  $\omega-3$  PUFAs are discussed in this chapter.

In the past two decades, the short-term success rate of solid organ transplantation including kidney, heart and liver reached unprecedented high levels. Eighty-five to ninety percent 1-year graft survival rate for kidney allograft has been achieved in most organ transplantation institutions all over the world, whereas the survival rates were about 60% in 1980 before the introduction of CsA. The survival rates for heart, liver, and lung transplantation have been similarly improved [Dunn *et al.*, 1990; Beers *et al.*, 1999]. This great achievement is mainly due to cyclosporine-based immunosuppressive regimens, and CsA became the cornerstone of modern immunosuppression for organ transplantation. Because of the success of CsA in solid organ transplantation, its usage was also extended to other refractory conditions, such as autoimmune diseases. Further research has shown that CsA also possesses anti-inflammatory [Hewitt, Black, 1996], antiproliferative [Blankenship *et al.*, 2003], antiparasitic and other properties [Dambrin *et al.*, 2000]. In cancer therapy, CsA is also used as a re-sensitizer together with anticancer agents for the treatment of those patients who develop resistance to chemotherapy [Nielsen *et al.* 2002].

## 1.1. Physicochemical properties of CsA

CsA is a fungal metabolite and possesses potent immunosuppressive effect. It was first isolated from the fermentation of the fungus *Beauveria nivea* (formally *Tolyposcladium inflatum* GAMS) [Csawell, A] in 1972 [Flechner, 1983]. It is a cyclic peptide containing 11 amino acids with a molecular weight of 1203 (Figure 1.1.). This structure includes a  $\beta$ -hydroxyl, singly unsaturated C9 amino acid that was never before observed in nature at position number 1, as well as a D-alanine at position 8. Seven of the amino acids at position 1, 3, 4, 6, 9, 10, 11 are N-methylated that make CsA extremely hydrophobic [Dreyfuss, 1976].

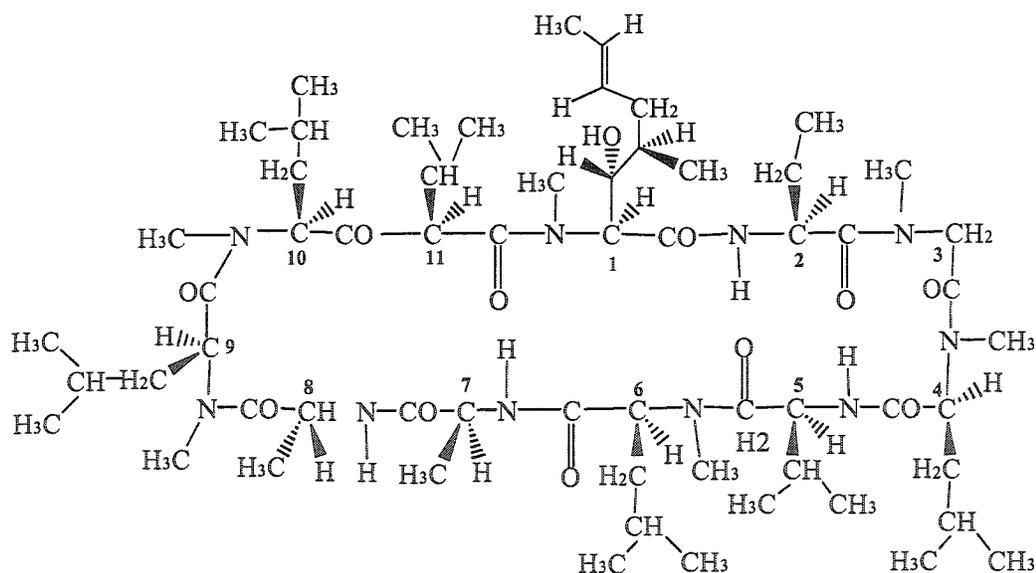


Figure 1.1. Chemical structure of cyclosporine A (adapted from *Helv Chim Acta* 1976; 59(5): 1480).

The compound is neutral and insoluble in water and *n*-hexane, soluble in ethanol and most other organic solvents and lipids. Therefore, it is usually administered by oral route in a lipid-based vehicle such as olive oil and peanut oil. Currently there are two commercial products of CsA: Sandimmun® and Neoral® available for clinical use.

## 1.2. Pharmacokinetic properties of CsA

Upon oral administration, CsA is absorbed from the upper small intestine and its absorption is influenced by the rate of bile flow, food intake, concomitant medications and excipients used for administrating CsA. The absorption of CsA from the gastrointestinal tract is variable and incomplete, averaging 34%, with wide variability within and among patients, ranging 20-50% [Lemaire *et al.*, 1990]. Initially, the mean absolute oral bioavailability from the first generation formulation of Sandimmune®, a solution of CsA in vegetable oil derivatives containing some other inactive excipients, was 25-30%, with up to 10-time inter-individual variations of CsA concentrations in blood [Fahr, 1993]. Then a new formulation named Neoral® was developed. With this formulation, CsA is incorporated into a microemulsion concentrate that contains lipophilic solvents, surfactants and ethanol. Because of the small particle size of the microemulsion and high degree of dispersion upon contacting with aqueous gastrointestinal fluid, absorption of Neoral® is significantly improved and more repeatable, with the oral bioavailability of 50-60%, lower inter- and intra-individual variations, and less food dependence [Mueller *et al.*, 1994; Kovarik *et al.*, 1994].

In blood, about 2/3 of the CsA are bound to red cells and 1/3 to plasma lipoproteins. Only about 5-10% of the drug molecules are in a free form. Therefore, its therapeutic efficacy and toxicity may easily be influenced by lipoprotein levels and hematocrit. Due to its extreme hydrophobicity, CsA has a large volume of distribution throughout the body and high concentrations in adipose tissue, liver, and pancreas. The drug does not preferentially accumulate in kidney [Kahan, 1985a].

CsA is metabolized extensively to several metabolites by the hepatic and intestinal cytochrome P450 3A (CYP3A4) mixed function oxidase enzymes, with the parameters as follows:  $T_{1/2}$ : 18 h,  $T_{max}$ : 1.4 h (ranging 1-8 h);  $V_c$  (central compartment volume): 1 L/kg;  $V_z$  (distribution volume of the elimination phase): 5.8 L/kg [Baraldo *et al.*, 2001]. Moreover, the exorption pump P-glycoprotein (P-gp) along the intestinal wall simultaneously pumps the absorbed drug molecules back to the lumen, preventing its accumulation inside the intestinal cells. These actions in a large part account for the low oral bioavailability of CsA. Because of the high dependence on cytochrome P450 enzyme, CsA interacts easily with other concomitant administered medicines. Drugs that inhibit cytochrome P450 can increase the concentrations of parent CsA, and therefore cause side effects. On the other hand, drugs that induce the enzymes can decrease the concentrations of parent CsA and lead to reduction of the immunosuppressive effects. The majority of the metabolites are excreted in the bile and only a small amount through renal route into urine [Barone *et al.*, 1996].

### 1.3. Immunosuppressive profiles of CsA

In 1977, Borel *et al.* [Borel *et al.*, 1977] reported the immunosuppressive effects of CsA. The clinical use of CsA to prevent organ transplantation rejection in oral or intravenous dosage forms was approved by the Food and Drug Administration (FDA, USA) in 1983. Studies showed that CsA was effective in inhibiting both humoral and cell-mediated responses. *In vivo*, CsA strongly suppresses humoral immune response, which involves the production of antibody in response to specific antigenic stimulation, but it is the production of antibody by B-lymphocytes against T-cells dependent antigens that is strongly suppressed [Borel *et al.*, 1994]. In addition, CsA also strongly suppresses cell-mediated immunity, which plays a major role in delayed-type hypersensitivity, autoimmune response, and the rejection of transplanted organs [Borel *et al.*, 1977]. Moreover, preferential action of CsA on T-cells rather than B-lymphocytes had been demonstrated in mice, rat, and porcine cells [Burchardt *et al.*, 1979].

CsA also inhibits cytokine production and its major immunosuppressive activities are exerted by blocking interleukine-2 (IL-2) synthesis [Schrieber, Crabtree, 1992]. IL-2 is a lymphokine produced by activated helper T-cells when stimulated with IL-1. It has a wide spectrum of effects on immune responses. IL-2 affects clonal expansion of activated T-cells and natural killer cells (NK), and plays a key role in immune responses. Researchers [Bunjes *et al.*, 1981] discovered that in human and mouse mixed lymphocyte reaction (MLR), which is an *in vitro* event reflecting host reaction to grafts, the production of IL-2 was decreased in the presence of CsA. Decreased IL-2 synthesis leads to reduced T-cell activation or prolonged periods of T-cell unresponsiveness [Schwartz,

1990] and thus inhibits allograft rejection. Besides IL-2, CsA was also found to inhibit the production of various immunomodulators, such as interferon- $\gamma$  (IFN- $\gamma$ ), IL-6, macrophage chemotactic factor (MCF), and macrophage activating factor (MAF) [Abbud-Filho *et al.*, 1984].

The unique characteristic of CsA is that it acts specifically and reversibly on lymphocytes, and preferentially on T- rather than B-lymphocytes. Unlike other immunosuppressive agents such as Azathioprine and cyclophosphamide, CsA has no functional effect on granulocytes, thrombocytes, macrophages or phagocytic cells [Paavonen *et al.*, 1981]. It was also found that CsA lacks of lymphocytotoxicity since it has no effect on the viability of unstimulated lymphocytes. Therefore, CsA acts early and reversibly on the triggering of lymphocyte proliferation, rather than interrupting the stimulation of antigen [White *et al.*, 1979]. One of the major advantages of CsA is lack of myelosuppression, which was a major limitation in using the conventional immunosuppressive agents.

#### **1.4. Mechanisms of action of CsA**

Study of the action of CsA at a molecular level demonstrated that its effect is dependent on binding to a substance to convert the drug to an active form, and undergo structural conversion. The substance that CsA binds to is a 16 kD cytoplasmic peptidyl-prolyl isomerase named cyclophilin (Cyp) [Handschumacher *et al.*, 1984]. The complex then binds to calcineurin (CaN) to form a pentameric unit to exert its immunosuppression.

CaN is a serine-threonine phosphatase with important regulatory effects on various genes, and requires calcium for its activation. In lymphocytes, CaN has a key role in the calcium-dependent pathway to the IL-2 gene. Normally, activation of a T-cell receptor at the surface of the cell causes increase of cytosolic  $\text{Ca}^{2+}$ , which subsequently causes an activation of CaN. Then CaN dephosphorylates a number of substrates within the cell. When dephosphorylated, these substrates enter the nucleus and regulate the transcription of various genes. One of these CaN substrates is nuclear factor of activated T-cells (NF-AT), which is the first regulatory protein critical for promotion of DNA transcription of mRNAs that encode IL-2, which induces maturation and proliferation of helper T-cells, and other proinflammatory cytokines, including interferon- $\gamma$  and TNF- $\alpha$  [Johansson *et al.*, 1990]. There was evidence suggested that CaN accompanies NF-AT into the nucleus, and itself participates in gene transcription by inhibiting the removal of NF-AT from the nucleus [Zhu *et al.*, 1999]. Once CaN is bound by CsA-Cpy complex, its activity is inhibited, and the downstream dephosphorylation and transport of NF-AT is consequently obstructed, leading to the interruption of IL-2 production [Liu *et al.*, 1992]. In this way, by impairing IL-2 production, CsA selectively suppresses IL-2 driven proliferation of activated T-cytotoxic lymphocyte, while sparing T-suppressor cells.

Besides the apparent inhibitory effects of CsA on CaN, the drug was also reported to enhance the expression of transforming growth factor- $\beta$  (TGF- $\beta$ ), a cytokine which not only has immunosuppressive effects, but also predisposes renal allografts to the progression of fibrosis [Shin *et al.*, 1998].

### 1.5. Renal effects of CsA

Compared to other immunosuppressive agents, CsA reversibly inhibits only some classes of lymphocytes, and does not affect haemopoietic tissues [Rehacek *et al.*, 1991]. Despite its unique advantage and immunosuppressive potency, the clinical use of CsA was limited by its frequent, diverse side effects. The reported side effects attributed to CsA include nephro-, neuro-, and hepatotoxicity, as well as hirsutism, gingival hyperplasia, hypertension and gastro-intestinal symptoms, among which nephrotoxicity and hypertension are the most serious and common [Myers, 1986; Klintmalm *et al.*, 1981; First *et al.*, 1994]. It is well known that CsA causes two kinds of nephrotoxicity: dose-related decrease in renal function, and morphological changes in both human and experimental animals.

The functional toxicity or acute nephrotoxicity includes dose-dependent reduction of renal plasma flow (RPF) and glomerular filtration rate (GFR), reflected by a decrease of creatinine clearance (Cl<sub>cr</sub>) and an increase in blood urine nitrogen (BUN). This is associated with altered hemodynamics, which is largely thought to be due to CsA-induced imbalance of production of vasoconstrictors and vasodilators that result in vasoconstriction of the afferent arterioles and glomerular capillaries [Remuzzi *et al.*, 1995; English *et al.*, 1987]. The mechanisms of its action are still under debate. There are many vasoactive factors found to be involved in CsA-induced nephrotoxicity: increased production of vasoconstriction factors, such as endothelin (ET) [Bunchman *et al.*, 1991; Darlametsos *et al.*, 2000], thromboxane A<sub>2</sub> (TXA<sub>2</sub>) [Rogers *et al.*, 1988], noradrenaline (NA) [Duruibe *et al.*, 1990], renin-angiotensin (R-A) [Siegl *et al.*, 1982]; reduced release

of vasodilator prostaglandins E<sub>2</sub>, I<sub>2</sub> (PGE<sub>2</sub>, PGI<sub>2</sub>) [Bennett *et al.*, 1988a]; and nitric oxide synthase inhibition, of which the vascular alterations produced by CsA are very similar to those observed at early stages of atherosclerosis with decreased ability of the endothelium to secrete nitric oxide [Gallego *et al.*, 1993]. These effects were reported to be reversible upon dose reduction [Skorecki *et al.*, 1992; Kahan *et al.*, 1985b]. These acute hemodynamic effects may not be the only consequences of CsA on the kidney. *In vitro* studies revealed that CsA exerted direct toxicity on cultured bovine vascular endothelial cells, and the injury to the cells could induce the release of a number of vasoactive compounds [Zoja *et al.*, 1986]. The injurious effects of CsA on cultured rat microvascular endothelial cells were practically due to inhibition of CsA on the regenerative response of the cells to injury, and the resultant alterations in prostacyclin production by these cells [Lau *et al.*, 1989]. Altogether, vasoconstriction and endothelial cell damage may contribute to CsA-associated functional impairments.

Structural or chronic nephrotoxicity may not be reversible and often is progressive, involving both renal arterioles and tubules. The morphological changes induced by CsA therapy in autoimmune disease patients, organ transplantation recipients, and experimental animals showed varied severity, including striped tubular atrophy, tubulointerstitial fibrosis, tubular vacuolization, tubular megamitochondria, microcalcification and afferent arteriopathy [Mihatsch *et al.*, 1988]. Mihatsch *et al.* [Mihatsch *et al.*, 1995] stated that the landmark of cyclosporine nephropathy rests on the demonstration of particular vascular lesions in the afferent arteriole. Clinical studies found that the vascular lesions of chronic CsA nephropathy are not necessarily dose-

related. According to Deray *et al.* [Deray *et al.*, 1992], in clinical practice, even doses of CsA as low as 2-4 mg/kg given to patients can not completely spare the kidney from its injurious effects. Perico *et al.* [Perico *et al.*, 1992] reported the impairment of renal hemodynamics from even a single dose of cyclosporine in chronically treated renal transplant patient. Therefore, some degree of renal vasoconstriction probably occurs in all patients who receive effective immunosuppressive therapy with CsA. Since CsA is a long-term used drug, especially for patients who have undergone organ transplantation, chronic cyclosporine nephropathy is of most concerned. It was defined by Mihatsch *et al.* [Mihatsch *et al.*, 1995] as a clinical pathologic entity produced by exposure of the patient to cyclosporine, characterized by tubulointerstitial fibrosis in a striped pattern, beginning in the medulla and progressing to the medullary rays of the cortex. It is associated with degenerative hyaline changes in the walls of afferent arteriolar-sized blood vessels extending from the pro-glomerular area proximally up to the afferent arteriole.

The mechanisms of structural nephrotoxicity may be different from those of functional impairments. It was reported that proximal tubular cell injury may play a major role in tubulointerstitial disease by releasing several vasoactive substance, influx of inflammatory cells into the interstitium, and fibroblast proliferation and matrix synthesis [Ong, Fine, 1994]. CsA has also been shown to activate apoptotic genes and increase apoptosis in tubular and interstitial cells, and CsA-induced apoptosis related with tubular atrophy and tubulointerstitial fibrosis [Yang *et al.*, 2002; Thomas *et al.*, 1998]. Angiotensin II (Ang II) and transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) also play predominant roles in renal tubular morphological changes [Shihab *et al.*, 1997; Young *et*

*al.*, 1995]. Collectively, CsA may advance functional change to structural change by promoting two interrelated pathophysiological processes, sustained renal ischemia, enhanced apoptosis, and enhanced generation of hormones leading to chronic nephrotoxicity. The functional and structural nephrotoxicity are not distinct but interrelated processes with renal vasculature abnormality as their basic pathological pathway.

#### **1.6. Discovery of the beneficial effects of fish oil**

The epidemiological studies of Dreyeberg *et al.* [Dreyeberg *et al.*, 1975] and Bang *et al.* [Bang *et al.*, 1976] in the middle of 1970s demonstrated an association between the low prevalence of atherosclerosis in Greenland Inuit and their high intake of marine derived food sources. The incidence of coronary vascular diseases in Greenland Inuit is only 1/10 of that of Danes or North American Whites, although their dietary lipid intakes are comparable. The distinct difference between their diets was found to be the composition of the dietary fat. The Danish diet contained twice as much saturated fat and more omega-6 ( $\omega$ -6) PUFAs than the diet of Inuit; while Inuit consume 5-10 g omega-3 ( $\omega$ -3) PUFAs from fat of marine mammal and fish per day. Epidemiological studies also showed a low prevalence of cardiovascular diseases in people of the fishing and farming communities of Japan, who consume large amounts of marine fish in their diet [Hirai *et al.*, 1980]. In a longitudinal study of risk factors and their relationship to chronic diseases, Kromhout *et al.* recorded the dietary habit of a group of men in the town of Zutphen, Netherlands and followed their mortality over a 20 years period. An inverse relationship

between fish consumption in 1960 and mortality from coronary artery diseases during 20 years of follow-up was observed [Kromhout, 1985a; Kromhout *et al.*, 1985b]. These benefits were thought to be ascribed to the unsaturated fatty acids composition of deep-sea cold-water fish, and triggered immense interest of the role of unsaturated fatty acids in human health.

### 1.7. Characteristics and classification of fatty acids

Fatty acids are products of hydrolysis of fats. They are long chain organic acids that contain a long, nonpolar hydrocarbon chain with a single carboxyl group on one end and a methyl group on the terminal end. In biological systems, nearly all natural fatty acids contain an even number of carbon atoms, typically between 14 and 24. They are either saturated or unsaturated, containing one or more double bonds. In fatty acids containing two or more double bonds, the double bonds are mainly conjugated (-CH=CH-CH=CH-). The double bonds of nearly all the naturally occurring unsaturated fatty acids exist in the *cis* geometrical configuration [Lehninger, 1982].

Fatty acids play two major physiological roles in mammalian tissues: a role in energy sources for fuel production and a role in structural components. Triacylglycerides (TAGs), esters of alcohol glycerol with three fatty acids molecules, function mainly as energy storage and fuel production in the cells. Another function of fatty acids is as building blocks of phospholipids and TAGs, and thus are important structural elements of cells. After intake into the body, instead of staying in free form in cells, fatty acids rapidly incorporate into the phospholipids of cell membrane, and consequently influence

membrane properties and function, such as membrane fluidity, ion transport and activities of membrane-associated proteins [Stubbs *et al.*, 1990]. In addition, PUFAs in membrane phospholipids also serve as substrate for generating biologically active mediators upon stimuli [Cook, 1991]. The biological functions of fatty acids may vary with different chemical structures. There is evidence that the presence of eicosapentaenoic acid (EPA) or docosahexanoic acid (DHA) in the phospholipids of cell membranes may affect the physical properties of cell membranes and modify the function of membrane-bound proteins (receptors, transport pathways, and enzymes) by altering the microenvironment within which these proteins act [Dratz *et al.*, 1986].

Fatty acids have been classified on the basis of their degree of saturation. Saturated fatty acids have no double bond; monounsaturated fatty acids have one double bond, and polyunsaturated fatty acids have more than one double bond. They are named on the basis of the length of the hydrocarbon chain, number and position of double bonds within the chain. Carbon atoms are numbered from the carboxyl carbon (carbon 1) to the terminal methyl carbon, which is known as  $\omega$ -carbon. The designation of omega ( $\omega$  or  $n$ ) is used to give the location of the first double bond in a fatty acid counting from the first carbon atom at the terminal methyl of hydrocarbon chain. Taking  $\omega$ -3 fatty acids for instance, its first double bond is located at the third carbon atom from the methyl end [Ackerman, 1995].

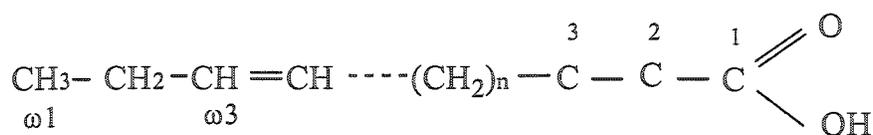


Figure 1.2. Illustration of fatty acid nomenclature.

In mammals, there are two distinctive classes of PUFAs:  $\omega$ -3 and  $\omega$ -6. Unlike saturated and monounsaturated fatty acids which can be synthesized by all mammals, including humans, the long chain PUFAs with double bonds more distal than the ninth carbon atoms counting from the terminal carboxyl of the hydrocarbon cannot be synthesized in the body. They are considered as the essential fatty acids, and therefore, must be intaken through the diet. The  $\omega$ -3 family includes alpha-linolenic acid (ALA, C18:3 n-3), EPA (C20:5 n-3) and DHA (C22:6 n-3). ALA is the parent compound, existing in flax seeds, soybean, nuts and canola oil. Elongation and further desaturation of ALA can yield long-chain  $\omega$ -3 EPA and DHA in animal and human very slowly; while both EPA and DHA can be obtained abundantly from marine source. They are originally formed in unicellular phytoplankton and multicellular sea algae and eventually pass through the food chain and become incorporated into the body of fish and higher marine species [Tinoco, 1982]. The  $\omega$ -6 PUFAs come mainly from plant sources: vegetable, seed and nut oils such as corn oil and sunflower oil. Arachidonic acid (AA, C20:4 n-6) is a long-chain  $\omega$ -6 PUFA that is present in meat and plant sources, or is synthesized from linoleic acid (LA, C18:2 n-6). The structures of these PUFAs are listed in Figure 1.3.

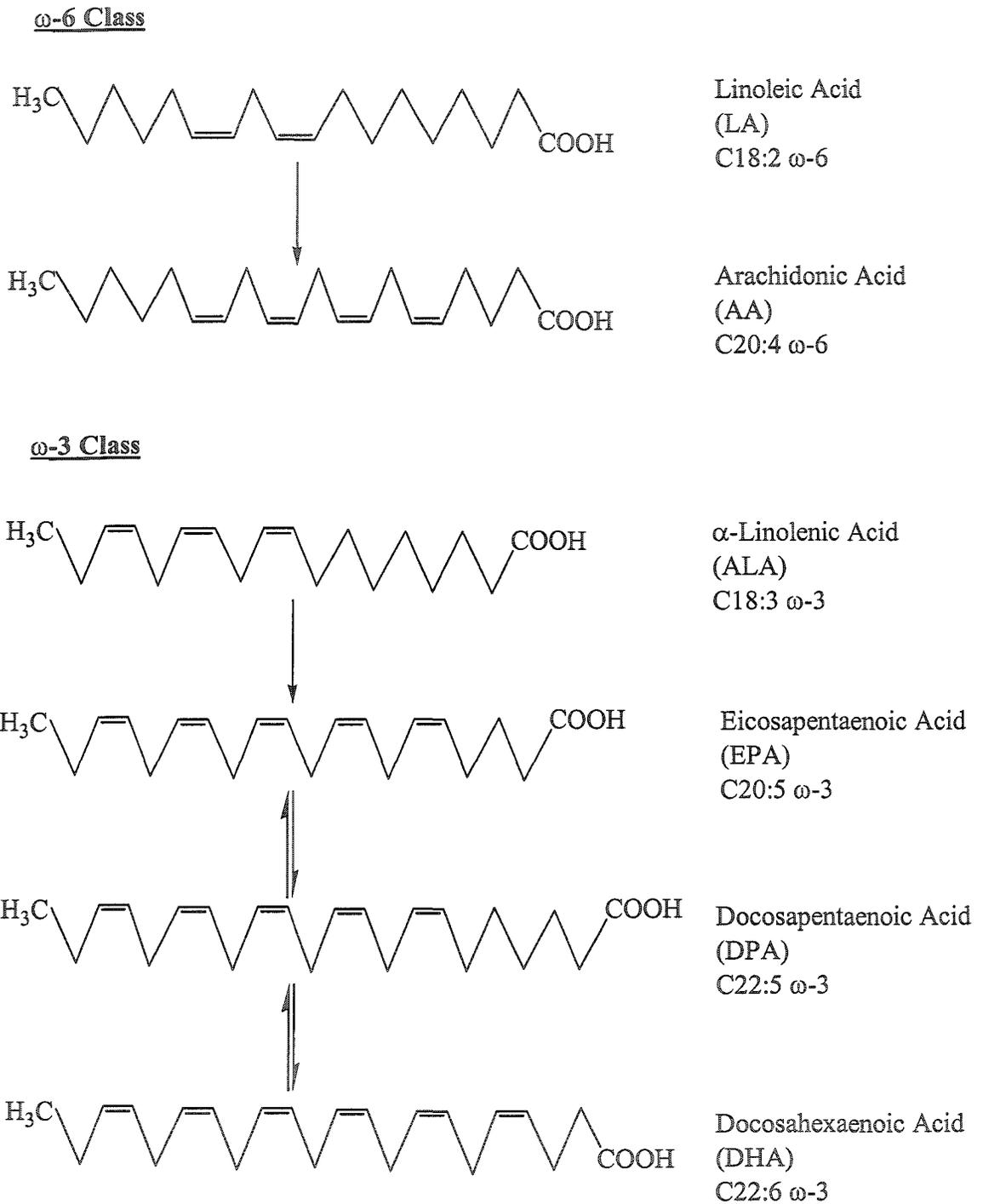


Figure 1.3. Important PUFAs of the ω-6 and ω-3 classes (adapted from *New England Journal of Medicine* 1988; 318(9): 550).

## 1.8. Fatty acids on eicosanoids metabolism and function

Both AA and EPA serve as key precursors of eicosanoids in tissues. Eicosanoids are a family of biologically active substances produced by oxygenation of 20-carbon PUFAs, in particular dihomo- $\gamma$ -linolenic acid (DGLA, C20:3  $\omega$ -6), AA and EPA. Eicosanoids include prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs), lipoxins, hydroperoxyeicosatetraenoic acids (HPETE), and hydroxyeicosatetraenoic acids (HETE). The fatty acid precursors for eicosanoid synthesis are released from cell membrane phospholipids, usually by phospholipase A<sub>2</sub> (PLA<sub>2</sub>) in response to a cellular stimulus. Because the membrane of most cells commonly contains predominantly AA, compared with DGLA and EPA, AA is usually the main precursor of eicosanoids production. AA produces the 2-series PGs (mainly PGI<sub>2</sub>, PGE<sub>2</sub>) and TXs (TXA<sub>2</sub>) through the cyclooxygenase (COX) pathway; and proinflammatory LTs (LTB<sub>4</sub>, C<sub>4</sub>, D<sub>4</sub>, E<sub>4</sub>) through the 5-lipoxygenase (LOX) pathway [Balter *et al.*, 1989] (see Figure 1.4.).

Eicosanoids are known to have vascular and haemostatic actions. In platelet, TXA<sub>2</sub> is a major product of AA from the COX pathway. TXA<sub>2</sub> has potent vasoconstriction and platelet aggregation effects. PGI<sub>2</sub> is the main COX product of AA in endothelial cells. It possesses vasodilatory and antiaggregatory effects and can therefore counteract the actions of TXA<sub>2</sub>. PGI<sub>2</sub> along with PGE<sub>2</sub> are considered to be beneficial to renal disorders in that they are vasodilators of glomerular vessels, and thereby increase single nephron GFR and plasma flow [Plotnick, 1996].

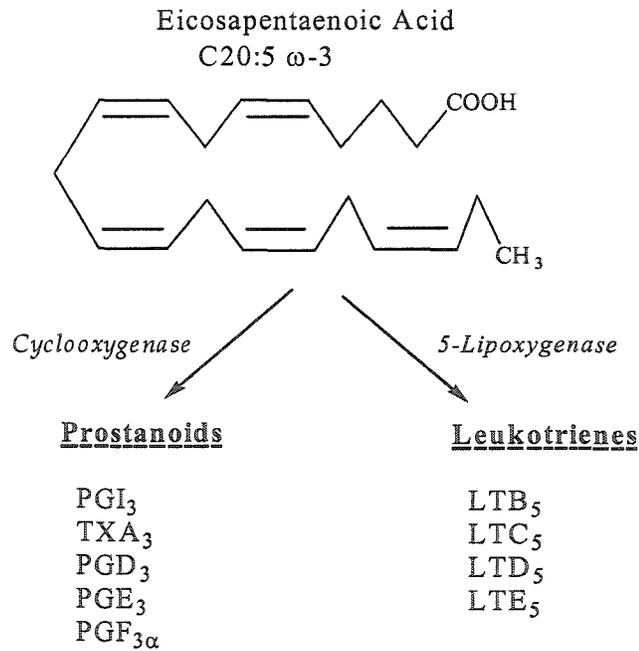
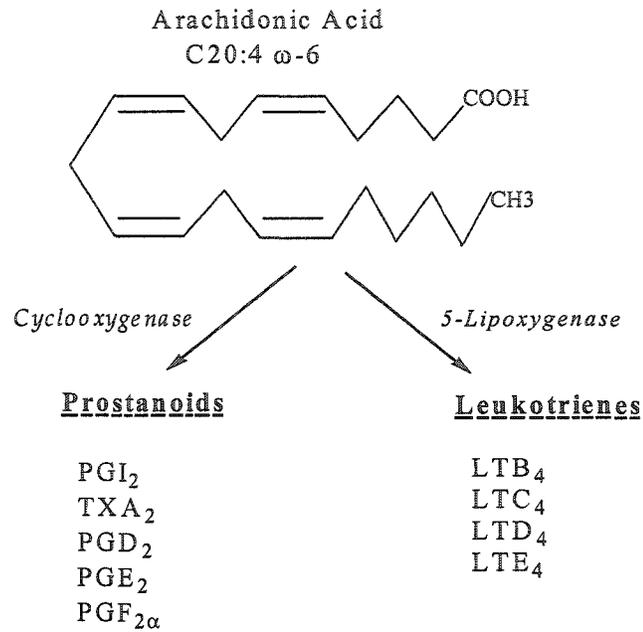


Figure 1.4. Synthesis of eicosanoids from the ω-6 precursor AA and the ω-3 precursor EPA (adopted from the *New England Journal of Medicine* 1988; 318(9): 550).

Most of the beneficial effects attributed to fatty acids are believed to be the modulation of the ratio of the eicosanoid products. When  $\omega$ -3 fatty acids are obtained from dietary sources, EPA and DHA are incorporated into the structure of phospholipids. EPA has the same structure as AA except for one additional double bond at the  $\omega$ -3 position. Thus, at the molecular level, EPA competes with AA in several ways: Firstly, it inhibits the formation of AA from LA [Holman, 1964]. Secondly, it competes with AA for the *sn*-2 position of membrane phospholipids, as AA is the predominant fatty acid occupying the 2-acyl position of phospholipids [Goodnight *et al.*, 1982], and therefore the cellular and plasma levels of AA are decreased. Thirdly, EPA competes with AA as a substrate of COX to produce the 3-series of metabolites: TXA<sub>3</sub> by platelets and PGI<sub>3</sub> by endothelial cells. TXA<sub>3</sub> is physically inactive and lacks vasoconstrictor properties [Siess, *et al.* 1980; Fischer, Weber, 1983]; while PGI<sub>3</sub> is as potent as its 2-series counterpart to serve as a vasodilator [Fischer, Weber, 1984]. In fact, EPA does not seem to be a good substrate for TXA<sub>3</sub> synthesis but allows normal or increased synthesis of PGI<sub>3</sub> [Dyerberg, 1986; Bunting *et al.*, 1983]. In this way, the net effect is a change in the haemostatic balance toward a more vasodilatory state, with less platelet aggregation.

The 4-series of LTs (LTB<sub>4</sub>, C<sub>4</sub>, D<sub>4</sub>, E<sub>4</sub>) produced from AA through the 5-LOX pathway have strong chemoattraction to circulating polymorphonuclear leukocytes and monocytes. They are involved in inflammatory, allergic, and immune responses [Samuelsson, 1983], and in the vascular response to ischemia [Mullane *et al.*, 1987]. When EPA is incorporated into the phospholipids of cell membranes, it competes the 5-LOX with AA, inhibiting the conversion of AA to the 4-series of LTs and producing the

less biologically active 5-series of LTs (LTB<sub>5</sub>, C<sub>5</sub>, D<sub>5</sub>, E<sub>5</sub>) in small amount. The 5-series of LTs compete with the 4-series of LTs at the receptor sites, and therefore reduce the chemoattractive effect of the 4-series of LTs [Goldman *et al.*, 1983]. In this way, supplementation of  $\omega$ -3 PUFAs in the diet manifests the anti-inflammatory effects towards inappropriate recruitment of neutrophils to inflammation sites.

### **1.9. Effects of $\omega$ -3 PUFAs on diseases and CsA-induced nephrotoxicity**

The beneficial effects of consuming  $\omega$ -3 PUFAs to the cardiovascular system had been widely recognized [Sassen *et al.*, 1994]. Diets rich in  $\omega$ -3 PUFAs have also been shown to improve inflammatory bowel disease [Stenson *et al.*, 1992], rheumatoid arthritis [Geusens *et al.*, 1994], and clinical status of cancer cachexia [Wigmore *et al.*, 1996]. Since decreased GFR associated with reduced blood flow that may be caused by renal vasoconstriction has been recognized as a main cause of CsA-induced nephrotoxicity, and beneficial effects of  $\omega$ -3 fatty acids on hemodynamics and the vascular system have been well documented, there has been considerable interest in the effect of dietary  $\omega$ -3 fatty acids supplementation on patients who undergo organ transplantation with CsA treatment.

A clinical study with patients of normal kidney function that were given CsA for psoriasis showed that when CsA was combined with fish oil, the decrease of GFR was not as severe as that in CsA only group. Effective renal blood flow was unchanged for the CsA plus fish oil group, whereas that in the CsA without fish oil group decreased more than 10%. Total renal vascular resistance did not change for the group with fish oil

administration, but there was a nearly 20% increase of the total renal vascular resistance in the CsA only group [Stoof *et al.*, 1989]. In another study, patients who had undergone renal transplantation receiving stable CsA treatment were enrolled [Homan van der Heide *et al.*, 1990]. In the group of patients given CsA with fish oil, GFR increased by nearly 20%; effective renal blood flow increased by 17%; mean arterial pressure decreased by 10%, and the calculated total renal vascular resistance decreased by 23%. However, no improvement of these parameters was observed in the group given CsA with corn oil. Two years later, a similar study [Homan van der Heide *et al.*, 1992] was conducted and revealed that the administration of fish oil to patients receiving CsA for kidney transplantation significantly reduced the incidence of rejection and improved blood pressure and GFR. In this study, patients were admitted to a randomized double blind trial. Half of the patients were treated with 6 g fish oil per day during the first postoperative year, whereas the control group was treated with 6 g coconut oil for 3 months, then it was stopped. One year after transplantation, the group supplemented with fish oil had higher values for GFR, lower mean arterial pressure and fewer cumulative rejection episodes (8 in fish oil group and 20 in the controls). One year graft survival was 97% in the fish oil group and 84% in the control group. Bennett *et al.* [Bennett *et al.*, 1995] also reported improved renal function, reduced blood pressure and graft rejection episodes in patients who underwent renal transplantation and received CsA regimen with fish oil supplementation, started after surgery. The results of these clinical studies revealed an attenuation of CsA-induced renal dysfunction when fish oil is supplemented to the patients receiving CsA treatment.

In animal experiments, Elzinga *et al.* [Elzinga *et al.*, 1987a] showed pretreatment of rats with either fish oil or olive oil for 14 days, followed by administration of 50 mg/kg CsA for additional 14 days along with different oil treatment, resulted in decreased GFR in both CsA treated groups compared with normal control, but the mean value in rats administered fish oil was significantly higher than that of the olive oil group. A striking reduction in proximal tubular morphological change in the fish oil group was observed, accompanied with significantly lower renal cortical TXB<sub>2</sub> content when compared with that of the olive oil group. Substituting fish oil for olive oil that is adopted for commercial products as a vehicle for CsA also reduced nephrotoxicity and TXA<sub>2</sub> content in the kidney in Fischer rats during CsA therapy [Elzinga *et al.*, 1987b]. Another study [Casas *et al.*, 1995] using either fish oil or olive oil as vehicle for CsA administration found that CsA-induced nephrotoxicity and the decreased ratio of 6-keto-PGF<sub>1α</sub>/TXB<sub>2</sub> was greatly improved in the fish oil vehicle group, and renal function was directly correlated with eicosanoids production.

Furthermore, when fish oil substituted for olive oil as a vehicle for CsA, fish oil potentiated the immunosuppressive activity of CsA, and thus enhanced the anti-rejection therapy of CsA [Kelly *et al.*, 1989]. Levy and Alexander [Levy, Alexander, 1995] showed that Lewis strain rats receiving ACI strain cardiac allografts had a remarkable prolongation in allograft survival when they were fed a complete immunonutrient diet containing arginine and fish oil compared with the rats fed on standard diet (survival time: 275 ± 53 days in fish oil diet versus 72 ± 7 days in standard diet).

According to both clinical and animal studies, it seems to indicate improved renal function and hemodynamics, and enhanced immunosuppressive effects when CsA based immunosuppressive regimens were administered in combination with long chain  $\omega$ -3 PUFAs from fish oil.

#### 1.10. Immunosuppressive effects of PUFAs from fish oil

It is known that PGE<sub>2</sub> has a number of proinflammatory effects, such as induction of fever and erythema, increasing vascular permeability and vasodilation. Since dietary fish oil leads to decreased PGE<sub>2</sub> and LTs production, it is supposed to have immunosuppressive effects. A number of reports about the actions of fatty acids on the immune responses of animals and humans also triggered considerable interests of researchers. Systemic investigation of a wide range of fatty acids *in vitro* has shown that fatty acids generally can suppress mitogen-stimulated proliferation of lymphocytes isolated from rat lymph nodes, spleen and lymphatic duct, from mouse spleen, or human peripheral blood [Tsang *et al.*, 1977; Buttke, 1984]. Although both saturated and unsaturated fatty acids inhibit lymphocyte proliferation, unsaturated fatty acids exert greater inhibitory effect, and the greatest inhibition on proliferation is observed with the  $\omega$ -3 EPA [Calder *et al.*, 1991; Soyland *et al.*, 1993]. It has been reported [Kelley *et al.*, 1985] that using dietary supplementation with fish oil as the exclusive source of lipid suppressed autoimmune lupus in MRL-*lpr* mice. A fatty acid component uniquely found in fish oil was speculated to be responsible for suppressed immunologic and/or inflammatory mediators of murine lupus by directly decreasing the production of PGE<sub>2</sub>,

TXB<sub>2</sub> and PGI<sub>2</sub>. Animal feeding studies indicate that high levels of fish oil decrease NK cell activity, cytotoxic T lymphocyte activity, expression of IL-2 receptor on activated lymphocytes, lymphocyte proliferation and production of IL-2 and IFN- $\gamma$  [Fritsche *et al.*, 1990; Yaqoob *et al.*, 1994; Nair *et al.*, 2002]. The animal studies suggest that fish oil impairs cell mediated immunity and induces a shift in T-lymphocyte away from cell-mediated immunity and chronic inflammation.

Dietary supplementation with 1.6 g EPA and DHA per day to human volunteers for 3 weeks led to decreased expression of major histocompatibility complex (MHC) class II on the surface of blood monocytes [Hughes *et al.*, 1996]. Another study showed that supplementation of fish oil with > 2.4 g EPA and DHA per day can decrease the production of TNF, IL-1 and IL-6 by mononuclear cells [Meydani *et al.*, 1991]. However, there are also reports of no effects on TNF, IL-2 production and NK cell activation of intaking 3.2 g EPA and DHA per day [Yaqoob *et al.*, 2000; Schmidt *et al.*, 1996]. Collectively, these studies indicated that high levels of fish oil supplementation to humans exert anti-inflammatory effects, and at least in some studies inhibit lymphocyte response.

#### **1.11. Seal oil: another source of long chain $\omega$ -3 PUFAs**

Harp seal is a marine mammal living in an ice-cold water environment. Blubber oil from harp seal is a rich source of long-chain  $\omega$ -3 PUFAs. The original interest in studying seal oil is based on the observation of the low prevalence of cardiovascular

diseases in a population of Greenland Inuit whose diet included predominantly seal oil and meat [Kromann *et al.*, 1980].

Studies demonstrated that seal oil is a superior source of  $\omega$ -3 PUFAs compared to fish oil in several ways. Firstly, seal oil contains a substantial amount of the less well-known  $\omega$ -3 PUFA, docosapentaenoic acid (DPA, 22:5  $\omega$ -3). DPA is present at very low levels relative to EPA or DHA in fish oils. Seal oil can supply up to ten times more DPA than fish oils. In blood vessel walls EPA may actually be converted to DPA to exert its beneficial effects [Murphy *et al.*, 1997]. Japanese researchers [Kanayasu-Toyoda *et al.*, 1996] have shown that EPA exerted stimulating effects on endothelial cell migration via DPA, and DPA may act as a potent anti-atherogenic factor. Although it has been widely accepted that EPA is beneficial to the cardiovascular system by elevating the ratio of vasodilators to vasoconstrictors, this study indicated that DPA may be 10-20 times more powerful than EPA in this effect. Compared to EPA and DHA, DPA was also found to be the most potent PUFA in suppressing TXA<sub>2</sub> formation by platelets which were exposed to collagen, thrombin, or AA [Akiba *et al.*, 2000].

Secondly, the intramolecular distributions of fatty acids in TAG of marine mammals differ from that in the TAG of fish. In seal oil, the  $\omega$ -3 fatty acids are found mainly in the *sn*-1 and *sn*-3 positions of the TAG molecules, which is the same as in humans, whereas in fish oil the  $\omega$ -3 fatty acids are mainly esterified in the *sn*-2 position [Puppione *et al.*, 1992]. It is known that fatty acids at the *sn*-2 position of dietary TAG are retained during absorption process, which may be due to positional specificity of pancreatic lipase. This lipase hydrolyzes fatty acids at *sn*-1 and -3 positions, and produces

2-monoglycerol and free fatty acids. After hydrolysis products enter into intestinal mucosal cells, most of the free fatty acids are reesterified to 2-monoglycerol, and resynthesized TAG is incorporated into chylomicrons, which are secreted into lymph [Nelson *et al.*, 1988]. Metabolic difference between fatty acids at the *sn*-2 and *sn*-1, -3 positions of the TAGs after absorption has been thought to be a cause of the different physiological functions [Yamamoto *et al.*, 1971].

A study conducted by Christensen *et al.* [Christensen *et al.*, 1996] showed that rats administered seal oil had significantly higher load of  $\omega$ -3 PUFAs esterified in the *sn*-1, -3 position of chylomicron TAG, compared with that of rats administered fish oil. Since TAG is degraded by *sn*-1, -3 specific lipoprotein lipase, the intramolecular fatty acid distribution of TAG in chylomicron may affect TAG metabolism in the systemic circulation. In another study [Yoshida *et al.*, 1996], dietary lipids mainly containing seal oil or fish oil and having constant  $\omega$ -6/ $\omega$ -3 ratio of fatty acids were fed to rats for three weeks. Seal oil more effectively reduced plasma and liver TAG than fish oil. Ratio of the productions of aortic PGI<sub>2</sub> to platelet TXA<sub>2</sub> was significantly higher in rats fed seal oil than in those fed fish oil. The results suggested that the different intramolecular distribution of EPA and DHA in dietary fat affected lipid metabolism differently in rats. The difference of fatty acids distribution in TAG may also influence the absorption and distribution of  $\omega$ -3 fatty acids in human [Nelson *et al.*, 1988]. Although no clinical trial data is available based on the previous animal experiments, it may be speculated that the absorption and utilization of seal oil into the human body could be easier and more thorough than that of fish oils due to the difference of their chemical structures.

Thirdly, as seals are mammals, their metabolic and digestive systems filter out many natural impurities found in fish. Seal oils therefore provide a naturally purified and high quality source of  $\omega$ -3 fatty acids. It was reported that the  $\omega$ -3 PUFAs content of harp seal oil is around 21% with DHA (9%), EPA (7%) and a relatively high portion of DPA (4-6%) [Shahidi *et al.*, 1996], which is higher than most fish oils. Moreover, Seal oil is virtually free of cholesterol, while many fish and fish oils are relatively high in cholesterol. In addition, because seal oil was extracted strictly from seal blubber, it is much purer than fish oils which are obtained by grinding, cooking and pressing fish offal, or whole fish [Ho].

Finally, seal oil is more stable than fish oil as a source of long chain PUFAs. Fish oil is susceptible to oxidization, while seal oil is more resistant to natural oxidative processes. Experiments [Nakhla, 1997] showed that the extent of oxidization of  $\omega$ -3 PUFAs in seal *in vitro* was less than half of that observed in fish oils.

#### **1.12. Studies of the effects of seal oil on cardiovascular system**

Ikeda *et al.* [Ikeda *et al.*, 1998] conducted a long term feeding of either seal or squid oil-rich fats that have different positional distribution of fatty acids to exogenously hypercholesterolemic rats for 160 days, and found that the AA levels in phospholipids of liver, platelet and aorta was lower in both marine oil groups than that of control, seal oil is more effective than squid oil; the platelet aggregation and platelet TXA<sub>2</sub> production induced by collagen were strikingly reduced in marine oils treated groups, with more pronounced action by seal oil; and seal oil-rich diet significantly increased the ratio of

aortic PGI<sub>2</sub> production to platelet TXA<sub>2</sub>. These results indicated that seal oil with the majority of its PUFAs at the *sn*-1, -3 positions, is more potent in modulating the production of prostanoids towards favorable vasodilation compared the *sn*-2 PUFAs positioned fish oil.

Besides studies on the effects of seal oil as well as fish oil in animal models, clinical studies with both normal and hypercholesterolemic subjects were conducted focusing on cardiovascular diseases. Vognild *et al.* [Vognild *et al.*, 1998] reported a clinical study of 266 healthy volunteers consuming 15 mL/day of cod-liver oil (CLO), whale blubber oil, a mixture of seal blubber oil and CLO, or a mixture of olive oil and CLO respectively for 12 weeks. Cardiovascular / thrombotic diseases associated parameters revealed that the consumption of various kinds of marine oils cause changes in platelet membrane towards anti-thrombotic effects, and the changes of platelet function are directly related to fatty acids composition in platelet membrane. There were no significant differences in triacylglycerols, total cholesterol, or high-density lipoprotein cholesterol (HDL) observed.

Another long term clinical study was also conducted to test the effects of dietary supplementation of seal oil and CLO on 120 subjects with moderate hypercholesterolemia [Brox *et al.*, 2001]. The results showed that in people on seal oil diet or CLO diet for 14 months serum fatty acids composition changed significantly compared with control. Seal oil supplementation elevated serum EPA levels additionally 30% higher than that of cod-liver oil. Both of the marine oil groups had lower whole blood platelet aggregation than control. No effects on serum total cholesterol, HDL

cholesterol, apolipoproteins, lipoproteins and TNF was observed in both marine oil treated groups. Both experimental animal and clinical studies revealed the promising beneficial effects of seal oil on the cardiovascular system, especially on platelet aggregation, by changing the composition of membrane fatty acids and the consequent changes of prostanoids production.

Harp seals are abundant in Newfoundland and Labrador, and the beneficial medical effects of the superior long chain  $\omega$ -3 PUFAs in seal oil have attracted extensive interest from researchers. Nephrotoxicity is almost an inherent shortcoming of CsA. Despite the development of the new formulation Neoral<sup>®</sup>, which has been claimed to give improved bioavailability of CsA and less inter- and intra-patients variation, risk of adverse effects had been reported on switching to it, especially nephrotoxicity remains a medical challenge [Olyaei *et al.*, 1997]. Based on the previous literatures, supplementation of fish oil in combination with CsA administration is beneficial to the toxicities associated with CsA immunosuppressive regimens. This project was undertaken to investigate the effects of supplementation of seal oil on CsA-induced nephrotoxicity in a rat model. Positive results of this study may be the key impetus leading to the development of novel formulation of CsA using seal oil as lipid phase.

## Chapter 2. Materials and Methods

### 2.1. Materials

#### 2.1.1. Chemicals and reagents

CsA was a generous gift from Huabei Pharmaceutical Corp., China. CsA injection (Sandimmune IV<sup>®</sup>, Ampoules, 50 mg/mL microemulsion) was a product of Novartis Pharm Canada Inc., Dorval, QC, Canada. Seal oil was generously provided by Caboto Seafoods Ltd., St. John's, NL, Canada. Corn oil and sunflower oil were purchased from Walmart. Cremophor RH-40 was provided by Baret Products Corp., Englewood Cliffs, NJ, USA. The ingredients used to prepare diets for animal studies including casein, cornstarch, dextrinized cornstarch, sucrose, fiber, AIN mineral mix and AIN vitamin mix were purchased from ICN Pharmaceuticals Inc., Montreal, QC, Canada. PUFAs standards, mixtures of PUFAs with 14 to 22 carbon atoms chains, were obtained from Supelco Ltd. Supelco Park, Bellefonte, PA, USA. Fetal bovine serum (FBS, Cat. No: 30071.03) was purchased from HyClone, Logan, UT, USA. Medium 199 (M199, Cat. No: M4530), 0.25% trypsin-EDTA solution (1×, Cat. No: T3924), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tertrazolubromide (MTT, Cat. No: M-2128), DHA and EPA (minimum 99% pure) were purchased from Sigma-Aldrich Canada Ltd., Oakville, ON, Canada. Nutriene<sup>™</sup> Tocotrienols (Vitamin E, V<sub>E</sub>) was obtained from Eastman Chemical Com., Kingsport, TN, USA. All other chemicals used are at least of analytical grade and were purchased from Sigma-Aldrich Canada Ltd., unless otherwise indicated.

Diagnostics<sup>®</sup> Urea Nitrogen kit (Cat. No: 640) and Diagnostics<sup>®</sup> Creatinine kit (Cat. No: 555) were purchased from Sigma-Aldrich Ltd., St. Louis, MO, USA. PPR NAG Test Kit was purchased from PPR Diagnostics Ltd., London, UK. CYCLO-Trac<sup>®</sup> SP-Whole Blood Radioimmunoassay for Cyclosporine kit was purchased from DiaSorin Inc., Stillwater, MN, USA. Bio-Rad DC Protein Assay reagents (Cat. No: 500-0116) were purchased from Bio-Rad Laboratories Inc., Hercules, CA, USA. OXI-TEK Thiobarbituric Acid Reactive Substances (TBARS) Assay kit (Cat. No: 0801192) was purchased from ZeptoMetrix Corp., Buffalo, NY, USA. Thromboxane B<sub>2</sub> Biotrak Enzymeimmunoassay (EIA) System (Cat. No: RPN 220) and 6-Keto-Prostaglandin F<sub>1α</sub> Enzymeimmunoassay Biotrak (EIA) System (Cat. No: RPN221) were obtained from Amersham Biosciences Corp., Piscataway, NJ, USA. LDH Cytotoxicity Detection Kit (Cat. No: MK401) was purchased from Fisher Scientific, Ottawa, ON, Canada.

### **2.1.2. Instruments**

EmulsiFlex<sup>®</sup>-C5 high pressure homogenizer was purchased from Avestin Inc., Ottawa, ON, Canada. Delsa 440SX Zeta Potential Analyzer (Beckman Coulter<sup>®</sup>) was obtained from Coulter Electronics Inc., Hialeah, FL, USA. Heparinized microhematocrit capillary tubes were from Allied Corporation Fisher Scientific, Pittsburgh, PA, USA. Flat top microcentrifuge tubes were purchased from Fisher Scientific Ltd., Nepean, ON, Canada. Vacutainer tubes were obtained from BD, Franklin Lakes, NJ, USA. Centrifuge tubes were from Sarstedt Inc., Newton, NC, USA. Glass tissue grinder was from Kontes Glass Com., Vineland, NJ, USA. Cell culture flasks (25 cm<sup>2</sup>) were obtained from Falcon,

Franklin Lakes, NJ, USA. 24 and 96-well plates were obtained from Corning Corp., Corning, NY, USA. Multichannel pipette was obtained from BrandTech Scientific Inc., Essex, CT, USA. Microplate reader (Bio-Rad Model 550) was produced by Bio-Rad Laboratories, Hercules, CA, USA. Pulse amplifier (Model 29) was from 11TC Inc., Woodland Hills, CA, USA. The 2-channel recorder (LKB Bromma, Type 2210) was obtained from LabX Com., Midland, ON, Canada. Centra-CL3 type centrifuge was provided by Thermo IEC, Needham Heights, MA, USA. Waters 2795 Alliance HT HPLC system and Micromass Quattro LC tandem mass spectrometer were from VCU Life Sciences, Richmond, VA, USA. C18 SecurityGuard cartridge (3mm × 4mm) was obtained from Phenomenex Inc., Torrance, CA, USA. Spectrophotometer (Beckman, DU62) was obtained from Beckman Instruments Inc., Fullerton, CA, USA. Spectrofluorophotometer (RF-1501, SHIMADZU) was obtained from Mandel Scientific Com., Guelph, ON, Canada. Hewlett Packard 5890 Series II gas chromatograph was purchased from Global Medical Instrumentation Inc., Albertville, MN, USA.

### **2.1.3. Animals**

Male Sprague-Dawley (SD) rats were obtained from the Animal Care Services of Memorial University of Newfoundland, St. John's, NL, Canada.

### **2.1.4. Cells**

LLC-PK1 cells (CRL 1392, passage 200-220) were purchased from American Type Culture Collection (ATCC), Manassas, VA, USA.

## 2.2. Preparation and characterization of CsA emulsions

CsA is highly lipophilic and poorly water soluble. CsA emulsions in seal oil and corn oil, as a drug delivery system, were prepared in this study. Emulsions with various amounts of surfactants (Cremophor RH-40 / Tween 80 = 2:1 (w/w)) ranging from 0.3 to 3.8% in 10% or 20% seal oil or corn oil were prepared as follows:

1. Under constant stirring, 50 mg of CsA powder was dissolved in 1 g of seal oil or corn oil to prepare the 10% of oil emulsions, or in 2 g of oils to prepare the 20% of oil emulsions.
2. Surfactants (Cremophor RH-40 / Tween 80 = 2:1 (w/w)) of 0.03, 0.06, 0.09, 0.12, 0.15, 0.18, 0.21, 0.24, 0.27, 0.30, 0.33 and 0.38 g were added, respectively, and mixed thoroughly with the oil phase to form emulsion pre-concentrates.
3. Deionized distilled H<sub>2</sub>O was added dropwise to the pre-concentrate to make the total weight to 10 g under constant stirring to form crude emulsion.
4. The particle size of the crude emulsions thus prepared was reduced by passing through an EmulsiFlex<sup>®</sup>-C5 high pressure homogenizer at 27,000 psi for four repetitions.

The particle size of the final emulsions was determined by a Delsa 440SX Zeta Potential Analyzer.

### 2.3. *In vivo* studies

#### 2.3.1. Effect of different oils in the formulation of CsA emulsions on CsA-induced nephrotoxicity in rats

Male SD rats weighing 200-230 g were housed under standard conditions, using a 12 h light/dark cycle, with standard rat chow and tap water *ad libitum*. Rats were randomly divided into five groups as follows:

- Group I (normal control): *p.o.* normal saline (NS) 10 mL/kg/day
- Group II: *p.o.* CsA in 20% of seal oil emulsion 50 mg CsA/kg/day
- Group III: *p.o.* CsA in 20% of corn oil emulsion 50 mg CsA/kg/day
- Group IV: *i.p.* CsA in 20% of seal oil emulsion 25 mg CsA/kg/day
- Group V: *i.p.* CsA in 20% of corn oil emulsion 25 mg CsA/kg/day

The rats were administered NS or CsA in 20% of oil emulsions as indicated above for 28 days. The protocol used in this study was approved by Memorial University of Newfoundland Committee on Animal Care and Use, and adhered to the *Guide to the Care and Use of Experimental Animals* as issued by the Canadian Council on Animal Care.

Blood samples were taken from the orbital vein with heparinized microhematocrit capillary tubes and 20-h urine samples were collected with metabolic cages one week prior to and once a week during the four-week *i.v.* administration of NS or CsA in 20% of oil emulsions for 28 days. Systolic blood pressure (SBP) was also measured once a week.

## **2.3.2. Effect of dietary supplementation of different oils on CsA-induced nephrotoxicity in rats**

### **2.3.2.1. Preparation of the diets**

Two kinds of diets, Diet A-93G and Diet B-93G, were prepared and kept in sealed container at -20 °C until use. Diet A-93G contained a mixture of 85% seal oil and 15% sunflower oil, whereas Diet B-93G contained 100% sunflower oil as their lipid, respectively. The two diets were otherwise identical in protein (supplied as casein), carbohydrates, fiber, minerals and vitamins content. The diets were designed to be isocaloric and isonitrogenous, and their compositions are listed in Table 2.1. [Reeves *et al.*, 1993]. The procedure of diet preparation is as follows:

1. Weigh all the solid ingredients and mix them up thoroughly in a big bowl.
2. Weigh the oils and add to the powder mixtures while stirring at low-speed.
3. Thoroughly mix all the components by high-speed stirring for 10 min with an electrical stirrer.

Table 2.1. Compositions of the various diets (AIN Purified Diets) (unit: g/kg diet)

	Diet A-93G*	Diet A-93M <sup>#</sup>	Diet B-93G*	Diet B-93M <sup>#</sup>
Casein ( $\geq 85\%$ protein)	200	140	200	140
Cornstarch	397.5	465.7	397.5	465.7
Dextrinized Cornstarch (90-94% tetrasaccharides)	132	155	132	155
Sucrose	100	100	100	100
Fiber	50	50	50	50
Sunflower oil	10.5	6	70	40
Seal oil	59.5	34		
AIN mineral mix	35	35	35	35
AIN vitamin mix	10	10	10	10
Choline bitartrate (41.1% choline)	2.5	2.5	2.5	2.5
L-Cystine	3	1.8	3	1.8
<i>tert</i> -butylhydroquinone	0.014	0.008	0.014	0.008

\* G stands for young (growing) rats and <sup>#</sup> M stands for old (mature) rats.

### 2.3.2.2. *In vivo* rat model of CsA-induced nephrotoxicity with dietary supplementation of different oils

Based on the literature [Thliveris *et al.*, 1991; Elzinga *et al.*, 1987; Rogers *et al.*, 1988; Yamauchi *et al.*, 1998], 3-week old male SD rats weighing 56 to 78 g were housed under standard conditions with free access to the diets we prepared and tap water.

After three days acclimation to the new environment, the rats were randomly divided into four groups, and maintained on their respective diets for four weeks. To minimize variation, the diets for growing rats (Diet A-93G and Diet B-93G) were used along the entire experiment. From the fifth week, while the rats were still maintained on their respective diets, they received the 10-fold dilution of CsA injection (Sandimmune IV<sup>®</sup>) with NS, administered intravenously once a day from the tail vein at a dosage of 15 mg/kg/day for four weeks. Equal volume of NS was administered to the rats as control as listed below:

- Seal oil diet + NS injection (control): rats were fed seal oil diet (Diet A-93G) for eight weeks, during which starting from the fifth week, NS was administered *i.v.* for four weeks (n = 6).
- Sunflower oil + NS injection (control): rats were fed sunflower oil diet (Diet B-93G) for eight weeks, during which starting from the fifth week, NS was administered *i.v.* for four weeks (n = 6).
- Seal oil diet + CsA injection: Rats were fed seal oil diet (Diet A-93G) for eight weeks, during which starting from the fifth week, CsA (Sandimmune IV<sup>®</sup>, 15 mg/kg/day) was administered *i.v.* for four weeks (n = 8).

- Sunflower oil diet + CsA injection: Rats were fed sunflower oil diet (Diet B-93G) for eight weeks, during which starting from the fifth week, CsA (Sandimmune IV<sup>®</sup>, 15 mg/kg/day) was administered *i.v.* for four weeks (n = 8).

#### 2.3.2.3. Collecting and processing of samples

Blood and urine samples were taken one week prior to and once a week during the four-week *i.v.* administration of NS or CsA (15 mg/kg/day). The “Bleed Jugular Vein” method, a standard bleeding method accepted by the Animal Protection Organizations in the USA and Canada, was used to take around 0.6 mL blood sample from jugular vein. Briefly, a plastic table with 30° incline was used to lie the rat on its back, and the rat was restrained with a soft rope around its paw or wrist to stretch its arms straight out on the table. A plastic cap with holes was placed over the rat’s head and twist slightly to the right or left side depending on which side the blood will be taken, twist to the left to take blood from the right, and vice versa. When the head being twisted, a triangular shape will appear on the neck, which can help locate the jugular vein. Then a sterilized syringe with a 23-gauge needle was inserted into the vein to take blood.

A timed urine collection over 20-h was performed following blood sampling. Each rat was placed in a metal metabolic cage, with chow and tap water *ad libitum*.

Blood samples were subjected to centrifugation at 3,000 g, 4 °C for 15 min. Urine samples were centrifuged at 10,000 g, 4 °C for 10 min. The supernatants were pipetted into 0.6 mL flat top microcentrifuge tubes. Aliquots of urine and serum samples were stored at –20 °C for analysis.

Systolic blood pressure (SBP) was also measured one week prior to and once a week during the course of administration of CsA or NS.

At the end of the study, the rats were sacrificed by inhaling an overdose of ethyl ether. Blood samples were withdrawn by direct cardiac puncture. Four mL of blood was collected into Vacutainer tubes containing EDTA for determination of CsA concentration. Approximately 2 mL of the blood was added to 8 mL ice-cold NS in a centrifuge tube and mixed. Red blood cells (RBCs) were pelleted by centrifugation at 3,000 rpm for 10 min, and the supernatant was discarded. The RBCs were then washed three times with NS and stored at -80 °C until further processed for analysis of fatty acid composition by gas chromatography (GC).

The abdomen of the rats was opened by a midline incision and the abdominal aorta was cannulated retrogradely below the renal artery with an 18-gauge needle. With the aorta occluded by ligation above the renal artery and the renal vein opened by a small incision for outflow, the two kidneys were perfused *in situ* with 60 mL ice-cold NS till the color of the kidneys changed to pale. Kidneys were excised, halves of the left kidneys were fixed in buffered (phosphate-buffered saline (PBS), pH=7.4) 4% paraformaldehyde for histological processing and examination. The other halves of the left kidneys and the right kidneys were cut into smaller pieces, frozen in liquid nitrogen immediately, and stored at -80 °C for biochemical analysis.

## 2.4. Assessment of the effects of CsA

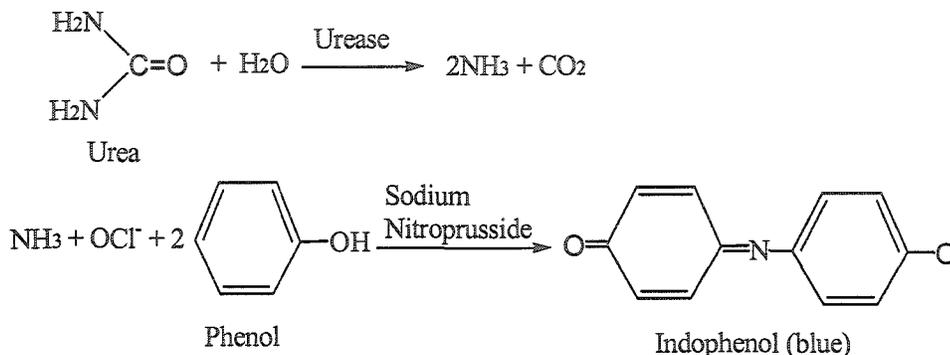
### 2.4.1. Determination of blood urea nitrogen (BUN)

The BUN test is used primarily to evaluate kidney function. Cells in the body need protein and blood carries proteins for use by cells. During digestion, protein is broken down to amino acids. Amino acids contain nitrogen, which is split off as  $\text{NH}_4^+$  (ammonium ion), while the rest of the molecule is used to produce energy or other substances needed by cells. The ammonia is combined with other small molecules to produce urea in the liver as the end product of protein metabolism, which is then secreted into blood and excreted in urine by the kidney. Most renal diseases affect urea excretion so that the BUN level increases.

For the first animal study protocol, the BUN levels at day 4, 11, 18 and 28 following the commence of administration of respective drug regimen were measured spectrophotometrically by a Beckman Autoanalyzer-2 performed in the clinical biochemistry diagnosis lab at the General Hospital, Health Care Corporation of St. John's (HCCS), St. John's, NL, Canada.

For the second animal study protocol, the BUN levels were monitored one week prior to and once a week during the four-week *i.v.* administration of NS or CsA (15 mg/kg/day) by a clinically used colorimetric method using a Diagnostics<sup>®</sup> Urea Nitrogen kit. The principle is that urea is hydrolyzed by urease to ammonia and carbon dioxide. Ammonia then reacts with alkaline hypochlorite and phenol in the presence of a catalyst, sodium nitroprusside ( $\text{Na}_2\text{Fe}[\text{CN}]_5\text{NO} \cdot 2\text{H}_2\text{O}$ ), to form indophenol [Fawcett, Scott, 1960; Horn, Squire, 1967]. The concentration of ammonia is directly proportional to the

absorbance of the blue color of indophenol, which can be measured spectrophotometrically at 570 nm.



The testing procedure is as follows:

1. To each properly labeled test tube, 100  $\mu\text{L}$  of urease solution was added.
2. To the blank, standards, or tests tubes, 5  $\mu\text{L}$  of  $\text{H}_2\text{O}$ , urea nitrogen standards, or test samples were added respectively. Mix contents by gently swirling.
3. Place tubes in a 37  $^\circ\text{C}$  water bath for 10 min allowing urea to be hydrolyzed to ammonia.
4. Add 200  $\mu\text{L}$  of phenol nitroprusside solution, 200  $\mu\text{L}$  of alkaline hypochlorite solution and 1 mL of  $\text{H}_2\text{O}$  to each tube in order. Mix after each addition.
5. Allow tubes to develop color at room temperature for 30 min.
6. After color development, 300  $\mu\text{L}$  of solution from each tube was added to wells in a 96-well plate, and the absorbance was read by a microplate reader at 570 nm.

#### 2.4.2. Measurement of creatinine clearance (Clcr)

Clcr is another measurement of kidney function. Creatinine, a waste product in the blood, is created by the normal breakdown of muscle during physical activity. Healthy kidneys remove creatinine from the blood and it is finally excreted out of the body in the urine. Creatinine accumulates in the blood when the kidney does not work properly. The Clcr test shows how fast the kidney removes creatinine from the blood, *i.e.* the amount of blood "cleared" of creatinine per time period. Clearance is commonly measured in milliliters per minute (mL/min). The determination of creatinine in both serum and a timed collection of urine is necessary [Bowers, Wong, 1980].

Clcr was calculated as follows:

$$\text{Clcr (mL/min)} = (\text{urine creatinine} / \text{serum creatinine}) \times \text{urine Vol. (mL)} / [\text{time (h)} \times 60]$$

For the first animal study protocol, the Clcr levels were measured spectrophotometrically using a Beckman Autoanalyzer-2, performed in the clinical biochemistry diagnosis lab at the General Hospital, HCCS, at day 4, 11, 18 and 28 following the start of administration of CsA in 20% of oil emulsions.

For the second animal study protocol, serum and urine creatinine concentrations were measured one week prior to and once a week during the four-week *i.v.* administration of NS or CsA (15 mg/kg/day), with a colorimetric method using a Diagnostics<sup>®</sup> Creatinine kit. The principle of this test is that yellow/orange color forms when creatinine reacts with alkaline picrate. The color derived from creatinine is destroyed at acidic conditions. The difference in color intensity measured at or near 500

nm before and after acidification is proportional to creatinine concentration [Heinegard, Tiderstrom, 1973; Cook, 1975].

In our study, this colorimetric reaction was carried out in a 96-well plate, instead of in cuvetts as suggested by the producer, because of the large number of samples. The procedure is as follows:

1. To the blank, standard and test wells, 25  $\mu\text{L}$  of  $\text{H}_2\text{O}$ , a series of dilutions of standard, serum and 12-fold diluted urine samples were added to the corresponding wells.
2. To each test well, 250  $\mu\text{L}$  of alkaline picrate solution was added. The plate was shaken by a microplate shaker for 10 min at room temperature.
3. The initial absorbance was read at 490 nm by a microplate reader.
4. To each well, 8.3  $\mu\text{L}$  of acid reagent was added. The plate was shaken for 60 min at room temperature.
5. The final absorbance was read at 490 nm.

$$\text{Absorbance of creatinine} = \text{Initial Absorbance} - \text{Final Absorbance}$$

#### 2.4.3. Measurement of urine volume

The volumes of urine samples collected over 20 h with metabolic cages were measured using a volumetric flask. The mean values were obtained from 6-8 animals in each group.

#### **2.4.4. Determination of protein contents in urine**

Protein concentrations in urine samples collected over 20 h were determined by a colorimetric assay using the Bio-Rad DC Protein Assay reagents. The reaction is a modified Lowry assay, which is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. The amino acids tyrosine and tryptophan, and to a lesser extent, cystine, cysteine, and histidine, react with copper in alkaline medium. The copper-treated proteins affect the reduction of the Folin reagent by loss of 1, 2, or 3 oxygen atoms. The consequent reduced products have a characteristic blue color which can be measured at 630-750 nm [Peterson, 1979]. The procedure of determination is as follows:

1. Pipette 5  $\mu\text{L}$  of  $\text{H}_2\text{O}$ , protein standards (0.2 – 1.5 mg/mL) and urine samples into the blank, standard and test wells in a clean and dry 96-well plate.
2. Add 25  $\mu\text{L}$  of Regent A into each well.
3. Add 200  $\mu\text{L}$  of Regent B into each well and mix for 5 sec with the mixing function of microplate reader.
4. After 15 min, a blue color developed, and the absorbance was read at 640 nm by the microplate reader.

#### **2.4.5. Measurement of urinary N-Acetyl-1- $\beta$ -D-glucosaminidase (NAG)**

Urinary NAG is recognized as a sensitive and reliable indicator of the presence of renal disease. The NAG test was reported to offer a greater sensitivity to reflect kidney

abnormality than the Clcr test, because serum creatinine levels do not become abnormally elevated until 50% of renal function has been lost [Price, Whiting, 1992].

The NAG levels in urine samples of rats were measured by a colorimetric procedure using a commercially available PPR NAG Test Kit. This test is based on the hydrolysis of its substrate (2-Methoxy-4-(2'-nitrovinyl)-phenyl 2-acetamido-2-deoxy- $\beta$ -D-glucopyranoside, (MNP-GlcNAc)) by the enzyme NAG to release a phenolic compound (2-Methoxy-4-(2'-nitrovinyl)-phenol). In the presence of alkaline buffer, this compound produces a red color in proportion to the hydrolysis created by the levels of NAG present [Yuen *et al.*, 1984]. According to the direction, the procedure is as follows:

1. To each properly labeled test tube, 50  $\mu$ L of H<sub>2</sub>O, NAG calibrant solution, or urine samples were added to the corresponding tubes of blank, standards, or tests at strict timed intervals.
2. To each tube, 750  $\mu$ L of Reaction Solution 1 was added. The content was mixed and incubated in a 37 °C water bath for 30 min.
3. To each tube, 250  $\mu$ L of Color Development Buffer was added. Mix the content and place the tubes at room temperature for 10 min.
4. The absorbance was read at 505 nm using a Beckman Spectrophotometer DU62.

All the adding of samples and reagents, reading of absorbance are at the strictly timed intervals. Enzyme activity is expressed as  $\mu$ mol/h.

#### **2.4.6. Measurement of SBP**

SBP of conscious restrained rats was measured using the tail plethysmography method. For this measurement, rats were placed in a quiet, temperature-controlled environment (33 °C) to prewarm for 30 min, so that they were in a relaxed mood when measuring the blood pressure. A tail cuff with a light sensor connected to a pulse amplifier was placed as proximal as possible and four to six blood pressure readings were recorded using a 2-channel recorder. For the first animal study protocol, SBP was measured prior to and at day 8, 15, 22, and 27 following the administration of CsA in 20% oil emulsions. For the second animal study protocol, SBP was monitored one week prior to and once a week following the start of i.v. administration of CsA (15 mg/kg/day) or NS. The SBP reported are the average of four to five measurements.

#### **2.4.7. Assessment of growth**

It had been reported, and observed in our experiments that administration of CsA to rats suppressed their growth. The body weight (BW) of rats was recorded daily, and the mean value of each group was calculated once a week.

#### **2.4.8. Renal histological change**

For the second animal study protocol, immediately after sacrifice of the rats, the kidneys were removed and bisected. Portions that were fixed in 4% paraformaldehyde were processed for light microscopic examination by standard histologic techniques. Paraffin sections were cut to 4 µm thicknesses, and stained with hematoxylin and eosin

(HE). The severity of cortical and medullary injury was assessed by a pathologist without knowledge of the treatment group.

#### 2.4.9. Measurement of CsA concentration in blood

In the first *in vivo* study, the CsA concentrations in whole blood taken 24 h after the last dosing were determined using CYCLO-Trac<sup>®</sup> SP-Whole Blood Radioimmunoassay for Cyclosporine kit. It was measured by the Renal Lab of the HCCS.

In the second *in vivo* study, the CsA levels in whole blood were determined by liquid chromatography-Tandem mass spectrometry (LC-MS).

Briefly, a CsA stock solution (0.5 g/L) was prepared in methanol, and a set of calibrators (25, 50, 100, 200, 400, 800 µg/L) was prepared by two-fold diluting of the stock solution. Ascomycin (100 µg/L) was used as an internal standard.

To a 100 µL of whole blood sample, 100 µL of acetonitrile containing ascomycin internal standard was added. Then the samples were deproteinised by adding 40 µL of 0.1 M ZnSO<sub>4</sub> solution. After vortex mixing and centrifugation at 13,000 g for 5 min, the clear, colorless supernatant was transferred to an autosampler vial for further analysis.

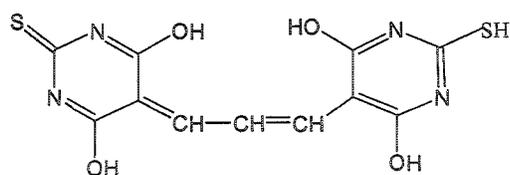
HPLC: Chromatography was performed on a Waters 2795 Alliance HT HPLC system. The processed whole blood samples were analyzed using an on-line solid phase extraction technique. Ten µL samples were injected into a C18 SecurityGuard cartridge (3 mm × 4 mm) maintained at 45 °C and equilibrated with 50% aqueous methanol containing 2 mM ammonium acetate and 0.1% formic acid at a flow rate of 0.5 mL/min. After washing for 0.1 min, the methanol concentration was maintained to 100% over 1

min to elute the test samples. The cycle time from injection to injection was approximately 2.5 min. The elute was connected directly to the electrospray probe of the mass spectrometer.

MS: A Micromass Quattro LC tandem mass spectrometer fitted with a Z Spray ion source was used for analyses. The instrument was operated in electrospray positive-ionization mode and was directly connected to the HPLC system. CsA and ascomycin were detected using multiple reaction monitoring (MRM) for the ammoniated species  $[M+NH_4]^+$ . The tuning parameters for CsA involved monitoring the  $m/z$  1220>1203 transition at 20 eV collision energy using argon gas. For ascomycin the  $m/z$  809.4>756.4 transition was monitored using a 26 eV collision energy. Cone voltage was held at 65 V throughout with RF lens setting at 35.0. System control and data acquisition were performed with MassLynx NT 3.4 software with automated data processing by the MassLynx Quantify program. Calibration curves were constructed using linear least-squares regression with  $1/x$  weighting.

#### 2.4.10. Measurement of renal lipid peroxidation

Lipid peroxidation in kidney was assessed by detecting malondialdehyde (MDA), the lipid peroxidation product. An MDA molecule form a 1:2 adduct with thiobarbituric acid and produces a compound,



which can be measured spectrophotometrically. The MDA content in 10% kidney homogenate of rats was measured with an OXI-TEK TBARS Assay kit.

Preparation of 10% kidney homogenate: in a glass tissue grinder that sat in an ice bath, around 100 mg kidney tissues was added 0.4 mL, 100 mM ice-cold potassium chloride containing 3 mM EDTA, and homogenized. Additional 0.6 mL of the potassium chloride buffer solution was used to wash the grinder 3 times. The combined tissue homogenate was centrifuged at 10,000 g, 4 °C for 10 min. The supernatants were withdrawn and stored at -20 °C to minimize oxidation [Wang, Salahudeen, 1994].

MDA content in the supernatant of kidney homogenate was measured as follows:

1. To each properly labeled test tube, 50  $\mu$ L of H<sub>2</sub>O, MDA standards, or test samples were added to the corresponding tubes of blank, standards, or tests.
2. SDS solution 50  $\mu$ L was added to each tube and swirled to mix.
3. TBA reagent 1.25 mL was added to each tube and mixed.
4. Cover each tube with a glass marble and incubate at a boiling water bath for 60 min.
5. After cooling to room temperature in an ice bath, the samples were centrifuged at 3,000 g for 15 min.
6. The absorbance of supernatants were read by a spectrofluorophotometer with excitation wavelength of 530 nm, emission wavelength of 550 nm, and the sensitivity be set at high with a slit width of 10 nm.

The protein concentration in kidney homogenate was determined by DC protein assay as stated in section 2.4.4. The MDA levels were presented as nmol /mg protein.



According to the directions, the measuring procedure is as follows:

1. Set up sufficient strips of wells and add 50  $\mu\text{L}$  of buffer, standards, and test samples to the corresponding wells of blank, standards and tests; 100  $\mu\text{L}$  of buffer for the non-specific binding.
2. Add 50  $\mu\text{L}$  of antiserum to all wells except for the non-specific binding.
3. Add 50  $\mu\text{L}$  of 6-keto-PGF<sub>1 $\alpha$</sub>  Peroxidase conjugate into all wells except for the blank.
4. Incubate at room temperature for 1 h while samples were kept shaking by a microplate shaker.
5. Aspirate and wash all wells four times with 400  $\mu\text{L}$  of washing buffer each time.
6. Add 150  $\mu\text{L}$  of enzyme substrate TMB to all the wells and shaking for exactly 15 min at room temperature.
7. Add 100  $\mu\text{L}$  of 1.0 M sulfuric acid to all the wells, mix the content and the absorbance was read by microplate reader at 490 nm within 30 min.

The principle and operation of TXB<sub>2</sub> assay are the same as that of 6-keto-PGF<sub>1 $\alpha$</sub> .

#### **2.4.12. Analysis of fatty acid compositions in diets and erythrocyte membrane of rats**

The fatty acid compositions in erythrocyte membrane of rats and the two diets containing different oils were analyzed by GC.

The RBCs and diet samples were homogenized with a glass grinder and total lipids were extracted using a modified Folch wash procedure [Folch *et al.*, 1957] as follows:

1. Add 20 mL of  $\text{CHCl}_3$  and 10 mL of MeOH to the RBCs pellet in grinder, homogenize for 2 min.
2. Suction filtration---collect filtrate into the grinder, keep the liquid portion.
3. Add another 20 mL of  $\text{CHCl}_3$  and 10 mL of MeOH to the grinder, homogenize for 3 min, and suction filtrate.
4. Wash with 20 mL of  $\text{CHCl}_3$  and then 10 mL of MeOH.
5. Transfer the collected liquid portion to a graduated cylinder with a glass stop.
6. Add  $\frac{1}{4}$  of the total volume of 0.88% KCl aqueous solution, shake thoroughly and allow to settle.
7. Remove top layer by aspiration, add  $\frac{1}{4}$  of the bottom layer (organic layer) volume of  $\text{H}_2\text{O}$ -MeOH (1:1, v/v), shake and settle.
8. Remove top layer by aspiration, transfer to beaker and blow down with nitrogen ( $\text{N}_2$ ).
9. Resuspend with 2 mL of  $\text{CHCl}_3$  and store in a small vial for transmethylation.

Isolated lipid samples were transmethylated using 2 mL Transmethylation Reagent (94% MeOH, 6% aqueous HCl) and a few crystals of hydroquinone in a transmethylation vial at 60 °C for 2 h. Fatty acid methyl esters thus formed were separated by GC using an Omegawax™ 320 capillary column (30 m × 0.32 mm × 0.2 μm thickness) in a Hewlett Packard 5890 Series II gas chromatograph equipped with a flame ionization detector. Separation was performed isothermally at 200 °C, with helium as the carrier gas at a flow rate of 1 mL/min. Identification of the peaks in the chromatograms

was made by comparison of retention time with known standards composed of different fatty acids. The percent composition data were normalized as:

$$\text{transformed data} = \text{arcSin}(\sqrt{\text{area\%/100}}) * 100$$

The transformed data were employed to conduct the statistical analysis.

## 2.5. *In vitro* studies

To further investigate the effects of seal oil on the nephrotoxicity associated with CsA, *in vitro* studies were also conducted. LLC-PK1, a pig proximal tubular epithelial cell line, was used as a model of the proximal tubule for the study of renal cytotoxicity induced by CsA.

LLC-PK1 cells were grown in M199 supplemented with 3% FBS at 37 °C, 5% CO<sub>2</sub> as suggested by ATCC. CsA that used for the *in vitro* study was Sandimmune IV<sup>®</sup> microemulsion containing 50 mg CsA /mL. Non-medicinal ingredients in the formulation include: dl- $\alpha$ -tocopherol, ethanol, hydrogenated castor oil, maize oil, and propylene glycol. The CsA microemulsion was diluted with the cell culture medium to appropriate concentrations shortly before experiment.

Seal oil was prepared as a 10% seal oil emulsion with 1.2% Lipoid 80 as surfactant, 2.25% glycerol, and 2.0  $\mu$ M K<sub>2</sub>HPO<sub>4</sub>, 4.0  $\mu$ M EDTA-2Na buffer as the aqueous components. The emulsions were prepared as described earlier (Section 2.2.), and sterilized by autoclaving at 121 °C for 30 min prior to use.

DHA, EPA and V<sub>E</sub> were dissolved in dimethylsulfoxide (DMSO) (50 mg/mL), and then diluted with the cell culture medium to appropriate concentrations.

### 2.5.1. Growth curve determination

Knowledge about the growth state of a cell line and its kinetic parameters is important in designing *in vitro* experiments using the cell line. Therefore, the growth cycle of LLC-PK1 cells was determined by direct counting the cell number following a certain time of incubation at various seeding densities in multiwell plates. The procedure is as follows:

1. After LLC-PK1 cells grown to confluent in plastic culture flasks, they were trypsinized with 0.25% trypsin-EDTA solution to prepare single cell suspension.
2. Three concentrations of cell suspension  $1 \times 10^4$ ,  $3 \times 10^4$ , and  $1 \times 10^5$  cells/mL in 25 mL of medium was prepared.
3. Cells were seeded in a 24-well plate at each concentration, with 1 mL/well. Cell suspension was added slowly from the center of the wells, so that the cells do not swirl and achieve even distribution of cells.
4. Plates were placed in a humidified 5% CO<sub>2</sub> incubator without stirring the cell suspension.
5. After 24 h incubation, medium was removed completely from three wells in each plate; the cell monolayer was washed three times with PBS; 200  $\mu$ L of 0.25% trypsin-EDTA solution was added into each of the wells; the plate was incubated for 15 min at 37 °C; cells were then dispersed with a pipette thoroughly in trypsin-EDTA and counted with a hemocytometer.

6. Plates were returned to the incubator as soon as the cell samples in trypsin were removed.
7. Sampling was repeated every 24 h until the stationary phase was reached. The medium was changed every three days or sooner depending on their growth.

### 2.5.2. MTT assay

Cytotoxicity of CsA in LLC-PK1 cells was determined using the MTT assay.  $IC_{50}$  was defined as the concentration of CsA causing 50% inhibition of cell growth.

The MTT assay is based on the enzymatic reduction of a yellow, water-soluble tetrazolium dye (MTT) in living, metabolically active cells, but not in dead cells. The reaction is carried out in 96-well plate, and produces a purple-colored formazan soluble in DMSO, which can be measured colorimetrically using a microplate reader.

Basically, the procedure used in this study was identical to the assay described by Ford [Ford and Richardson, 1989], which is a modification of a method described originally by Mosmann [Mosmann, 1983].

MTT was dissolved in PBS at a concentration of 5 mg/mL as a stock, sterile filtered and stored in a dark environment at 4 °C for up to three weeks.

LLC-PK1 cells were grown at 37 °C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> in plastic culture flasks to confluence. To find a proper range of cell density for which absorbance at 570 nm versus cell density is linear, the cells were trypsinized with 0.25% trypsin-EDTA solution to prepare single cell suspension in medium at  $2.6 \times 10^6$  cells/mL. A 96-well plate was filled entirely with 100  $\mu$ L/well medium, to which 100  $\mu$ L

of the cell suspension was added into eight wells of the first column, and the cells were titrated 100  $\mu\text{L}$ /well by two-fold serial dilutions across the plate using a multichannel pipette, *i.e.*, titrated 100  $\mu\text{L}$ /well cell suspension with a multichannel pipette from the wells of Column 1 into wells of Column 2 containing 100  $\mu\text{L}$ /well medium, and mix the cell suspension thoroughly. Then transfer 100  $\mu\text{L}$ /well of the two-fold diluted cell suspension from these wells into the next wells of Column 3 containing 100  $\mu\text{L}$ /well medium. This step was repeated till Column 11. Wells of Column 12 containing only medium was served as blank. After the plate was incubated at 37 °C for 24 h, 50  $\mu\text{L}$  of medium was added into each well, followed by additional 48 h incubation. Then medium was aspirated and 100  $\mu\text{L}$  of MTT solution (0.5 mg/mL) was added to each well. After 4 h incubation with MTT at 37 °C, the supernatant was carefully aspirated, and then 100  $\mu\text{L}$  of DMSO was added to each well. The plate was agitated on a plate shaker for 20 min at room temperature, then read spectrophotometrically at 570 nm with 630 nm as reference wavelength by a microplate reader.

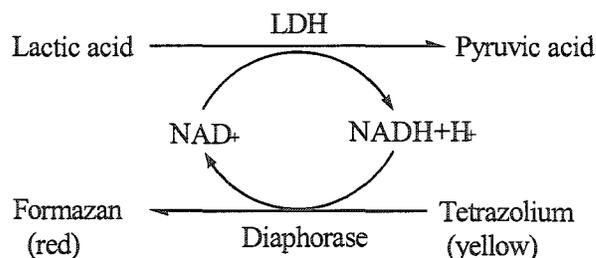
To choose a proper concentration of seal oil emulsion that is not toxic to the LLC-PK1 cells, a single cell suspension at  $2 \times 10^4$  cells/mL was seeded 50  $\mu\text{L}$ /well into a 96-well plate. After incubation at 37 °C for 24 h, 100  $\mu\text{L}$  of various concentrations of seal oil emulsion (0.000032%-0.004%) was added to the plate, followed by 24 h incubation. Then the medium was aspirated, MTT solution (0.5 mg/mL) was added and the plate was incubated for an additional 4 h, and finally DMSO was added to the cells after removal of MTT solution to read absorbance at 570 nm. Cells incubated only in medium were used as a control; Wells containing 0.004% seal oil emulsion with no cells served as blank.

To determine the  $IC_{50}$  value of CsA under the current experiment condition, a LLC-PK1 single cell suspension at a density of  $2 \times 10^4$  cells/mL was seeded 50  $\mu$ L/well into 96-well plates using a multichannel pipette. The plates were incubated at 37 °C for 20 h to allow cells to reattach and re-equilibrate, followed by addition of 50  $\mu$ L of medium and incubation for 4 h. Without removing any medium, 50  $\mu$ L/well of various concentrations of CsA (0, 1.88, 3.75, 7.5, 15.0, 30.0  $\mu$ M) in medium were added to the plate. After 24 h incubation with CsA, the medium was aspirated from each well and MTT solution (0.5 mg/mL) was added for additional 4 h incubation, and finally DMSO was added to read absorbance at 570 nm. Wells containing medium in combination with 10  $\mu$ M CsA (final concentration) but no cells were used as blank. Results were expressed as a percentage of the absorbance of the control, and  $IC_{50}$  values were calculated.

The effect of DHA, EPA and  $V_E$  on the cytotoxicity of CsA was also determined using the MTT assay, respectively. As stated above, LLC-PK1 cells were incubated in 96-well plates for 20 h, then 50  $\mu$ L of various concentrations of DHA (0.24-30  $\mu$ M), EPA (0.3-30  $\mu$ M) or  $V_E$  (0.3-30  $\mu$ M) in cell culture medium (three times the final concentration) were added to each well, respectively, followed by 4 h incubation. Then 50  $\mu$ L of 10  $\mu$ M CsA (final concentration: 3.3  $\mu$ M,  $IC_{50}$ ) was added to each well. After 24 h incubation following drug exposure, the cells were incubated with MTT solution (0.5 mg/mL) for 4 h, and then added DMSO to read absorbance at 570 nm.

### 2.5.3. Measurement of cytotoxicity by lactate dehydrogenase (LDH) leakage

Cytotoxicity was also assessed using an indicator of membrane lysis. LDH is a stable cytoplasmic enzyme found in most cells, leaking out upon damage of the cell membrane [Welder, Acosta, 1994]. The release of intracellular LDH to the extracellular medium was measured by determining the activity of this enzyme using a LDH Cytotoxicity Detection Kit. The principle of this enzymatic test is that lactic acid is converted to pyruvic acid by catalysis of LDH, with  $\text{NAD}^+$  reduced to  $\text{NADH} / \text{H}^+$ . In the second step,  $\text{H}^+$  is transferred from  $\text{NADH} / \text{H}^+$  to tetrazolium by the catalyst diaphorase to form formazan, a red colored substance whose absorbance can be read colorimetrically at 450 nm [Cook, Mitchell, 1989; Goergen *et al.*, 1993].



Briefly, LLC-PK1 cells were seeded and incubated in 96-well plates in M199 supplemented with 1.5% serum (it is suggested to conduct the assay with low serum concentration to minimize background absorption) in the presence or absence of 0.0001% seal oil emulsion for 24 h. Then the cells were exposed to various concentrations of CsA (final concentrations: 0-10  $\mu\text{M}$ ) and incubated for another 24 h. At the end of the incubation, the 96-well plate was centrifuged at 250 g for 10 min. The supernatant in each well was carefully transferred (100  $\mu\text{L}$ /well) into corresponding wells of another optically clear flat bottom 96-well plate. Each well was then added 100  $\mu\text{L}$  of the reaction

mixture and the plate was incubated at room temperature for 30 min. During this incubation, the plate was protected from light. The absorbance in each well of the plate was read at 490 nm by a microplate reader.

The effects of DHA and  $V_E$  on CsA-induced LDH release in LLC-PK1 cells were also tested, respectively. Various concentrations of DHA (final concentration: 0.08-10  $\mu$ M) or  $V_E$  (final concentrations: 0.1-10  $\mu$ M) were added to the cells 4 h prior to the exposure to CsA, and coincubated with 3.3  $\mu$ M CsA for 24 h. The supernatants were collected to measure LDH levels the same as stated above.

#### **2.5.4. Measurement of lipid peroxidation in CsA-treated LLC-PK1 cells**

The effect of EPA, DHA or  $V_E$  on lipid peroxidation in the process of CsA-induced cytotoxicity in LLC-PK1 cells was also studied. The content of lipid peroxidation product, MDA, in the cell lysate was determined by TBARS assay.

LLC-PK1 single cell suspension was prepared by trypsinization with 0.25% trypsin-EDTA solution, and seeded in two 24-well plates at  $3 \times 10^4$  cells/mL, 1 mL/well. After incubation at 37 °C for 24 h, various concentrations (final) of EPA (0.01, 0.1, 1  $\mu$ M), DHA (0.01, 0.1, 1, 10  $\mu$ M), or  $V_E$  (0.1, 1, 10  $\mu$ M) were added to the cells and incubated for 4 h, respectively. Then 3.3 and 6.7  $\mu$ M CsA (final concentration) were added to each plate, respectively, followed by coincubation for 24 h. To one well in each plate, which carries no cell, was added medium and 3.3 or 6.7  $\mu$ M CsA to serve as blank. Cells incubated only with medium without drug treatment were used as normal control.

To release the cytoplasmic MDA, 2% Triton X-100 (final concentration) was added to each well to lyse the cells. After most of the cells were found lysed under a microscope, the plates were centrifuged at 1,000 rpm, 4 °C for 5 min. MDA content in the cell lysate supernatant was measured using TBARS assay as described in Section 2.4.10. The MDA levels were presented as nmol/ mg protein.

## **2.6. Statistical analysis**

Data of the *in vivo* study, effect of dietary supplementation of different oils on CsA-induced nephrotoxicity in rats (n= 6-8), were averaged within groups and presented as mean  $\pm$  SD. All *in vitro* experiments were repeated at least twice with three to five replicates. The results were expressed as mean  $\pm$  SD. Statistical analysis was performed using unpaired Student's *t* test. A *P* value less than 0.05 was considered statistically significant. All the calculations and statistics were performed by using SigmaPlot-2001 statistical analysis software.

## Chapter 3. Results

### 3.1. Preparation and characterization of CsA emulsions

The CsA in 10% and 20% of seal oil or corn oil emulsions containing 0.3-3.8% of Cremophor RH-40 and Tween 80 (2:1) were prepared using high pressure homogenization. The particle size of the CsA emulsions was determined by a Delsa 440SX Zeta Potential Analyzer. It was found that there was no significant difference with respect to particle size between CsA in 10% or 20% of seal oil emulsions and CsA in 10% or 20% of corn oil emulsions when the amount of surfactants was kept the same. The particle sizes of CsA in seal oil and CsA in corn oil emulsions with various concentrations of surfactants are shown in Figure 3.1. and Figure 3.2., respectively. It can be seen that the particle size decreased as the amount of surfactants increased from 0.3 to 1.5% and further increase in the amount of surfactants did not result in further reduction in particle size. The particle size of CsA in 10% and 20% of seal oil emulsions was found to be around 340 nm in diameter in the presence of 1.5% or higher amount of surfactants.

The stability of CsA in seal oil or corn oil emulsions was monitored by measuring the particle size of the emulsions weekly following preparation for four weeks. The results are presented in Table 3.1., and it can be seen that the particle size of CsA in 20% of seal oil or corn oil emulsions remained unchanged during the four weeks.

CsA in 20% of seal oil and 20% of corn oil emulsions with 1.5% of surfactants (Cremophor RH-40: Tween 80 = 2:1) were used for the following *in vivo* experiment.

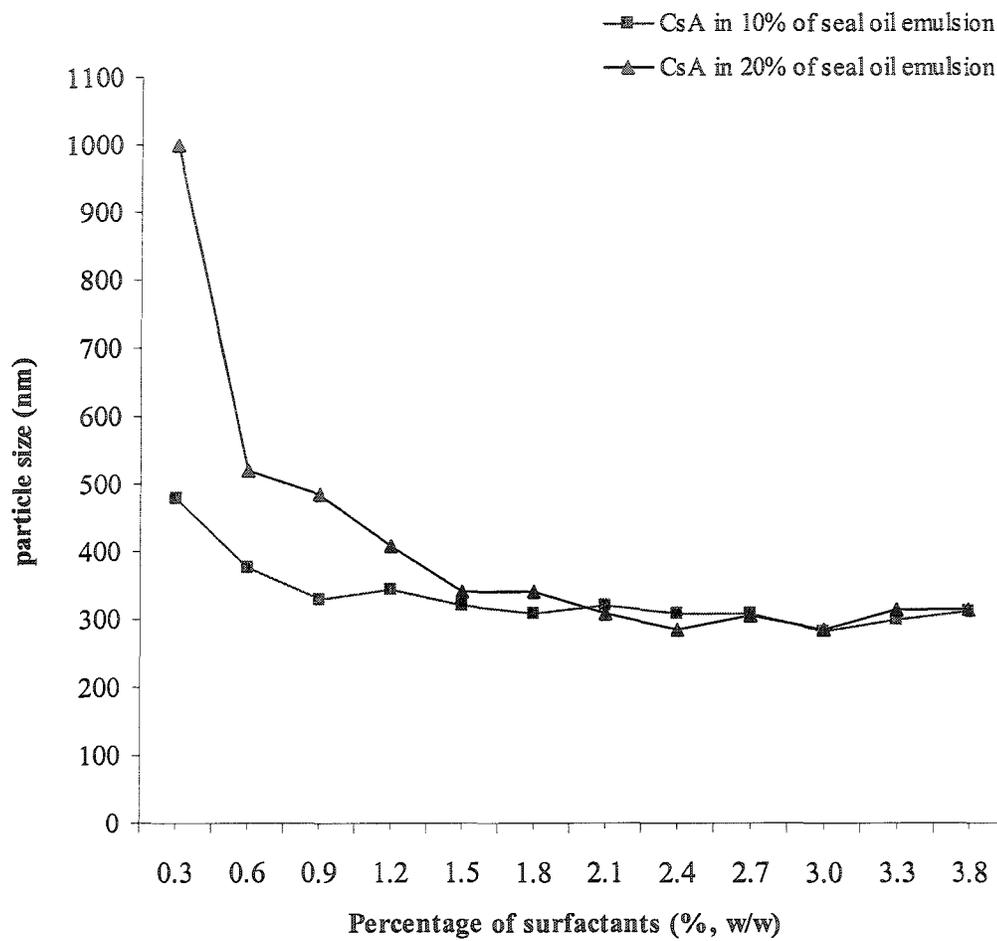


Figure 3.1. Particle size of CsA in 10% and 20% of seal oil emulsions containing various concentrations of Cremophor RH-40 and Tween 80 at a ratio of 2:1.

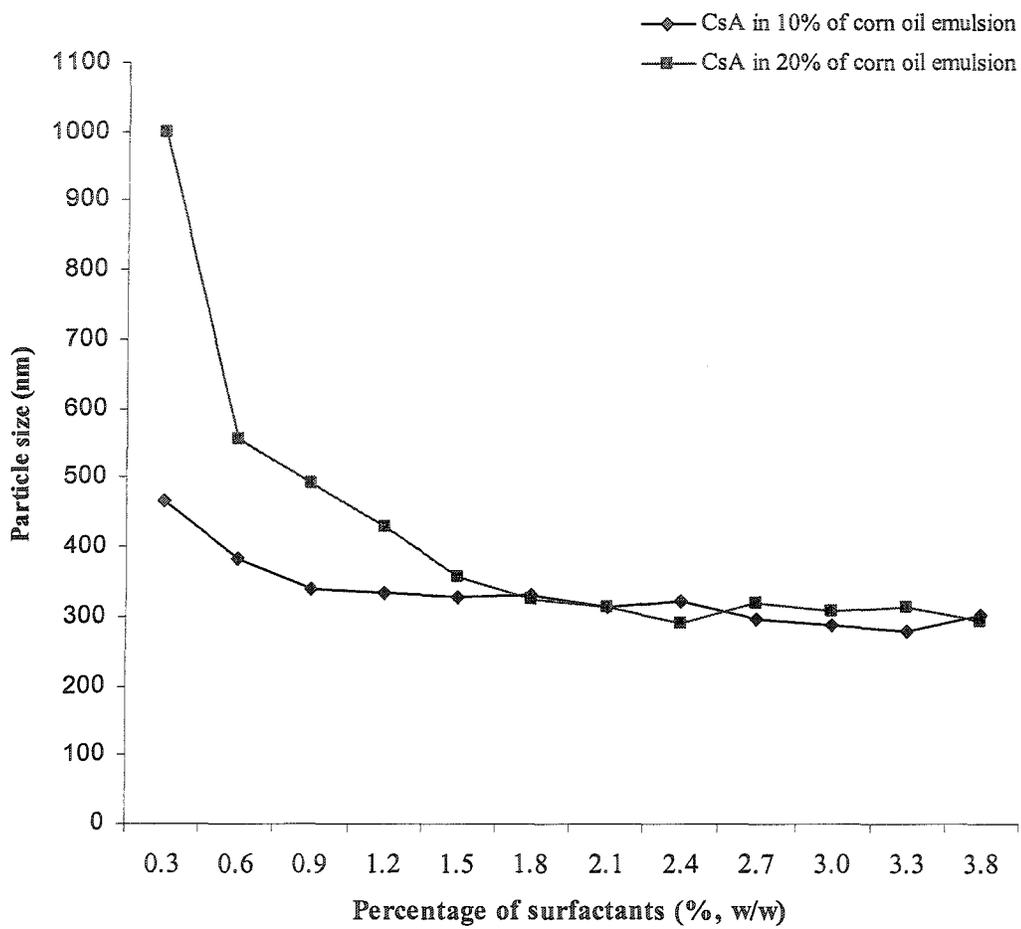


Figure 3.2. Particle size of CsA in 10% and 20% of corn oil emulsions containing various concentrations of Cremophor RH-40 and Tween 80 at a ratio of 2:1.

Table 3.1. Particle size of CsA in 20% of seal oil and 20% of corn oil emulsions, respectively, determined at different time intervals following preparation \*

Time	Particle size in diameter (nm)	
	CsA in 20% of corn oil emulsion	CsA in 20% of seal oil emulsion
0 week	331 ± 49	337 ± 53
1 week	346 ± 54	333 ± 50
2 weeks	324 ± 42	331 ± 38
3 weeks	336 ± 32	343 ± 39
4 weeks	341 ± 37	348 ± 45

\* Both CsA emulsions contained 1.5% of Cremophor RH-40 and Tween 80 at a ratio of 2:1.

### **3.2. *In vivo* studies**

#### **3.2.1. The comparison of kidney functions of rats administered with CsA in 20% of seal oil emulsion and those administered with CsA in 20% of corn oil emulsion**

Rats (three rats in each group) were administered CsA in 20% of seal oil or CsA in 20% of corn oil emulsion via *p.o.* (50 mg/kg/day) or *i.p.* (25 mg/kg/day) route, respectively, for 28 days. Various parameters of kidney function were monitored. The results of rats given CsA in 20% of seal oil emulsion were compared with those given CsA in 20% of corn oil emulsion.

##### **3.2.1.1. The level of BUN following administration of CsA emulsions**

BUN and Cl<sub>cr</sub> are commonly used clinical parameters of renal function. BUN levels of the rats measured at day 4, 11, 18 and 28 are shown in Figure 3.3. It can be seen that BUN levels increased following oral administration of 50 mg/kg/day CsA in both seal oil and corn oil emulsions in comparison with those administered with NS. The increased BUN levels in rats given CsA emulsions indicated impairment of kidney function by CsA. However, the mean values of BUN of rats given CsA in 20% of seal oil emulsion at day 18, 28 (11.8, 12.8 mmol/L) were lower than those of rats given CsA in 20% of corn oil emulsion (14.0, 13.6 mmol/L). The results suggest that oral administration of CsA (50 mg/kg/day) in 20% of seal oil emulsion induced less kidney functional impairment in comparison with CsA in 20% of corn oil emulsion.

Between the rats given CsA in 20% of seal oil and 20% of corn oil emulsions via *i.p.* route, there was no apparent difference found at all time intervals.

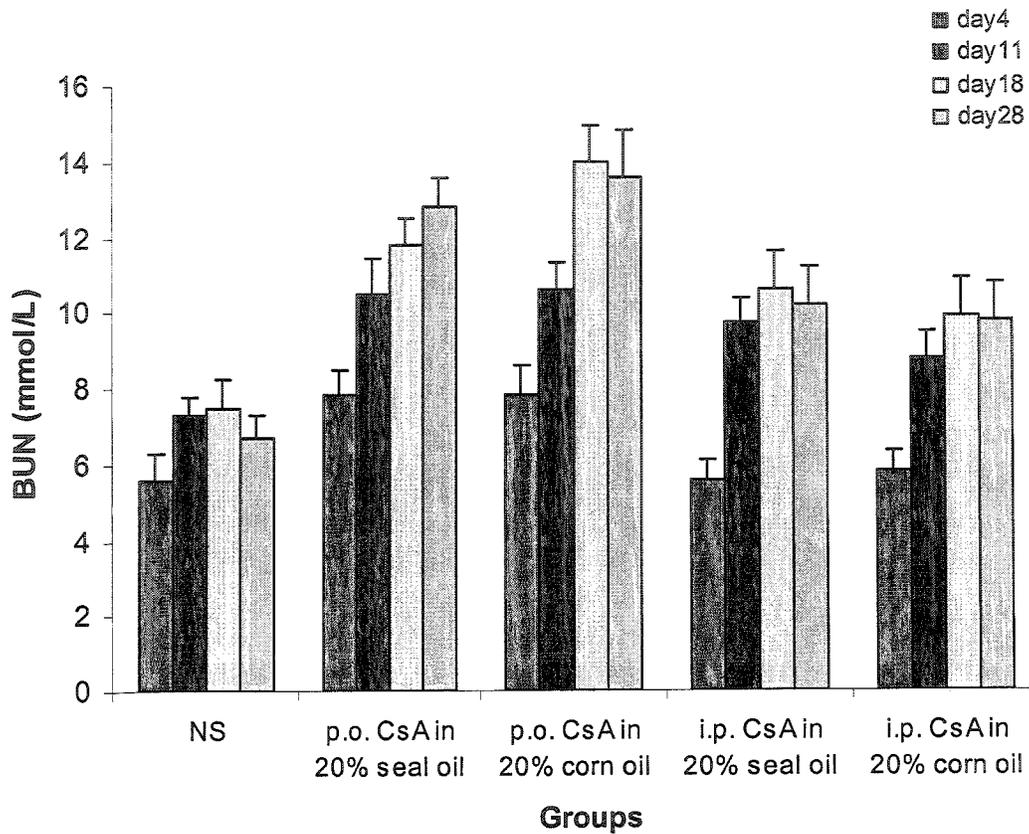


Figure 3.3. BUN levels in rats measured at day 4, 11, 18, and 28 following *p.o.* (50 mg/kg/day) or *i.p.* (25 mg/kg/day) administration of CsA in 20% of seal oil emulsion or CsA in 20% of corn oil emulsion, respectively. Data shown are mean  $\pm$  SD (n=3).

### 3.2.1.2. The level of Clcr following administration of CsA emulsions

The level of Clcr reflects the ability of the kidney to remove the metabolic waste from the body. The Clcr levels of rats following *p.o.* (50 mg/kg/day) or *i.p.* (25 mg/kg/day) administration of CsA in 20% of seal oil emulsion or CsA in 20% of corn oil emulsion for 28 days are shown in Figure 3.4.

It was found that administration of CsA in 20% of seal oil or 20% of corn oil emulsion via both *p.o.* and *i.p.* route for four days apparently resulted in decreased Clcr levels in comparison with the control group in which NS was given. With the two groups in which CsA emulsions were administered via *p.o.* route, it was found that the mean Clcr levels of rats given CsA in 20% of seal oil emulsion were higher than those given CsA in 20% of corn oil emulsion, suggesting less impaired waste eliminating function of kidney in rats given CsA in 20% of seal oil emulsion than those given CsA in 20% of corn oil emulsion.

However, there was no apparent difference of Clcr levels following *i.p.* administration of CsA in 20% of seal oil emulsion and 20% of corn oil emulsion.

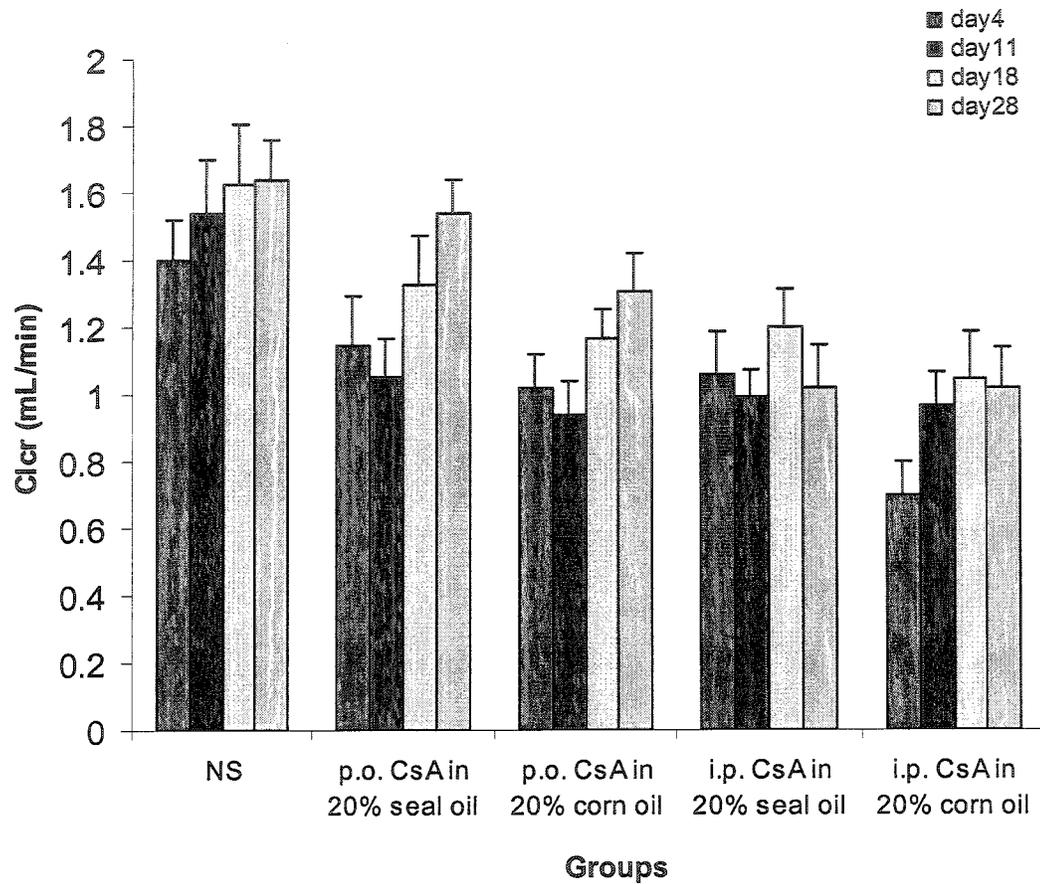


Figure 3.4. Clcr levels in rats measured at day 4, 11, 18, and 28 following *p.o.* (50 mg/kg/day) or *i.p.* (25 mg/kg/day) administration of CsA in 20% of seal oil emulsion or 20% of corn oil emulsion, respectively. Data shown are mean  $\pm$  SD (n=3).

### 3.2.1.3. The level of urinary NAG following administration of CsA emulsions

Urinary NAG is a sensitive indicator of renal tubular cell injury. The urinary NAG levels following *p.o.* (50 mg/kg/day) or *i.p.* (25 mg/kg/day) administration of CsA in 20% of seal oil or 20% of corn oil emulsion were determined at day 4, 11, 18 and 28, respectively. The results are shown in Figure 3.5. It was found that the urinary NAG levels were markedly elevated following administration of CsA for 11 days, which were consistent with the change of BUN.

On day 28 the NAG levels following *p.o.* administration of CsA in 20% of seal oil and 20% of corn oil emulsions were found to be 2.7 and 4 times of that of the control group. Urinary NAG levels in rats given CsA in 20% of seal oil emulsion were much lower than those given CsA in 20% of corn oil emulsion at both day 18 and 28.

A similar trend was observed with the two groups where CsA emulsions were given via *i.p.* route. The NAG levels in rats administered with CsA in 20% of seal oil emulsion were found to be lower than those given CsA in 20% of corn oil emulsion. The results indicated that both *p.o.* and *i.p.* administration of CsA in 20% of seal oil emulsion induced less renal tubular toxicity in rats than those of CsA in 20% of corn oil emulsion.

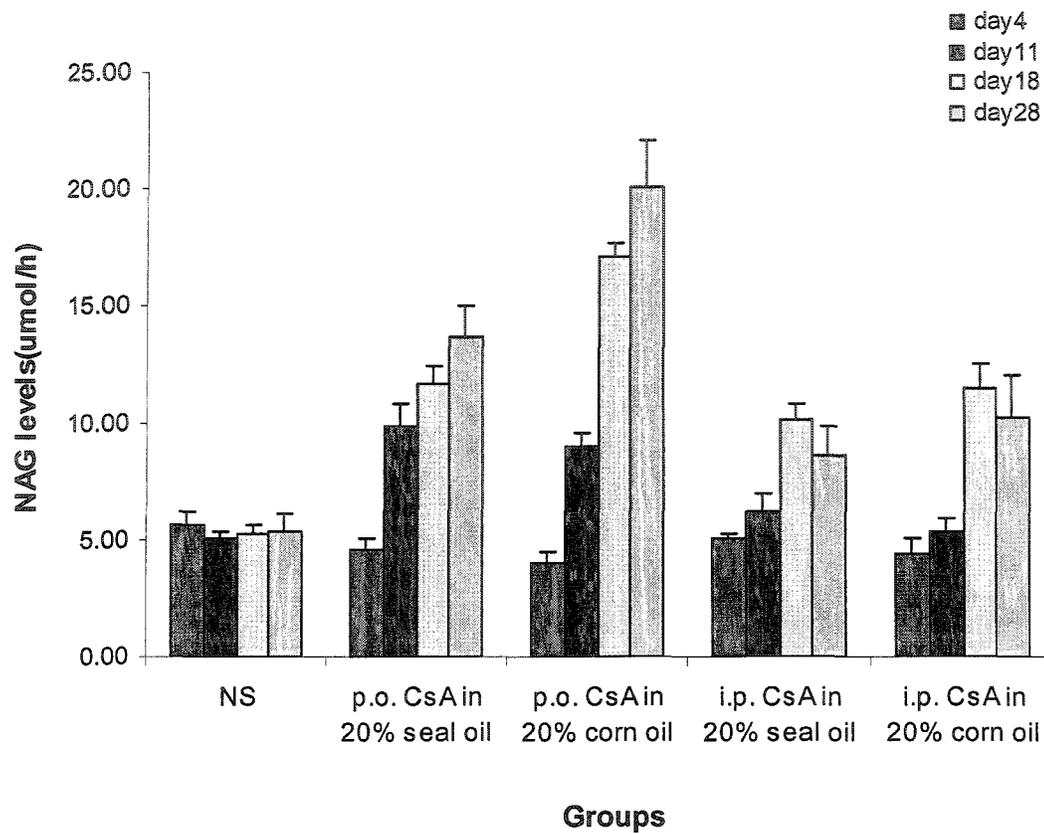


Figure 3.5. Urinary NAG levels in rats measured at day 4, 11, 18, and 28 following *p.o.* (50 mg/kg/day) or *i.p.* (25 mg/kg/day) administration of CsA in 20% of seal oil emulsion or 20% of corn oil emulsion, respectively. Data shown are mean  $\pm$  SD (n=3).

#### 3.2.1.4. The level of SBP following administration of CsA emulsions

Hypertension is one of the side effects of CsA. Therefore, the SBP of rats was monitored prior to and at day 8, 15, 22 and 27 following *p.o.* (50 mg/kg/day) or *i.p.* (25 mg/kg/day) administration of CsA in 20% of seal oil or 20% of corn oil emulsion. The results are shown in Table 3.2. It is clear that both *p.o.* and *i.p.* administration of CsA in 20% of seal oil or 20% of corn oil emulsion resulted in an increase of SBP in comparison with the control group. The mean SBP level increased to 152 mmHg following oral administration of CsA in 20% of corn oil emulsion at day 8, compared to 125 mmHg of the control group, and the increase persisted throughout the entire experiment. The mean SBP in rats given CsA in 20% of seal oil emulsion via *p.o.* route was found to have increased to 158 mmHg at day 8, following which it was decreased at day 22 and 27 to 131 and 123 mmHg, respectively.

In the two groups where CsA was given via *i.p.*, SBP increased continuously during the course of *i.p.* administration of CsA in 20% of corn oil emulsion, with SBP being 165 and 153 mmHg at day 22 and 27; whereas the mean SBP in rats receiving *i.p.* administration of CsA in 20% of seal oil emulsion was found to be lower, with SBP being 147 and 137 mmHg at day 22 and 27, respectively.

The results suggested less vascular toxicity in rats receiving CsA in 20% of seal oil emulsion than those receiving CsA in 20% of corn oil emulsion via both *p.o.* and *i.p.* routes.

Table 3.2. SBP in rats measured at day 8, 15, 22, and 27 following *p.o.* (50 mg/kg/day) or *i.p.* (25 mg/kg/day) administration of CsA in 20% of seal oil emulsion or CsA in 20% of corn oil emulsion, respectively.

Groups Time	NS	<i>p.o.</i> CsA in 20% seal oil	<i>p.o.</i> CsA in 20% corn oil	<i>i.p.</i> CsA in 20% seal oil	<i>i.p.</i> CsA in 20% corn oil
Day 8	130±15	158±20	152±24	136±14	147±19
Day 15	129±15	155±21	151±15	151±20	156±23
Day 22	125±12	131±17	152±21	147±21	165±24
Day 27	118±13	123±16	147±22	137±18	153±20

Note: Data shown are mean ± SD (n=3).

### 3.2.1.5. The CsA concentrations in blood following administration of CsA emulsions

At the end of the 28-day CsA administration, the CsA concentration in blood was measured by radioimmunoassay. The results are presented in Figure 3.6.

The CsA concentrations following oral administration of CsA (50 mg/kg/day) in 20% of seal oil and CsA in 20% of corn oil emulsion were found to be  $3816 \pm 523$  and  $4083 \pm 1226$   $\mu\text{g/L}$ , respectively. The CsA concentrations in blood following *i.p.* administration of CsA (25 mg/kg/day) in 20% of seal oil emulsion and CsA in 20% of corn oil emulsion were found to be  $3018 \pm 1195$  and  $3063 \pm 415$   $\mu\text{g/L}$ , respectively.

Oral administration of CsA at 50 mg/kg/day resulted in a higher concentration of CsA in blood than *i.p.* administration of CsA at 25 mg/kg/day. There was no significant difference between the two groups given CsA in 20% of seal oil emulsion and CsA in 20% of corn oil emulsion with the same dosage and administration route. The results showed a large variation among individual rats within each group.

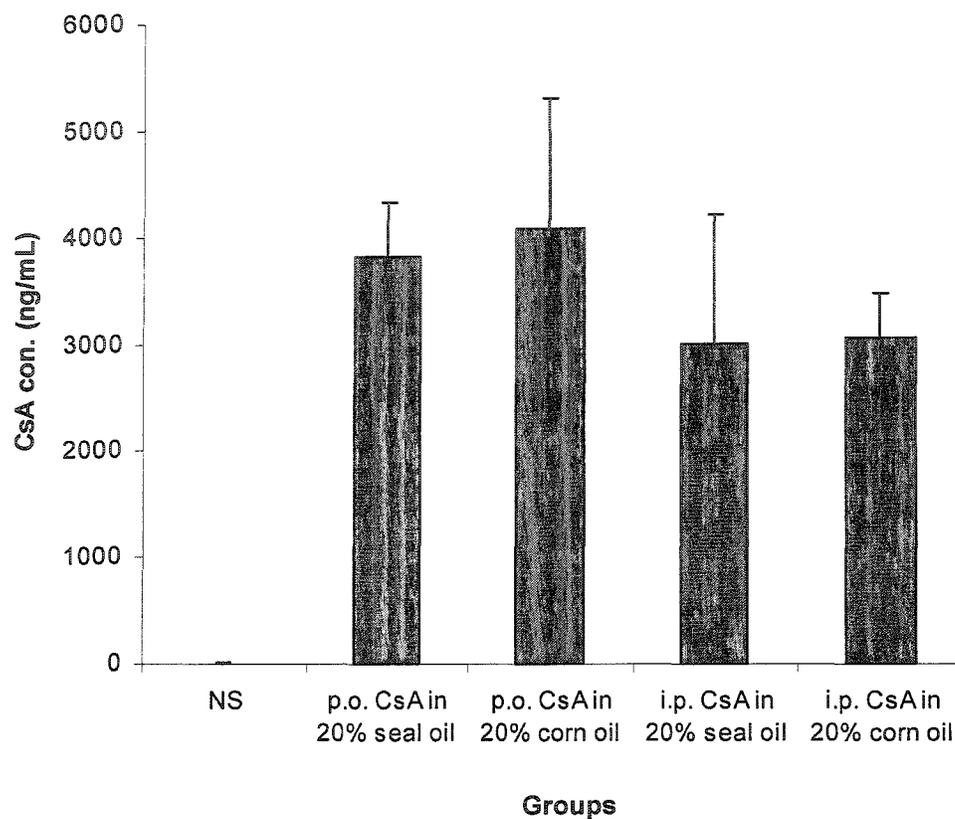


Figure 3.6. CsA concentrations in blood in rats following *p.o.* (50 mg/kg/day) or *i.p.* (25 mg/kg/day) administration of CsA in 20% of seal oil emulsion or CsA in 20% of corn oil emulsion, respectively, for 28 days. Data shown are mean  $\pm$  SD (n=3).

### 3.2.2. The effect of dietary supplementation of seal oil on the nephrotoxicity induced by CsA

Although the first *in vivo* experiment showed some interesting and promising findings, the significant effects of the seal oil emulsion formulation on the reduction of kidney impairment induced by CsA was not clearly demonstrated. This may be due to the insignificant supplementation of seal oil from the 20% of seal oil emulsion, as rats were still taking large quantity of vegetable oil from their regular chow diet. To eliminate the effect of dietary fat intake on the experimental results, the second *in vivo* experiment was designed so that the animal diet was carefully controlled in order to reflect the effect of seal oil.

Rats were randomly divided into four groups and were fed two different diets (one with predominantly seal oil and the other with only sunflower oil) we prepared for four weeks, followed by *i.v.* administration of NS or CsA (Sandimmune IV<sup>®</sup>, 15 mg/kg/day), respectively, for four weeks, while the rats were still kept on their respective diets.

Various parameters of kidney function were assessed. The effect of seal oil on the toxicity induced by CsA was determined by comparing the results of rats fed seal oil enriched diet and those fed regular diet containing sunflower oil.

### 3.2.2.1. The level of BUN following *i.v.* administration of CsA or NS

As mentioned in Section 3.2.1.1, BUN and Clcr are commonly used clinical parameters of renal function. BUN levels were measured one week prior to and once a week following the start of *i.v.* administration of CsA (15 mg/kg/day) or NS. The results of BUN levels are shown in Figure 3.7.

BUN levels in the two groups of rats administered CsA for one week were found to be higher than those of rats which did not receive CsA. The elevated BUN levels were found to be more significant ( $P < 0.01$ ) as CsA administration continued, especially in rats fed sunflower oil diet. The BUN levels in rats fed seal oil diet at week 3 and 4 were found to be lower than those in rats fed sunflower oil diet, and the difference of BUN levels between these two groups was significant ( $P < 0.05$ ) at week 4 of CsA administration. The baseline BUN value of rats was found to be  $17.58 \pm 2.26$  mg/dL, and the BUN levels following four weeks of CsA administration were found to be  $33.85 \pm 9.50$  and  $45.73 \pm 7.27$  mg/dL in rats on seal oil diet and sunflower oil diet, respectively, suggesting less renal functional impairment induced by CsA in rats maintained on seal oil diet than those maintained on sunflower oil diet.

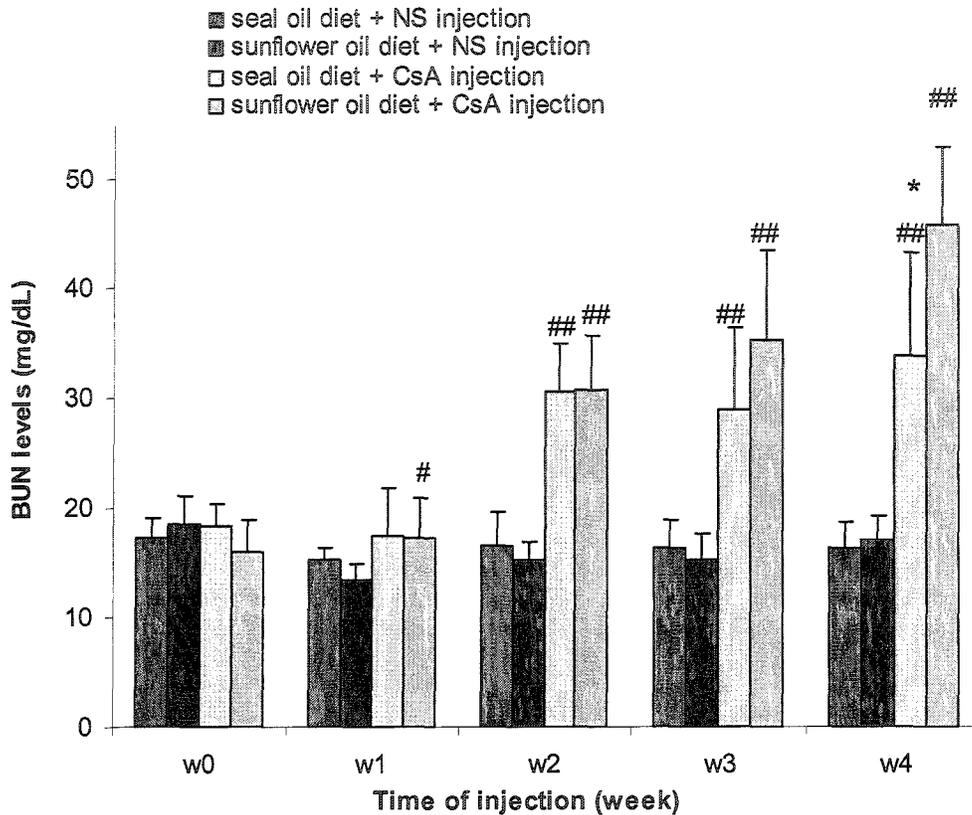


Figure 3.7. BUN levels in rats measured one week prior to and once a week during the *i.v.* administration of NS or CsA (Sandimmune IV<sup>®</sup>, 15 mg/kg/day) for four weeks. Rats were fed seal oil diet or sunflower oil diet for four weeks prior to and during the four-week *i.v.* administration of NS or CsA. Data shown are mean  $\pm$  SD (n=6-8).

\* $P < 0.05$ , \*\* $P < 0.01$ : seal oil diet + CsA injection *v.s.* sunflower oil diet + CsA injection

# $P < 0.05$ , ## $P < 0.01$ : NS injection *v.s.* CsA injection

### 3.2.2.2. The level of Clcr following *i.v.* administration of CsA or NS

The level of Clcr which reflects the rate of eliminating metabolic waste by kidney from blood to urine in rats was measured one week prior to and once a week during the *i.v.* administration of CsA (15 mg/kg/day) for four weeks. The results are presented in Figure 3.8.

It can be seen that the changes of Clcr following the administration of CsA were consistent with those of BUN. Clcr levels were found to have decreased in rats administered with CsA (both rats fed seal oil diet and rats fed sunflower oil diet), compared with the control groups where NS was given. Following two weeks of *i.v.* administration of CsA (15 mg/kg/day), the Clcr of rats fed seal oil diet and sunflower oil diet were found to be  $0.95 \pm 0.24$  and  $0.93 \pm 0.25$  mL/min, respectively, significantly decreased in comparison with  $1.50 \pm 0.37$  and  $1.49 \pm 0.28$  mL/min in the control groups. The reduced Clcr continued into week 3 and 4. However, at week 3 and 4, the reduction of Clcr levels in rats fed seal oil diet seemed to be less significant than those in rats fed sunflower oil diet, and the difference of Clcr levels between the two groups became significant ( $P < 0.05$ ) at week 4 of CsA administration.

The decreased Clcr levels in rats given CsA clearly indicated the kidney functional impairment induced by CsA administration. Rats maintained on seal oil diet for eight weeks showed less renal functional impairment in comparison with those maintained on sunflower oil diet at week 3 and 4 of *i.v.* administration of CsA (15 mg/kg/day).

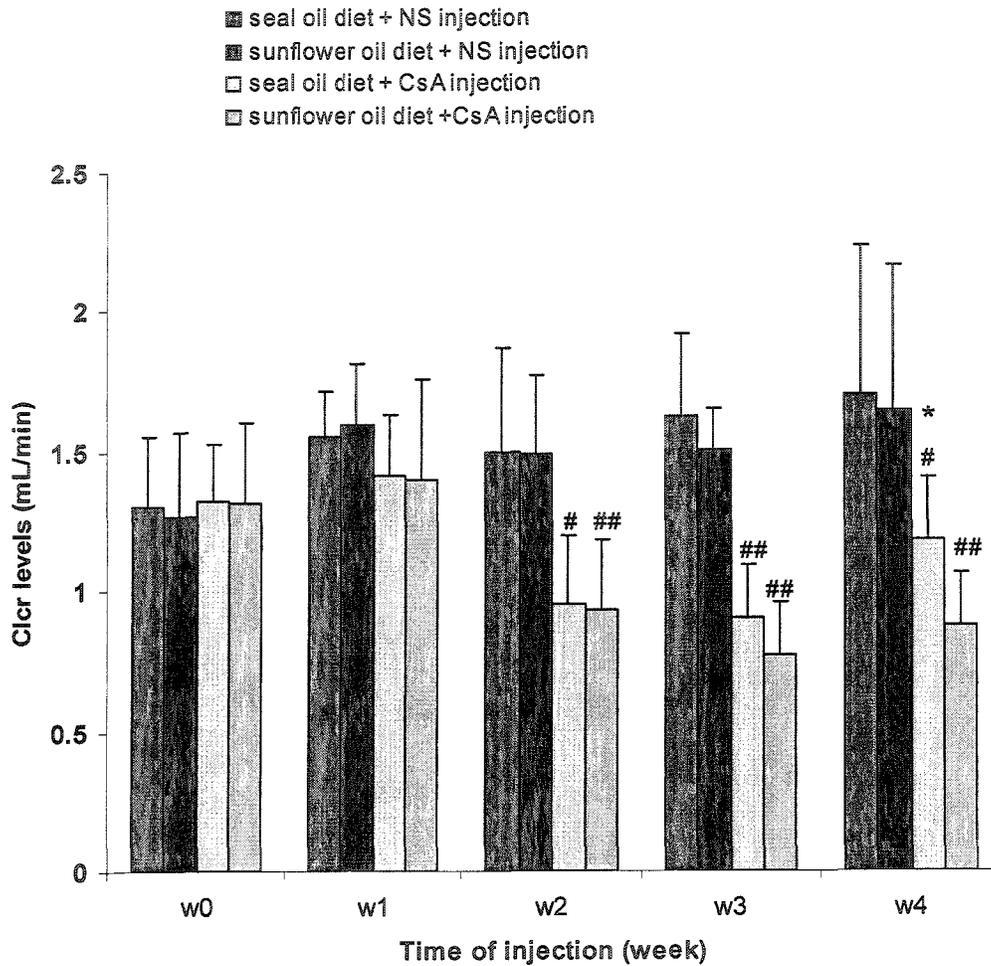


Figure 3.8. Clcr levels in rats measured one week prior to and once a week during the *i.v.* administration of NS or CsA (Sandimmune IV<sup>®</sup>, 15 mg/kg/day) for four weeks. Rats were fed seal oil diet or sunflower oil diet for four weeks prior to and during the four-week *i.v.* administration of NS or CsA. Data shown are mean  $\pm$  SD (n=6-8).

\* $P < 0.05$ , \*\* $P < 0.01$ : seal oil diet + CsA injection *v.s.* sunflower oil diet + CsA injection

# $P < 0.05$ , ## $P < 0.01$ : NS injection *v.s.* CsA injection

### 3.2.2.3. The volume of urine excreted in 20 h following *i.v.* administration of CsA or NS

Since the main function of the kidney is to excrete and condense urine, urine volume within a certain period of time is an indicator of kidney function. The volume of urine collected over 20 h using metabolic cages was measured one week prior to and once a week during the *i.v.* administration of CsA (15 mg/kg/day). The results are shown in Figure 3.9.

It can be seen that rats fed with either seal oil diet or sunflower oil diet demonstrated increased urine volume following the administration of CsA (15 mg/kg/day) during the course of the experiment. The average baseline urine volume prior to the administration of CsA was  $10.5 \pm 4.7$  mL/20h, which was increased to  $33.4 \pm 19.3$  and  $50.0 \pm 14.5$  mL/20h in rats maintained on seal oil and sunflower oil diets, respectively, at week 4. Rats, following administration of NS, did not demonstrate significant change in urine volume. Following administration of CsA, the mean urine volume of the rats maintained on seal oil diet was significantly lower than that of the rats kept on sunflower oil diet at all time intervals except for the first week, suggesting less interruption of the renal tubular reabsorption function in rats maintained on seal oil diet for eight weeks.

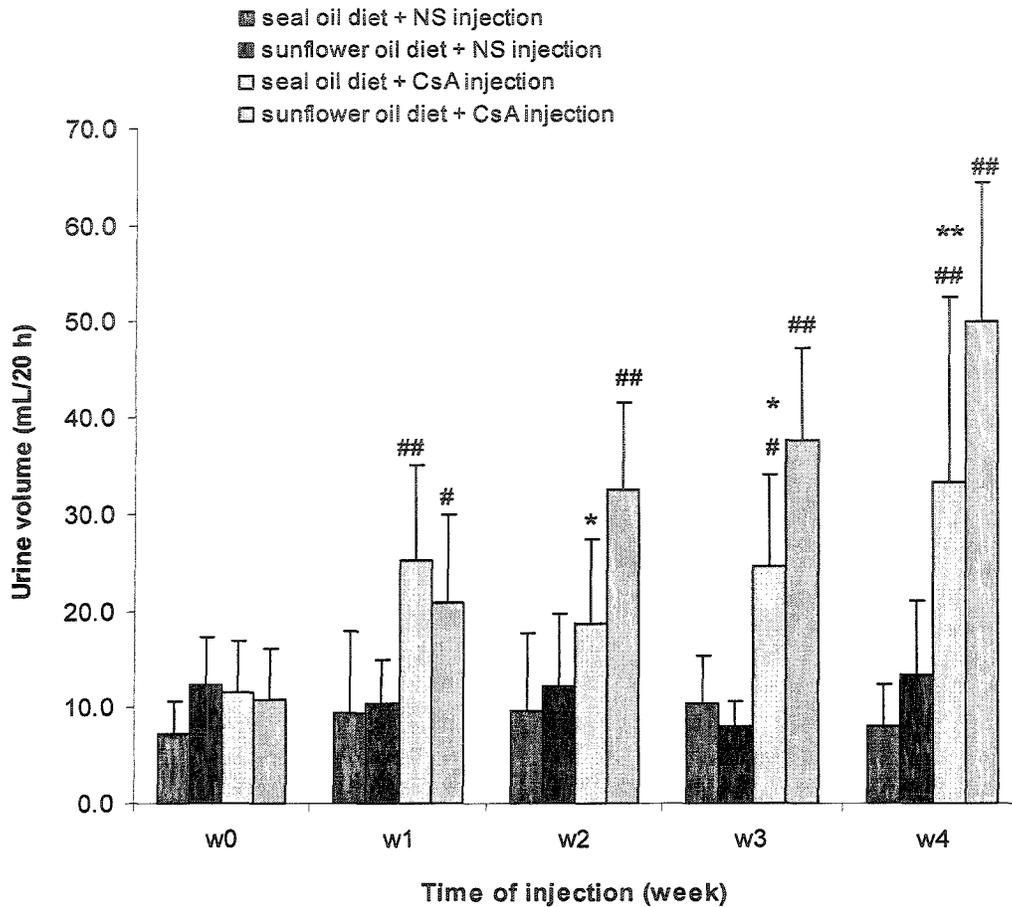


Figure 3.9. The volume of urine collected over 20 h from rats one week prior to and once a week during the *i.v.* administration of NS or CsA (Sandimmune IV<sup>®</sup>, 15 mg/kg/day) for four weeks. Rats were fed seal oil diet or sunflower oil diet for four weeks prior to and during the four-week *i.v.* administration of NS or CsA. Data shown are mean  $\pm$  SD (n=6-8).

\* $P < 0.05$ , \*\* $P < 0.01$ : seal oil diet + CsA injection *v.s.* sunflower oil diet + CsA injection

# $P < 0.05$ , ## $P < 0.01$ : NS injection *v.s.* CsA injection

#### 3.2.2.4. The amount of protein excreted in urine following *i.v.* administration of CsA or NS

The amount of protein excreted in urine within a certain period of time is an indicator reflecting the reabsorption function of kidney. The amount of protein in the urine was assessed by analyzing the protein content in the urine samples collected over 20 h from rats during *i.v.* administration of CsA (15 mg/kg/day) for four weeks. The results are compared with those obtained from rats administered NS. The protein amounts found in the urine samples of rats kept on seal oil diet or sunflower oil diet are shown in Figure 3.10.

It was found that following *i.v.* administration of CsA the protein excretion in the urine samples collected over 20 h rose significantly ( $P < 0.05$ ) in rats both kept on seal oil diet and on sunflower oil diet when compared with rats administered with NS. The protein excretion levels in the urine of rats fed on seal oil diet and sunflower oil diet following the administration of NS were comparable,  $41.0 \pm 9.1$  and  $49.0 \pm 20.5$  mg/20h, respectively. The protein excreted in the urine of rats fed on seal oil diet and sunflower oil diet following *i.v.* administration of CsA were  $70.8 \pm 26.5$  and  $85.4 \pm 45.1$  mg/20h, respectively.

The results indicated that the reabsorption of filtered protein by kidney was decreased following *i.v.* administration of CsA (15 mg/kg/day) for four weeks. However, rats maintained on the seal oil diet had lower protein content in urine, *i.e.* better reabsorption than those maintained on sunflower oil diet.

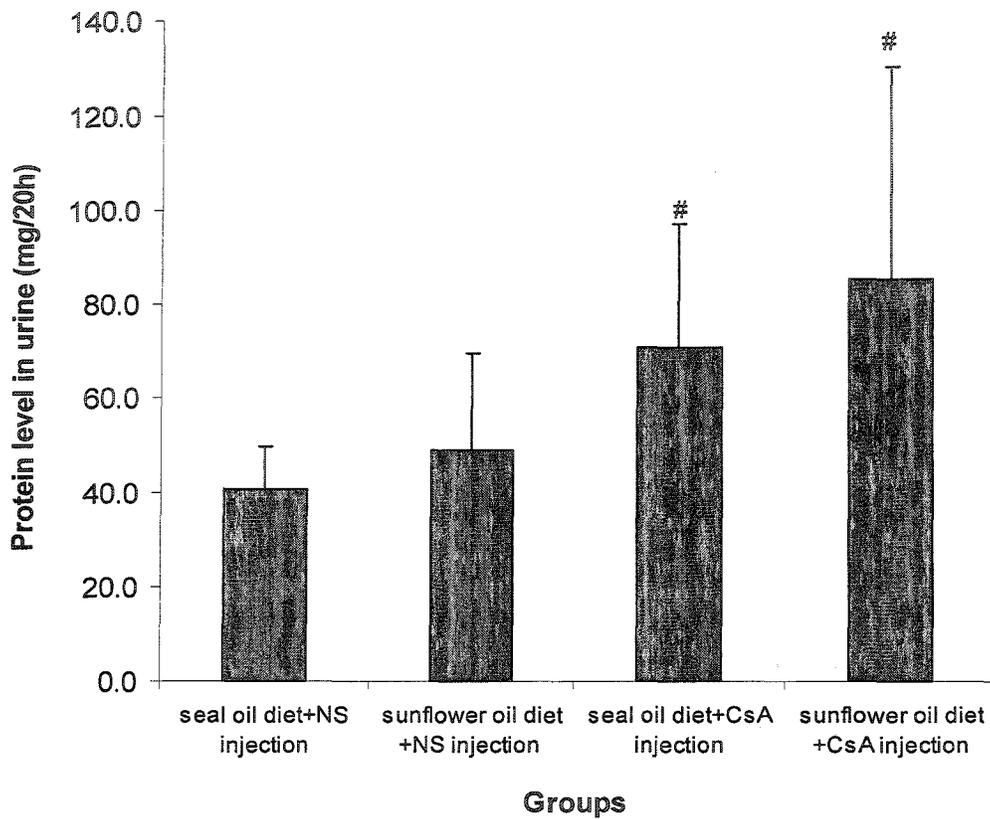


Figure 3.10. The protein excretion levels in urine samples collected over 20 h from rats following *i.v.* administration of NS or CsA for four weeks. Rats were fed seal oil diet or sunflower oil diet for four weeks prior to and during the four-week *i.v.* administration of NS or CsA (Sandimmune IV<sup>®</sup>, 15 mg/kg/day) for four weeks. Data shown are mean  $\pm$  SD (n=6-8).

\* $P < 0.05$ , \*\* $P < 0.01$ : seal oil diet + CsA injection *v.s.* sunflower oil diet + CsA injection

# $P < 0.05$ , ## $P < 0.01$ : NS injection *v.s.* CsA injection

### 3.2.2.5. The urinary NAG levels following *i.v.* administration of CsA or NS

As mentioned previously, urinary NAG is a sensitive indicator of renal tubular cell injury. NAG levels in the urine samples of rats were measured one week prior to and once a week during the *i.v.* administration of CsA (15 mg/kg/day) for four weeks. The results are shown in Figure 3.11.

The changes of urinary NAG levels following the *i.v.* administration of CsA were similar to those of BUN. Urinary NAG levels were found to have increased in rats administered with CsA (both rats fed seal oil diet and rats fed sunflower oil diet), compared with their respective control groups where NS was given. The elevation of urinary NAG levels in rats appeared significant ( $P < 0.01$ ) following two weeks of administration of CsA and continued throughout the entire experiment. Especially in rats maintained on sunflower oil diet, the value increased almost three times from the baseline of  $9842 \pm 1318$  to  $26755 \pm 2817$   $\mu\text{mol/h/L}$  at week 4. However, the urinary NAG levels in rats maintained on seal oil diet were lower than those of rats on sunflower oil diet at all time intervals during CsA administration, and the difference became significant by the third week of CsA administration, suggesting less renal tubular toxicity induced by *i.v.* administration of CsA in rats maintained on seal oil diet than those on sunflower oil diet.

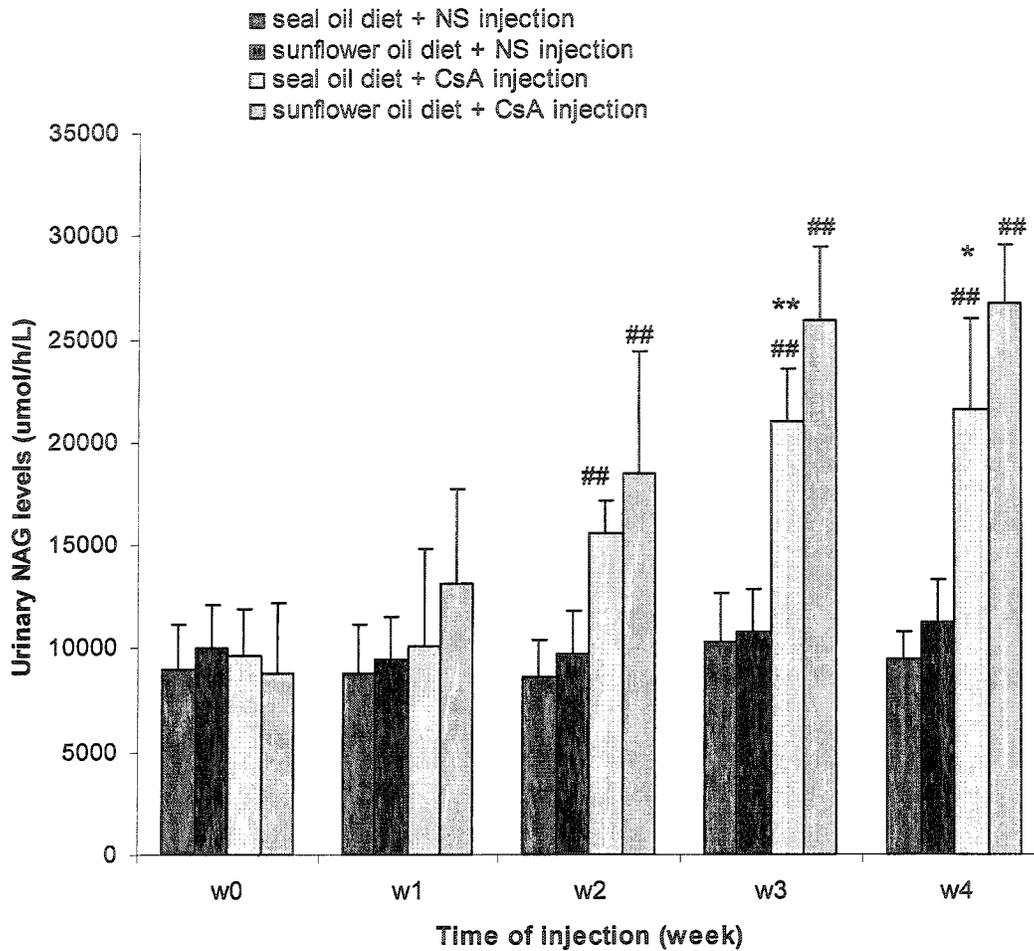


Figure 3.11. Urinary NAG levels in rats measured one week prior to and once a week during the *i.v.* administration of NS or CsA (Sandimmune IV<sup>®</sup>, 15 mg/kg/day) for four weeks. Rats were fed seal oil diet or sunflower oil diet for four weeks prior to and during the four-week *i.v.* administration of NS or CsA. Data shown are mean  $\pm$  SD (n=6-8).

\* $P < 0.05$ , \*\* $P < 0.01$ : seal oil diet + CsA injection *v.s.* sunflower oil diet + CsA injection

# $P < 0.05$ , ## $P < 0.01$ : NS injection *v.s.* CsA injection

### 3.2.2.6. Kidney histological changes following *i.v.* administration of CsA or NS

At the end of the four-week *i.v.* administration of NS or CsA (15 mg/kg/day), the rats were sacrificed, the left kidneys of rats were taken and kidney slides were prepared for histological analysis.

Light microscopic examination of kidney slides found no morphological changes between rats maintained on seal oil diet and sunflower oil diet following the *i.v.* administration of NS. However, focal alterations within the proximal tubule in rats administered CsA were observed. Most notably, the presence of numerous intracellular vacuoles was similar to those described in CsA nephrotoxic models [Blair *et al.*, 1982]. The abnormality found in rats maintained on seal oil diet following *i.v.* administration of CsA (15 mg/kg/day) for four weeks appeared less severe than that of rats kept on sunflower oil diet. In addition, brush border loss and single cell necrosis were noted in the latter.

Due to the unexpected health condition of the histologist, the morphological changes of kidney could not be graded and photos could not be taken as we designed.

### 3.2.2.7. The level of SBP following *i.v.* administration of CsA or NS

In order to evaluate if dietary supplementation with seal oil has any effect on hypertension associated with the administration of CsA, SBP of rats maintained on both seal oil and sunflower oil diets were measured one week prior to and once a week during the *i.v.* administration of NS or CsA (15 mg/kg/day). Results are shown in Table 3.3.

Increased SBP was observed in rats given CsA as early as one week following the start of administration of CsA, and continued throughout the entire course of the experiment. The SBP values in rats kept on sunflower oil diet and given CsA were  $136.6 \pm 9.1$ ,  $146.3 \pm 4.6$ ,  $142.9 \pm 3.1$ ,  $147.9 \pm 7.8$ ,  $145.3 \pm 8.2$  mmHg at week 0, 1, 2, 3, and 4, significantly ( $P < 0.01$ ) higher than those of the control, which were  $133.5 \pm 2.7$ ,  $133.2 \pm 4.7$ ,  $131.5 \pm 6.2$ ,  $135 \pm 6.1$ ,  $127.5 \pm 6.0$  mmHg, respectively, at corresponding time points.

Compared with the control group where NS was administered, the increase of mean SBP levels in rats maintained on seal oil diet following the administration of CsA (15 mg/kg/day) was not statistically significant, except for the value at the first week of CsA administration ( $P < 0.05$ ). The mean SBP values in rats kept on seal oil diet increased less than those in rats kept on sunflower oil diet at all time intervals following the *i.v.* administration of CsA, suggesting dietary supplementation of seal oil is beneficial in alleviating the elevated SBP associated with CsA administration .

Table 3.3. SBP in rats measured one week prior to and once a week during the *i.v.* administration of NS or CsA (Sandimmune IV<sup>®</sup>, 15 mg/kg/day) for four weeks.

Groups Time	seal oil diet + NS injection	sunflower oil diet + NS injection	seal oil diet + CsA injection	sunflower oil diet + CsA injection
Week 0	134.7±9.6	133.5±2.7	135.0±8.5	136.6±9.1
Week 1	130.2±3.7	133.2±4.7	138.8±6.6 #	146.3±4.6 ##
Week 2	133.7±5.9	131.5±6.2	139.3±4.5	142.9±3.1 ##
Week 3	134.7±5.2	135.0±6.1	142.1±11.9	147.9±7.8 ##
Week 4	133±8.8	127.5±6.0	140.7±7.8	145.3±8.2 ##

Note: The rats were fed seal oil diet or sunflower oil diet for four weeks prior to and during the four-week *i.v.* administration of NS or CsA. Data shown are mean ± SD (n=6-8).

\* $P < 0.05$ , \*\* $P < 0.01$ : seal oil diet + CsA injection *v.s.* sunflower oil diet + CsA injection

# $P < 0.05$ , ## $P < 0.01$ : NS injection *v.s.* CsA injection

### 3.2.2.8. The body weight (BW) of rats during the entire experiment

BW is a commonly used parameter of growth. Rats were fed seal oil diet or sunflower oil diet for four weeks, followed by four weeks of *i.v.* administration of NS or CsA (Sandimmune IV<sup>®</sup>, 15 mg/kg/day), respectively, while the rats were still maintained on their respective diets. The BW of rats was recorded everyday during the entire experiment.

The mean weekly BW of each group was calculated and the results are shown in Figure 3.12. There was no difference with respect to BW among the four groups of rats, before the administration of CsA or NS. The mean BW of rats prior to the administration of NS or CsA was  $294 \pm 17$  g. After the first week of administration of NS or CsA, it was found that the rats given CsA showed less BW increase, compared to the rats given NS. Moreover, this difference of BW between rats given NS and CsA became significant ( $P < 0.01$ ) after the second week. At the end of the fourth week, the BW of rats kept on seal oil diet or sunflower oil diet and given NS were  $451 \pm 30$  and  $455 \pm 29$  g; while the BW of rats kept on seal oil diet or sunflower oil diet and given CsA were found to be  $370 \pm 14$ , and  $383 \pm 18$  g, respectively.

The results indicated that *i.v.* administration of CsA (15 mg/kg/day) suppressed the growth of the rats. There was no difference of growth between rats supplemented with seal oil and sunflower oil with administration of CsA or NS.

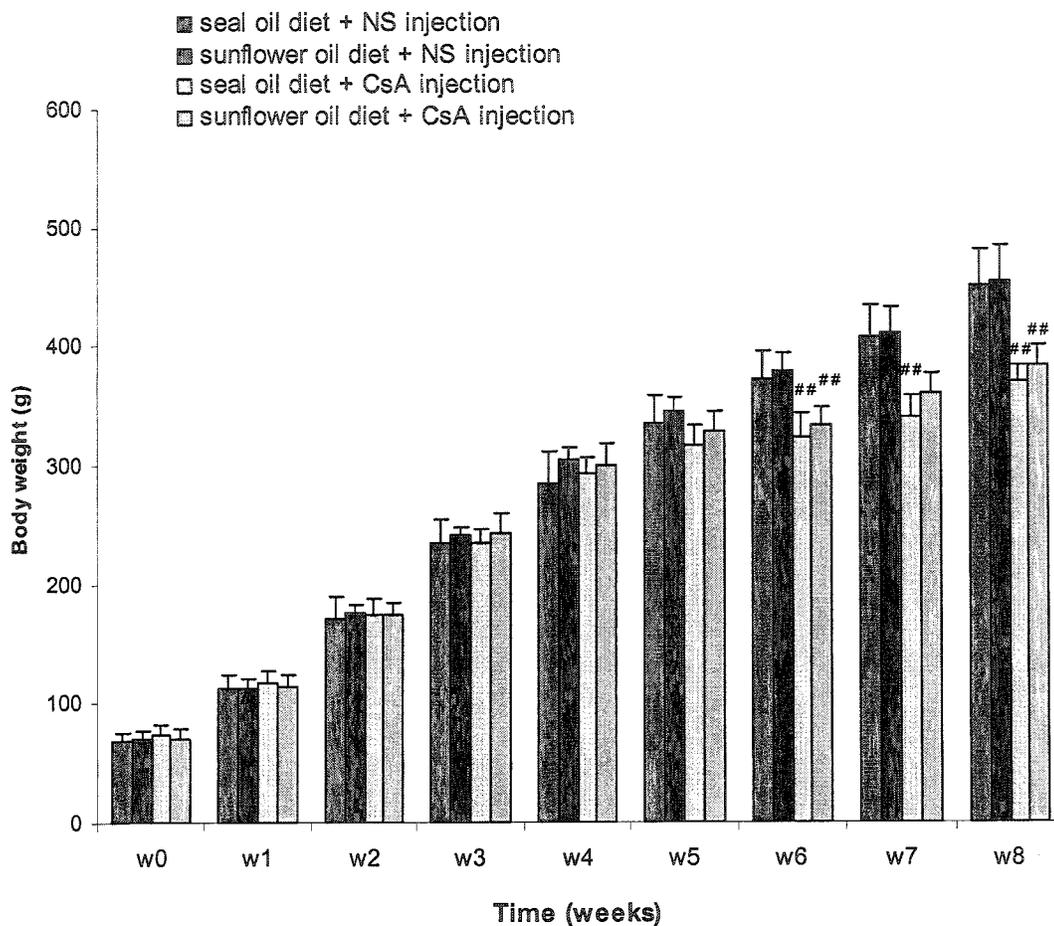


Figure 3.12. Mean BW of rats determined once a week throughout the entire experiment.

Rats were fed seal oil diet or sunflower oil diet for four weeks prior to and during the four-week *i.v.* administration of NS or CsA (Sandimmune IV<sup>®</sup>, 15 mg/kg/day). Data shown are mean  $\pm$  SD (n=6-8).

\* $P < 0.05$ , \*\* $P < 0.01$ : seal oil diet + CsA injection *v.s.* sunflower oil diet + CsA injection

# $P < 0.05$ , ## $P < 0.01$ : NS injection *v.s.* CsA injection

### 3.2.2.9. The CsA concentrations in blood

Rats were fed seal oil diet or sunflower oil diet for four weeks, followed by four weeks of *i.v.* administration of NS or CsA (Sandimmune IV<sup>®</sup>, 15 mg/kg/day), respectively, while still maintained on their respective diets. Blood was taken by cardiac puncture upon sacrifice at the end of the experiment.

CsA concentrations in the whole blood were measured by LC-MS, and the results are presented in Figure 3.13. The CsA concentrations in blood taken 24 h after the last dosing of the four-week *i.v.* administration of CsA were  $4417 \pm 1003$  and  $4231 \pm 664$   $\mu\text{g/L}$  in rats maintained on seal oil diet and sunflower oil diet, respectively. There was no statistical difference between them, suggesting dietary supplementation of different oils (seal oil and sunflower oil) had no effect on the pharmacokinetics of CsA following *i.v.* administration.

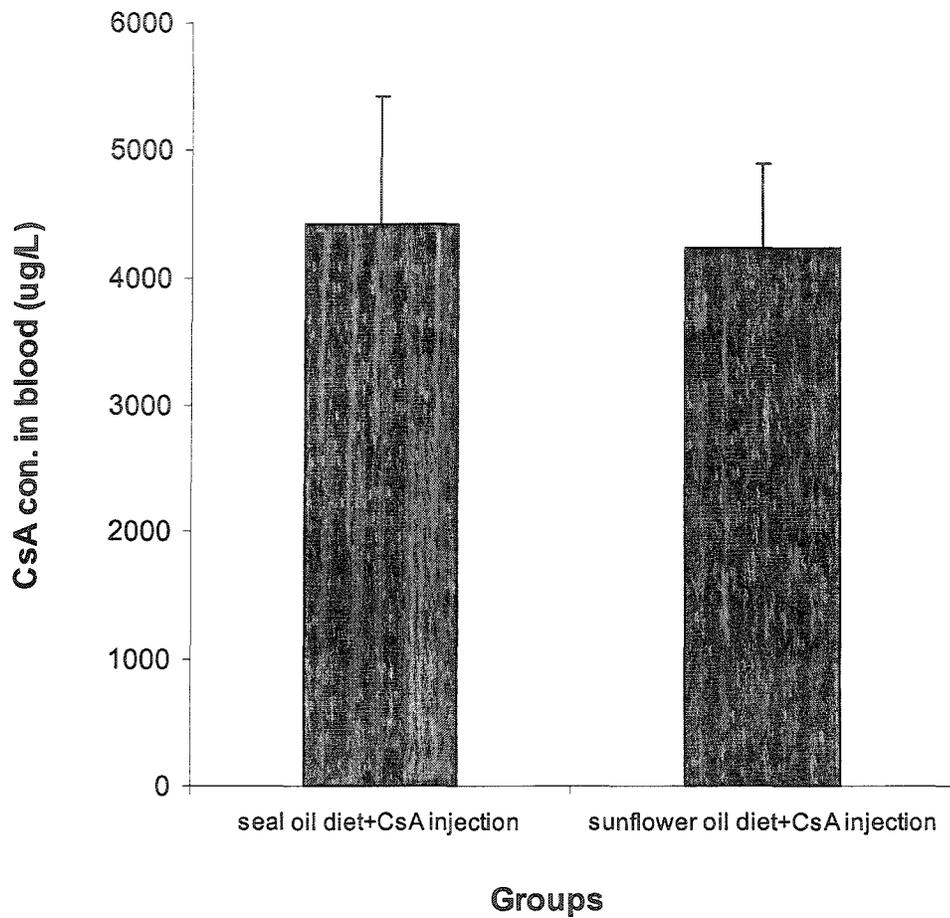


Figure 3.13. CsA concentrations in blood taken 24 h after the last dosing of a four-week *i.v.* administration of CsA in rats. Rats were fed seal oil diet or sunflower oil diet for four weeks prior to and during the four-week *i.v.* administration of NS or CsA (Sandimmune IV<sup>®</sup>, 15 mg/kg/day). Data shown are mean  $\pm$  SD (n=6-8).

\* $P < 0.05$ , \*\* $P < 0.01$ : seal oil diet + CsA injection *v.s.* sunflower oil diet + CsA injection

### 3.2.2.10. MDA content in kidney

MDA, a product of lipid peroxidation, is a parameter often used to monitor the involvement of ROS in certain pathological changes. Rats were fed seal oil diet or sunflower oil diet for four weeks, followed by four weeks of *i.v.* administration of NS or CsA (Sandimmune IV<sup>®</sup>, 15 mg/kg/day), respectively, while still maintained on their respective diets. At the end of experiment, rats were sacrificed and the right kidney was excised. Kidney homogenate was prepared. The level of MDA in the kidney homogenate was determined by TBARS assay and the results are shown in Figure 3.14.

The MDA contents in rats kept on seal oil diet or sunflower oil diet following the administration of CsA were significantly ( $P < 0.05$ ) elevated ( $197.1 \pm 37.3$  and  $208.7 \pm 49.2$  nmol/mg protein, respectively), compared to those in rats following the administration of NS ( $149.1 \pm 41.0$  and  $149.6 \pm 36.1$  nmol/mg protein, respectively). However, there was no statistical significance between the rats fed seal oil diet and those fed sunflower oil diet.

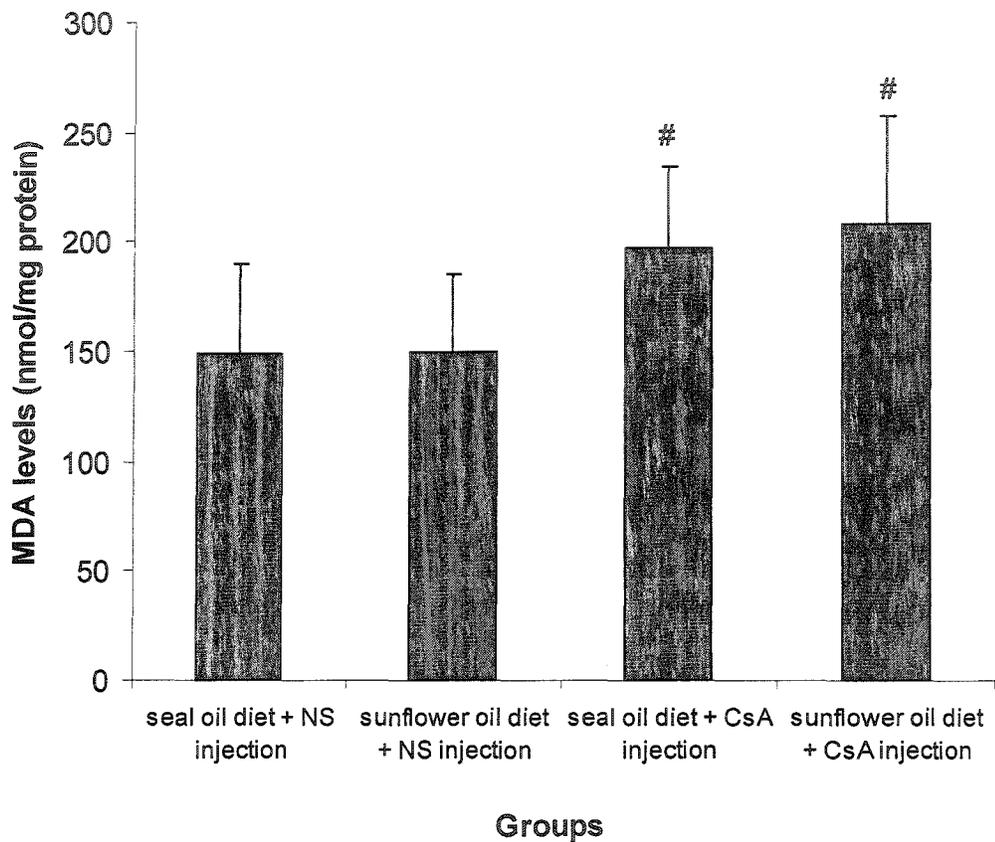


Figure 3.14. MDA content in kidney tissue in rats kept on seal oil diet or sunflower oil diet and administered NS or CsA (Sandimmune IV<sup>®</sup>, 15 mg/kg/day) *i.v.* for four weeks. Rats were fed seal oil diet or sunflower oil diet for four weeks prior to and during the four-week *i.v.* administration of NS or CsA. Data shown are mean  $\pm$  SD (n=6-8).

\* $P < 0.05$ , \*\* $P < 0.01$ : seal oil diet + CsA injection *v.s.* sunflower oil diet + CsA injection

<sup>#</sup> $P < 0.05$ , <sup>##</sup> $P < 0.01$ : NS injection *v.s.* CsA injection

### 3.2.2.11. The level of urinary 6-keto-PGF<sub>1α</sub> / TXB<sub>2</sub>

6-keto-PGF<sub>1α</sub> and TXB<sub>2</sub> are the stable metabolites of PGI<sub>2</sub> and TXA<sub>2</sub>, respectively. The 6-keto-PGF<sub>1α</sub> and TXB<sub>2</sub> levels in urine samples of rats maintained on seal oil diet or sunflower oil diet for four weeks, followed by *i.v.* administration of NS or CsA (15 mg/kg/day), respectively, for four weeks, were measured by EIA. The ratios of urinary 6-keto-PGF<sub>1α</sub> to TXB<sub>2</sub> are presented in Figure 3.15.

The ratios of urinary 6-keto-PGF<sub>1α</sub>/TXB<sub>2</sub> in rats kept on seal oil diet or sunflower oil diet following the administration of NS were  $1.23 \pm 0.33$  and  $1.01 \pm 0.23$ , respectively. The ratios in rats kept on seal oil diet or sunflower oil diet following the administration of CsA (15 mg/kg/day) for four weeks were  $0.53 \pm 0.09$  and  $0.43 \pm 0.08$ , respectively, significantly ( $P < 0.01$ ) lower than those administered with NS. The ratios were found higher in rats fed seal oil diet than those of rats fed sunflower oil diet, following the administration of either NS or CsA. Between the decreased ratios of urinary 6-keto-PGF<sub>1α</sub>/TXB<sub>2</sub> in the two groups given CsA, the rats maintained on seal oil diet had significantly ( $P < 0.05$ ) higher value than rats on sunflower oil diet.

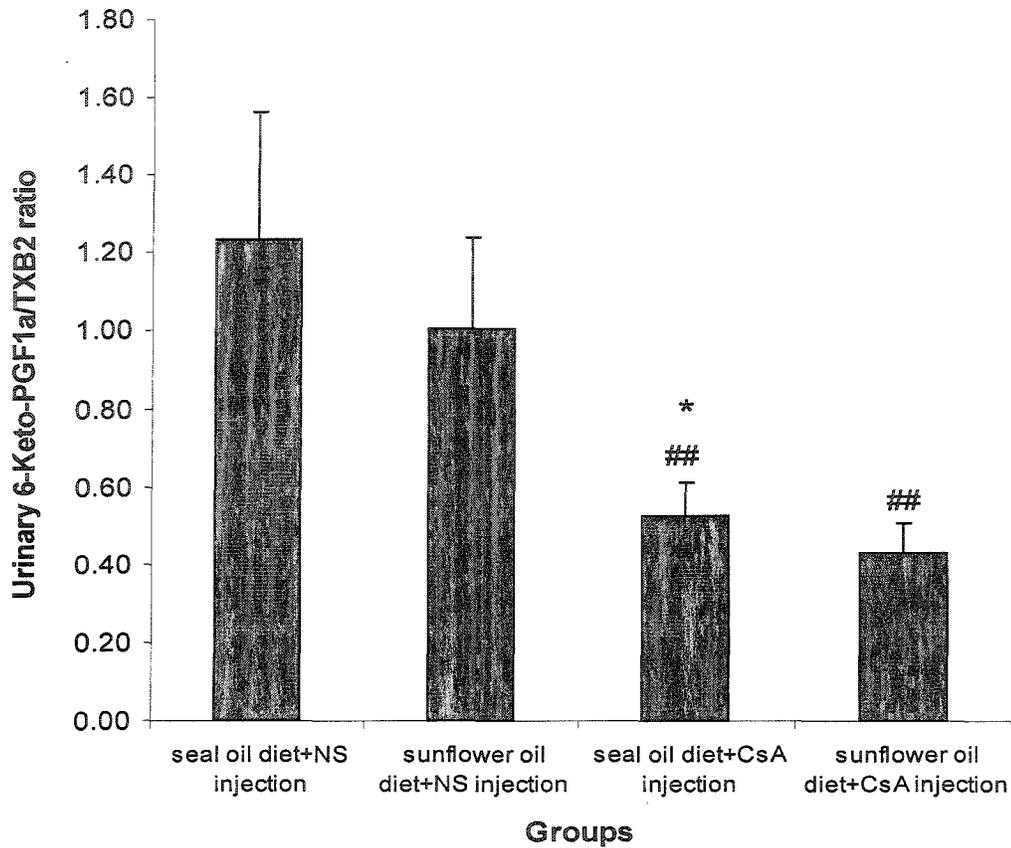


Figure 3.15. Urinary 6-keto-PGF<sub>1α</sub>/TXB<sub>2</sub> values in rats kept on seal oil diet or sunflower oil diet and administered NS or CsA (Sandimmune IV<sup>®</sup>, 15 mg/kg/day) *i.v.* for four weeks. Rats were fed seal oil diet or sunflower oil diet for four weeks prior to and during the four-week *i.v.* administration of NS or CsA. Data shown are mean ± SD (n=6-8).

\* $P < 0.05$ , \*\* $P < 0.01$ : seal oil diet + CsA injection *v.s.* sunflower oil diet + CsA injection

# $P < 0.05$ , ## $P < 0.01$ : NS injection *v.s.* CsA injection

### 3.2.2.12. The effect of fatty acid compositions of diet on the fatty acid compositions of erythrocyte membrane of rats

The fatty acid compositions from four randomly collected samples of seal oil diet and sunflower oil diet were determined using GC. The fatty acid compositions of lipids extracted from diets containing 100% sunflower oil (referred as sunflower oil diet), and 85% seal oil plus 15% sunflower oil (referred as seal oil diet) are presented in Table 3.4. It was found that the diets did not differ in the percentage composition of myristic (14:0), palmitic (16:0) and stearic (18:0) acids. However, contents of all other fatty acids were significantly different ( $p < 0.0001$ ). The sunflower oil diet consisted of a higher content of linoleic (18:2n6) and oleic (18:1n9) acids, and the total content of  $\omega$ -6 fatty acids was 31.31%; whereas the total content of  $\omega$ -6 fatty acids in seal oil diet was only 7.67%. However, the seal oil diet had a markedly higher level (17.34%) of  $\omega$ -3 fatty acids including 20:5n3, 22:5n3, 22:6n3; whereas there was no detectable level of any of these  $\omega$ -3 fatty acids found in the sunflower oil diet.

The phospholipid fatty acid compositions in the erythrocyte membrane of rats fed the two different diets for eight weeks were also analyzed to assess the effect of diets on the tissue fatty acid composition. The contents of the fatty acids found in the membrane of RBCs are summarized in Table 3.5.

It was found that rats maintained on sunflower oil diet had a significantly higher level of 20:4n6 and 22:4n6 in the erythrocyte membrane than those kept on seal oil diet. Rats kept on the seal oil diet had a significantly ( $p < 0.01$ ) higher level of palmitoleic (16:1n7), vaccenic (18:1n7), 11-eicosenoic acid (20:1n9), and the total amount of  $\omega$ -3

fatty acids was approximately six times higher than that of rats kept on sunflower oil diet. The difference of fatty acid compositions in the erythrocyte membrane of rats maintained on the two different diets appears to be consistent with the difference of fatty acid compositions in the two diets. There was no significant difference of fatty acid compositions in the erythrocyte membrane between rats maintained on seal oil diet and sunflower oil diet following *i.v.* administration of either NS or CsA for four weeks.

The results indicated that the fatty acid composition in the diet plays an important role in the fatty acid composition found in the erythrocyte membrane and CsA administration had no significant effect on the phospholipid fatty acid compositions of the erythrocyte membrane of rats.

Table 3.4. Levels of various fatty acids found in the two diets: sunflower oil diet and seal oil diet

Fatty acid	Diet	
	Seal oil* (n=4)	Sunflower oil** (n=4)
Myristic (14:0)	6.31±2.03	5.26±0.27
Palmitic (16:0)	8.25±1.21	4.40±0.97
Palmitoleic (16:1n7)	16.23±0.85 <sup>##</sup>	---
Stearic (18:0)	1.42±0.12	3.01±0.30
Oleic (18:1n9)	26.09±1.56 <sup>##</sup>	49.36±5.24
Vaccenic (18:1n7)	3.77±0.22 <sup>##</sup>	0.69±0.02
Linoleic (18:2n6)	7.29±0.64 <sup>##</sup>	31.31±4.30
α-Linolenic (18:3n3)	0.77±0.05 <sup>##</sup>	---
11-Eicosenoic (20:1n9)	5.81±0.20 <sup>##</sup>	---
Arachidonic (20:4n6)	0.38±0.16 <sup>##</sup>	---
Eicosapentaenoic (20:5n3)	6.65±0.49 <sup>##</sup>	---
Docosapentaenoic (22:5n3)	3.09±0.19 <sup>##</sup>	---
Docosahexaenoic (22:6n3)	6.83±0.74 <sup>##</sup>	---
Σ saturates	15.98	12.67
Σ mono-unsaturates	46.09	50.05
Σ n6	7.67	31.31
Σ n3	17.34	---

Values are mean ± SD, # P<0.0005; <sup>##</sup> P<0.0001

\* Seal oil diet consists of 85% seal oil plus 15% sunflower oil.

\*\* Sunflower oil diet consists of 100% sunflower oil.

Table 3.5. Levels of fatty acids found in the erythrocyte membrane of rats fed the two different diets, the seal oil diet and sunflower oil diet <sup>a</sup>

Fatty acid	Erythrocyte membrane			
	Seal oil+NS <sup>1</sup> (n=6)	Sunflower oil+NS <sup>2</sup> (n=6)	Seal oil+CsA <sup>3</sup> (n=7)	Sunflower oil+CsA <sup>4</sup> (n=8)
14:0	0.82±0.25	0.84±0.57	0.74±0.20	0.42±0.06**
16:0	31.11±8.55	23.95±3.33	32.56±2.16	31.13±5.26
16:1n7	1.38±0.40	0.72±0.40*	1.92±0.46	0.49±0.08**
18:0	11.90±4.40	11.71±3.96	11.01±0.71	13.19±5.60
18:1n9	7.22±1.77	6.89±1.17	11.20±1.07	10.68±1.58
18:1n7	3.37±0.73	2.82±0.36	4.37±0.21	2.47±0.41**
18:2n6	3.99±1.28	5.42±0.95	5.20±0.31	6.29±1.21
20:1n9	0.56±0.11	0.29±0.21	0.69±0.10	0.32±0.04*
20:4n6	6.60±3.45	18.62±1.77*	6.56±1.46	14.84±5.15*
20:5n3	6.90±4.65	0.71±0.59 **	5.07±1.20	0.32±0.06**
22:4n6	---	1.59±0.31**	0.17±0.01	1.58±0.53**
22:5n3	2.05±0.98	0.40±0.11**	2.24±0.58	0.38±0.20**
22:6n3	3.02±1.38	1.31±0.23*	3.48±1.10	0.97±0.18**
Σ saturates	43.83	36.50	44.31	46.63
Σ mono	12.53	10.72	18.18	11.49
Σ n6	10.59	25.63	11.93	22.71
Σ n3	12.28	2.42	10.79	1.67

Values are mean ± SD, \*P<0.05; \*\*P<0.01 seal oil diet v.s. sunflower oil diet

Note: <sup>a</sup> fatty acids of less than 0.1% of the total are not included.

<sup>1</sup> Rats fed on diet containing 85% seal oil plus 15% sunflower oil for four weeks, followed by four weeks of *i.v.* administration of NS while still kept on the same diet

<sup>2</sup> Rats fed on diet containing 100% sunflower oil for four weeks, followed by four weeks of *i.v.* administration of NS while still kept on the same diet

<sup>3</sup> Rats fed on diet containing 85% seal oil plus 15% sunflower oil for four weeks, followed by four weeks of *i.v.* administration of CsA 15 mg/kg/day while still kept on the same diet

<sup>4</sup> Rats fed on diet containing 100% sunflower oil for four weeks, followed by four weeks of *i.v.* administration of CsA 15 mg/kg/day *i.v.* while still kept on the same diet

### 3.3. *In vitro* studies

#### 3.3.1. Growth curve of LLC-PK1 cell line

The growth curves of LLC-PK1 cells seeded at different cell densities:  $1 \times 10^4$ ,  $3 \times 10^4$ , and  $1 \times 10^5$  cells/mL, in 24-well plates are shown in Figure 3.16 A. It was found that LLC-PK1 cells seeded at a density of  $1 \times 10^4$  cells/mL showed a typical growth curve with a lag phase, a log phase (exponential growth period) and a stationary phase. The cells entered the exponential growth period after the first day, which lasted for the next four to five days. As shown in Figure 3.16 B, there is a linear relationship between cell number and time of culture from the second to the sixth day following seeding at  $1 \times 10^4$  cells/mL. The curve can be fitted as:

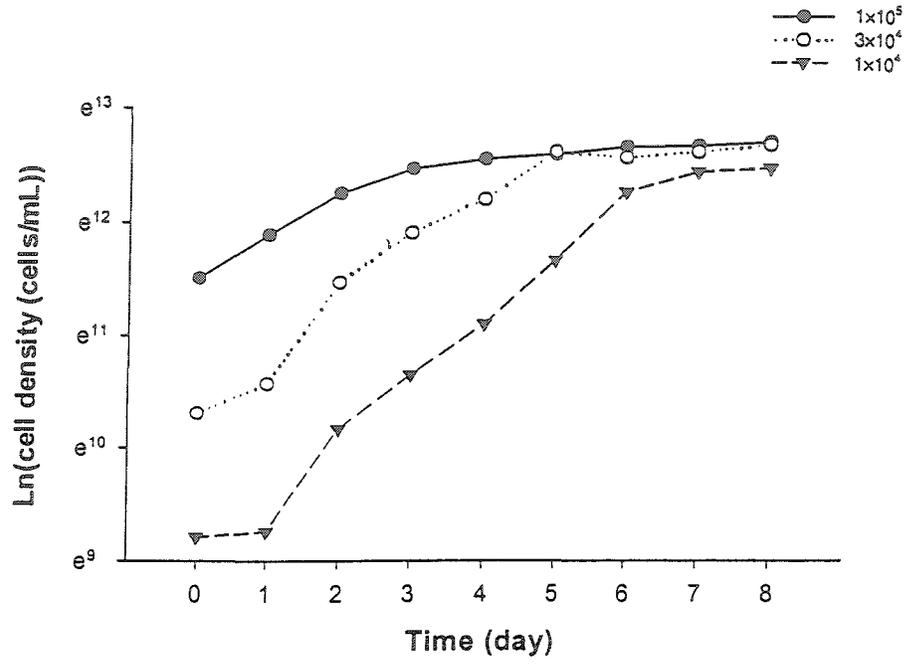
$$\ln(\text{cell number}) = 8.852 + 0.5704 * \text{Time}$$

$$R_{\text{sqr}} = 0.988$$

From the equation, the cell Population Doubling Time (PDT) was found to be 1.22 days (29 h).

Starting from the seventh day, the growth of cells was found to be leveled off. Based on the above results, experiments were conducted using cells from the second to the sixth day following subculturing.

**A**



**B**

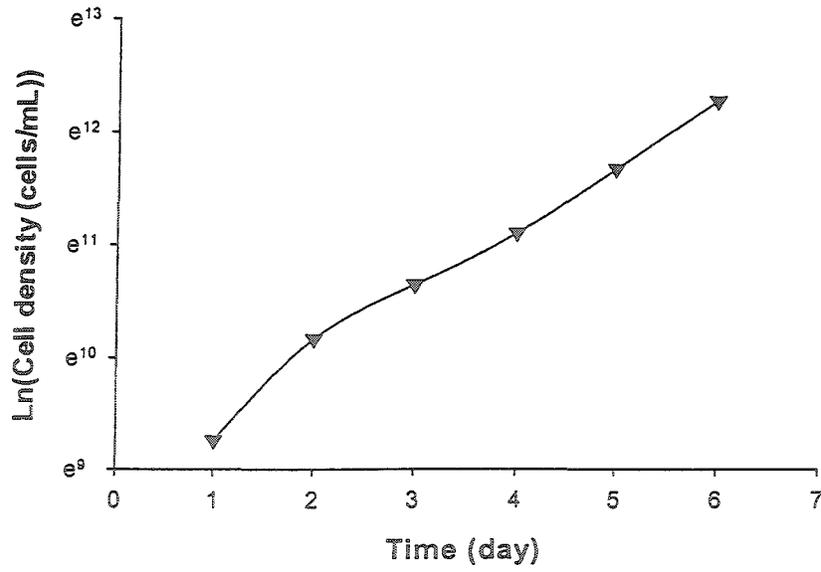


Figure 3.16. (A) Growth curves of LLC-PK1 cells seeded at different cell densities:  $1 \times 10^4$ ,  $3 \times 10^4$ , and  $1 \times 10^5$  cells/mL, in 24-well plates for eight days. (B) Linear range of cell density versus time following seeding at  $1 \times 10^4$  cells/mL. The cell numbers were determined using a hemocytometer.

Prior to using the MTT assay for cytotoxicity, it was necessary to determine a cell density range for which absorbance at 570 nm upon MTT assay versus cell number following a certain time of incubation is linear.

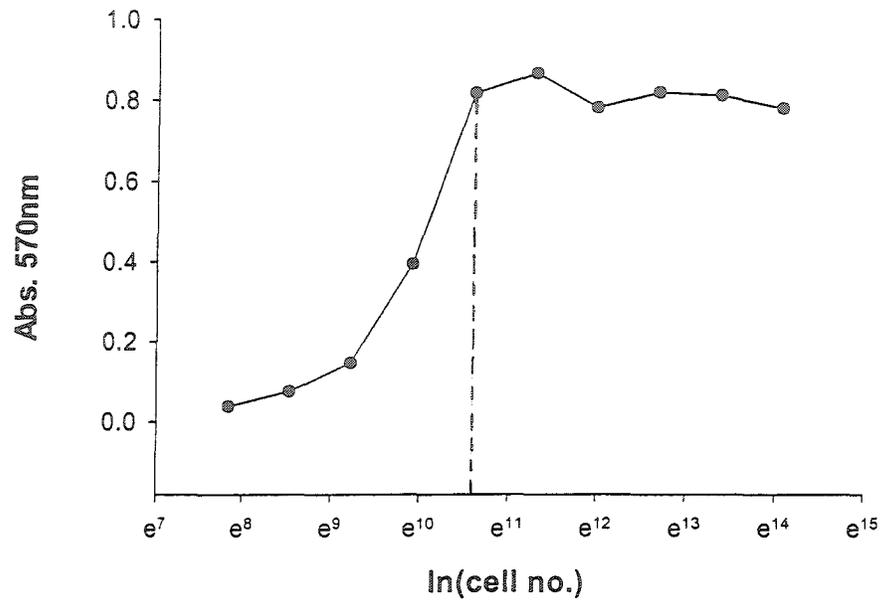
Cells of a series of two-fold dilutions ranging from  $1.3 \times 10^6$  to  $2.5 \times 10^3$  cells/mL were seeded into 96-well plates and incubated for 72 h. The number of living cells after 72 h incubation was reflected by the intensity of absorbance at 570 nm. The results of absorbance at 570 nm detected using MTT assay versus various seeding densities are presented in Figure 3.17.A. When the seeding density was higher than  $4 \times 10^4$  cells/mL ( $\ln(\text{cell number}) = e^{10.6}$ ), the absorbance at 570 nm leveled off, *i.e.*, the conversion of MTT dye by living cells is not linearly proportional to the number of cells. As shown in Figure 3.17. B, only when seeding density was in the range of  $2.5 \times 10^3$  to  $4 \times 10^4$  cells/mL, there was a linear relationship between absorbance at 570 nm and cell numbers after 72 h incubation.

The linear curve in (B) can be fitted as:

$$\text{Abs}_{.570\text{nm}} = 3.421 \times 10^{-2} + 2.084 \times 10^{-5} * \text{cell-density},$$

$$\text{Rsqr} = 0.997$$

**A**



**B**

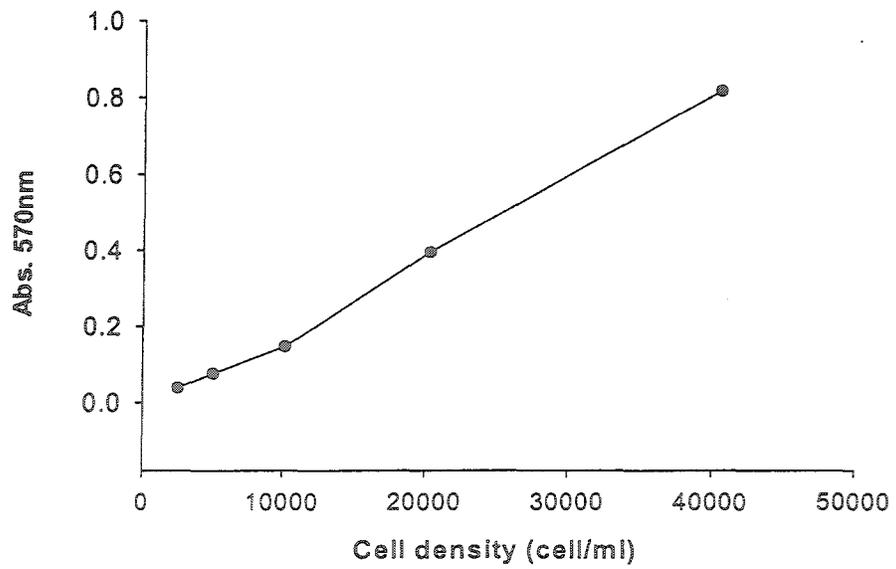


Figure 3.17. The relationship between seeding density of LLC-PK1 cells in 96-well plates and absorbance at 570 nm by MTT assay after 72 h incubation. (A) The absorbance at 570 nm versus seeding density of LLC-PK1 cells ranging from  $1.3 \times 10^6$  to  $2.5 \times 10^3$  cells/mL. (B) The linear range of absorbance at 570 nm by MTT assay versus seeding density.

### **3.3.2. Determination of cytotoxicity of the seal oil emulsion in LLC-PK1 cell line**

To assess the effect of seal oil on CsA induced cytotoxicity in LLC-PK1 cell line, it was important to choose a concentration of seal oil emulsion that is not toxic to the LLC-PK1 cells.

The cytotoxicity of seal oil emulsion at various concentrations (0.000032% - 0.004%) in the LLC-PK1 cell line was determined using the MTT assay. The results are shown in Figure 3.18.

Compared to the control, it is clear that seal oil emulsion of less than 0.0008% had negligible effect on the cells. Although the cell viability is lower in the presence of 0.000032% seal oil emulsion than that in the presence of 0.00016% seal oil emulsion, there was no statistical significance. Therefore, 0.0001% seal oil emulsion was used for further study.

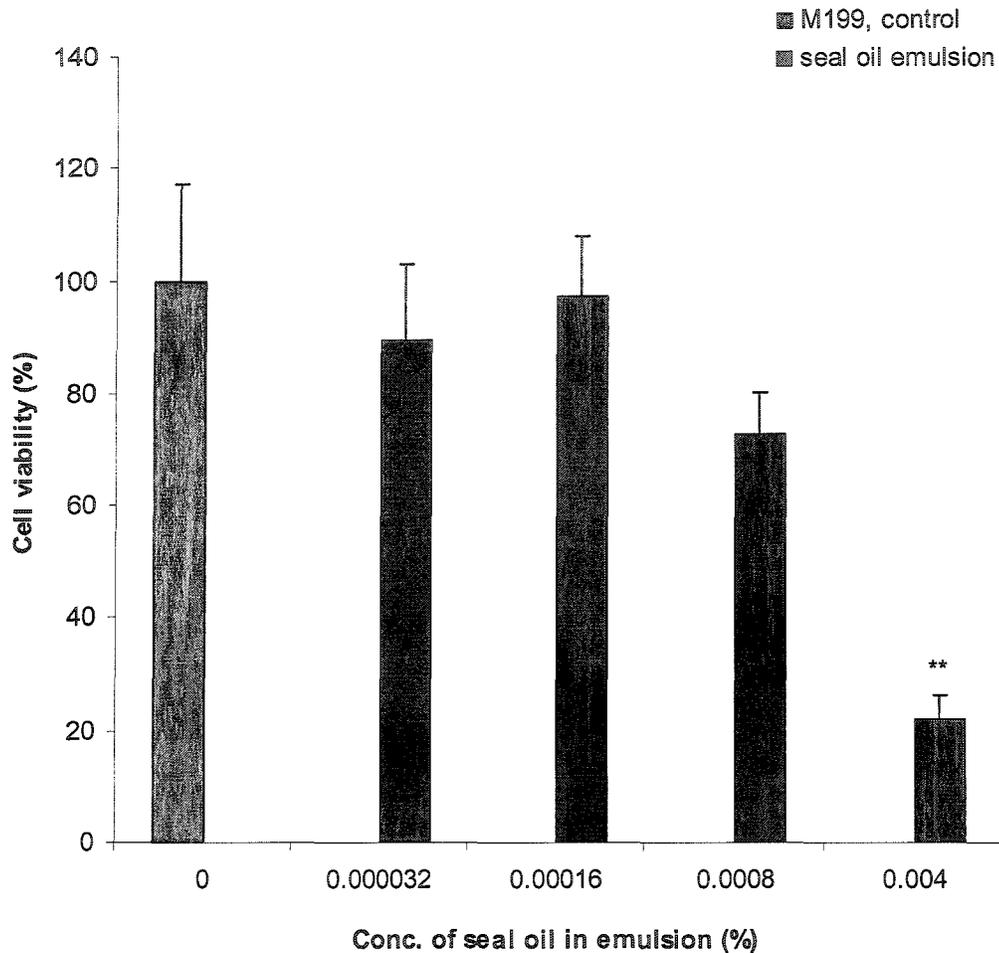


Figure 3.18. Viability of LLC-PK1 cells treated with various concentrations of seal oil emulsion (0.000032% - 0.004%) for 24 h. The data are presented as the percentage of absorbance at 570 nm of the cells treated with various concentrations of seal oil emulsion in comparison with that of control.

\*  $P < 0.05$ ; \*\*  $P < 0.01$  v.s. M199, control

### 3.3.3. The effect of seal oil emulsion on CsA-induced cytotoxicity measured by MTT assay

The cytotoxicity of CsA in the presence and absence of 0.0001% seal oil emulsion was determined using MTT assay in LLC-PK1 cell line. The cells were incubated in the presence and absence of 0.0001% seal oil emulsion for 24 h, followed by addition of various concentrations (0-10  $\mu$ M) of CsA and incubation for another 24 h. The result of one representative MTT assay out of five repeats is shown in Figure 3.19. The curves of cell viability versus concentrations of CsA can be fitted into equations using SigmaPlot software as follows:

- In the absence of seal oil, cell viability =  $244.0 - 92.51 * \ln(\text{CsA Conc.} + 4.54)$
- In the presence of 0.0001% seal oil, cell viability =  $109.4 - 46.39 * \ln(\text{CsA Conc.} + 1.21)$

Based on these equations,  $IC_{50}$  values of CsA can be calculated

- In the absence of seal oil,  $IC_{50} = 3.6 \mu\text{M}$
- In the presence of 0.0001% seal oil,  $IC_{50} = 2.4 \mu\text{M}$

The results indicated that when LLC-PK1 cells were exposed to various concentrations of CsA for 24 h in the presence of 0.0001% seal oil emulsion, the  $IC_{50}$  value of CsA was decreased.

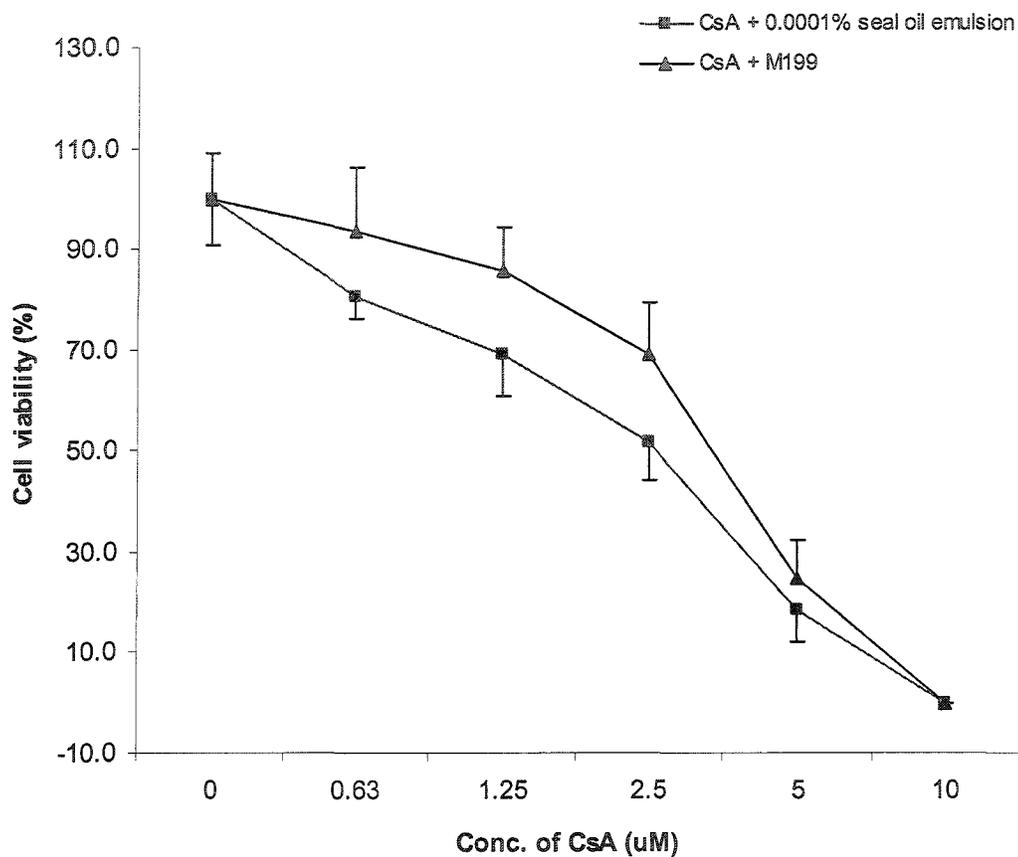


Figure 3.19. Viability of LLC-PK1 cells treated with different concentrations of CsA in the presence or absence of 0.0001% seal oil emulsion for 24 h using MTT assay. The data are presented as the percentage of absorbance at 570 nm of the cells treated with CsA in comparison with that of control.

#### **3.3.4. The effect of seal oil emulsion on CsA-induced LDH release**

The effect of seal oil emulsion on the toxicity of CsA was also assessed by measuring the level of LDH in the cell culture supernatant of LLC-PK1 exposed to various concentrations of CsA for 24 h.

One representative result of LDH levels out of five repeats is shown in Figure 3.20. It is clear that with the increase of CsA concentration, the level of LDH in the cell culture supernatant was also elevated. In the presence of 0.0001% of seal oil emulsion, the LDH value was higher than that in the absence of seal oil emulsion.

The results of both cell viability study and LDH levels indicated that in the presence of seal oil emulsion, the cytotoxicity of CsA was enhanced.

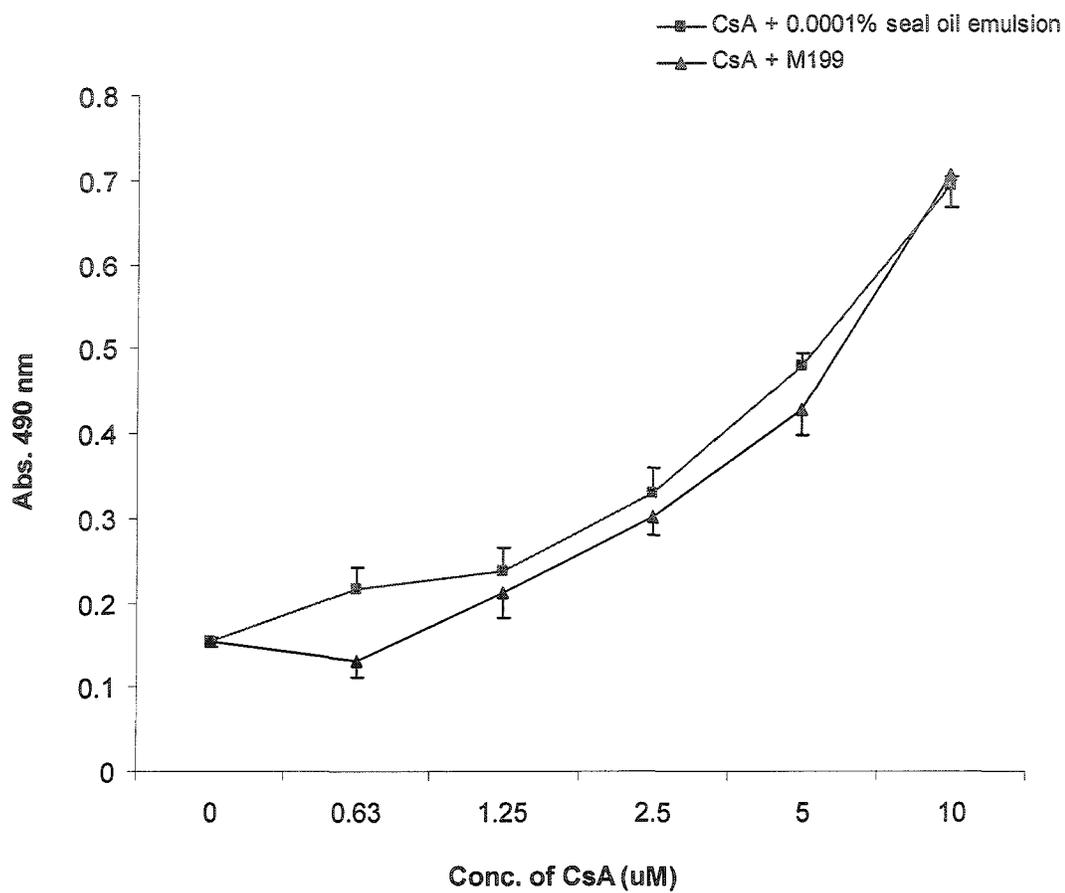


Figure 3.20. LDH levels in the cell culture supernatant of LLC-PK1 cells exposed to different concentrations of CsA, in the presence or absence of 0.0001% seal oil emulsion for 24 h. The LDH levels were presented as values of absorbance at 490 nm.

### 3.3.5. The effect of DHA, $V_E$ , and EPA on CsA-induced cytotoxicity measured by MTT assay

*In vitro* models have their limitations. The *in vitro* studies using LLC-PK1 cell culture may have underlined the effect of seal oil as the cells lack the proper lipase to release the PUFAs from their TAGs. If the benefits of seal oil are ascribed to the PUFAs, it was considered necessary to study the effects of PUFAs in the *in vitro* model. Lipid peroxidation has been recognized as a potential mechanism of toxic cell injury.  $V_E$ , a well known antioxidant, was also used to test its effect on the cytotoxicity of CsA.

Various concentrations of DHA (0.08-10  $\mu\text{M}$ ),  $V_E$  (0.1-10  $\mu\text{M}$ ) and EPA (0.1-10  $\mu\text{M}$ ) were added 4 h prior to exposure to CsA, respectively, and the cells were then coincubated with 3.3  $\mu\text{M}$  CsA and each of the substances for 24 h. Cell viability was measured using MTT assay and presented as a percentage of that of the control. Results are shown in Figure 3.21. and Figure 3.22.

It was found that incubated with 3.3  $\mu\text{M}$  CsA for 24 h, the viability of LLC-PK1 cells was suppressed to 54% of that of the cells incubated without CsA. However, cell viability was significantly increased in the presence of various concentrations of DHA (0.4-10  $\mu\text{M}$ ) after coincubated with 3.3  $\mu\text{M}$  CsA for 24 h, in a concentration-dependent manner. The similar trend was observed with different concentrations of  $V_E$  (0.1-10  $\mu\text{M}$ ) in combination with 3.3  $\mu\text{M}$  CsA for 24 h. In the presence of  $V_E$ , not only the cell viability was increased more significantly than that in the presence of DHA in a concentration-dependent manner, but also the cell growth was stimulated at a concentration of 10  $\mu\text{M}$  of  $V_E$ .

However, there was no significant change of the cell viability observed when incubated in various concentrations of EPA (0.1-10  $\mu\text{M}$ ) for 4 h prior to and in combination with 3.3  $\mu\text{M}$  CsA for 24 h. (Since the EPA was in 2 months back-order, it did not being tested together with DHA and  $V_E$ ).

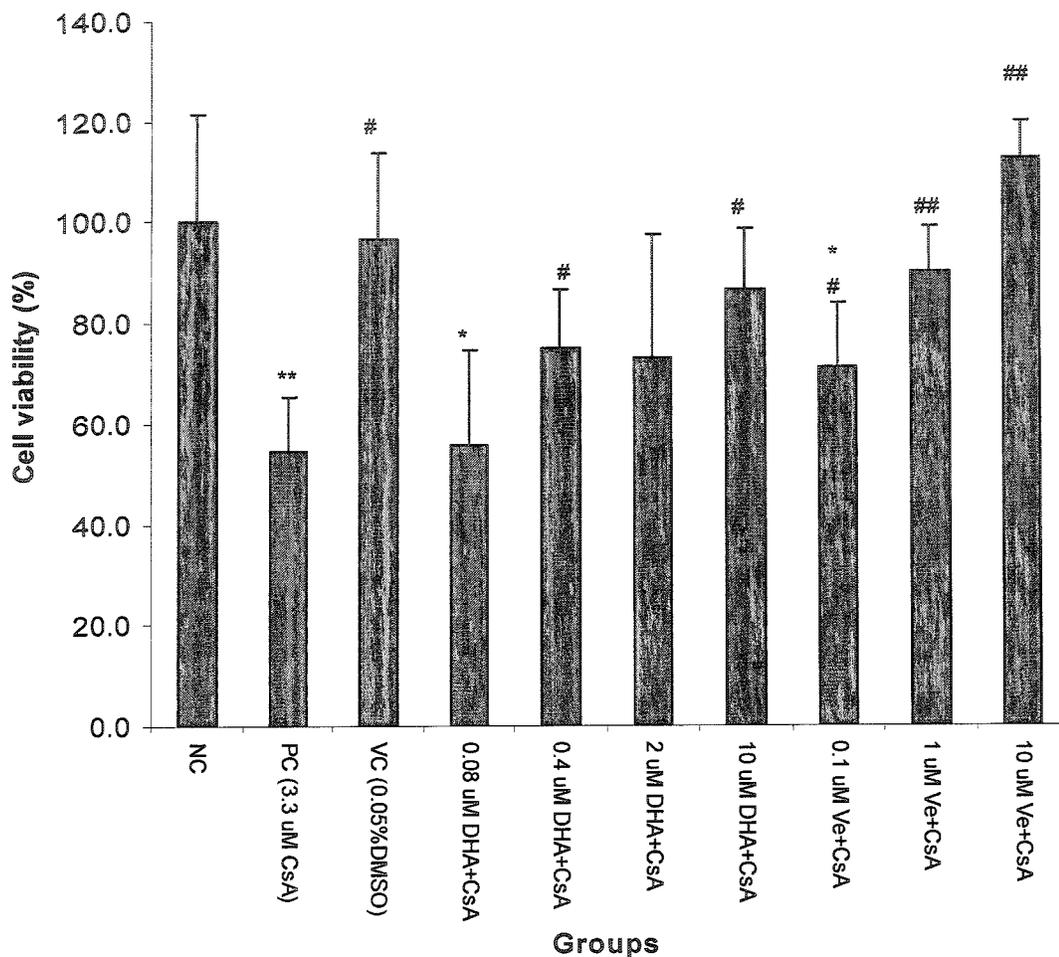


Figure 3.21. Viability of LLC-PK1 cells treated with 3.3  $\mu\text{M}$  CsA in combination with various concentrations of DHA (0.08-10  $\mu\text{M}$ ) or  $\text{V}_\text{E}$  (0.1-10  $\mu\text{M}$ ) using MTT assay. The data are presented as the percentage of absorbance at 570 nm of the cells treated with CsA combining serial concentrations of DHA or  $\text{V}_\text{E}$  in comparison with that of normal control. Data shown are mean  $\pm$  SD (n=5-8).

\*  $P < 0.05$ ; \*\*  $P < 0.01$  v.s. NC

#  $P < 0.05$ ; ##  $P < 0.01$  v.s. PC

NC: normal control; PC: positive control; VC: vehicle control; Ve: vitamin E

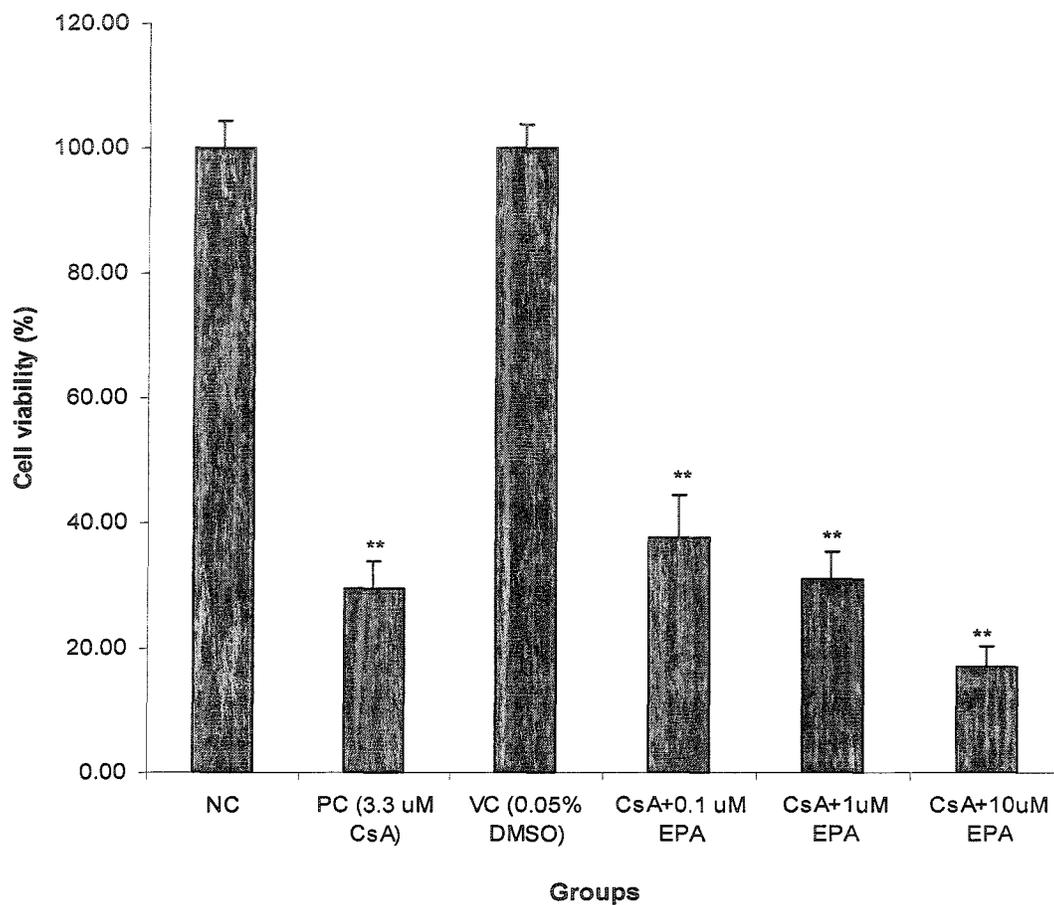


Figure 3.22. Viability of LLC-PK1 cells treated with 3.3  $\mu\text{M}$  CsA in combination with various concentrations of EPA using MTT assay. The data are presented as the percentage of absorbance at 570 nm of the cells treated with CsA and serial concentrations of EPA (0.1-10  $\mu\text{M}$ ) in comparison with that of normal control. Data shown are mean  $\pm$  SD (n=5-8).

\*  $P < 0.05$ ; \*\*  $P < 0.01$  v.s. NS

#  $P < 0.05$ ; ##  $P < 0.01$  v.s. PC

NC: normal control; PC: positive control; VC: vehicle control

### 3.3.6. The effect of DHA and $V_E$ on CsA-induced LDH release

LDH levels in the supernatant of LLC-PK1 cells that incubated with various concentrations of DHA (0.08-10  $\mu\text{M}$ ) or  $V_E$  (0.1-10  $\mu\text{M}$ ) for 4 h prior to and during the coincubation with 3.3  $\mu\text{M}$  CsA for 24 h were measured by a colorimetric method using a kit. The results are shown in Figure 3.23.

The effect of DHA and  $V_E$  on LDH release is consistent with the improvement of cell viability by DHA and  $V_E$ . Exposure to 3.3  $\mu\text{M}$  CsA for 24 h significantly elevated LDH release into culture media ( $P < 0.01$ ). 0.02% DMSO, the highest concentration of solvent that DHA,  $V_E$  may bring to the culture system, had no influence to LDH release compared to control. The elevated LDH levels were decreased significantly when incubated with various concentrations of DHA (0.4-10  $\mu\text{M}$ ) or  $V_E$  (0.1 - 10  $\mu\text{M}$ ) for 4 h prior to and during the coincubation with 3.3  $\mu\text{M}$  CsA for 24 h, respectively; and the decrease was in a concentration-dependent manner.

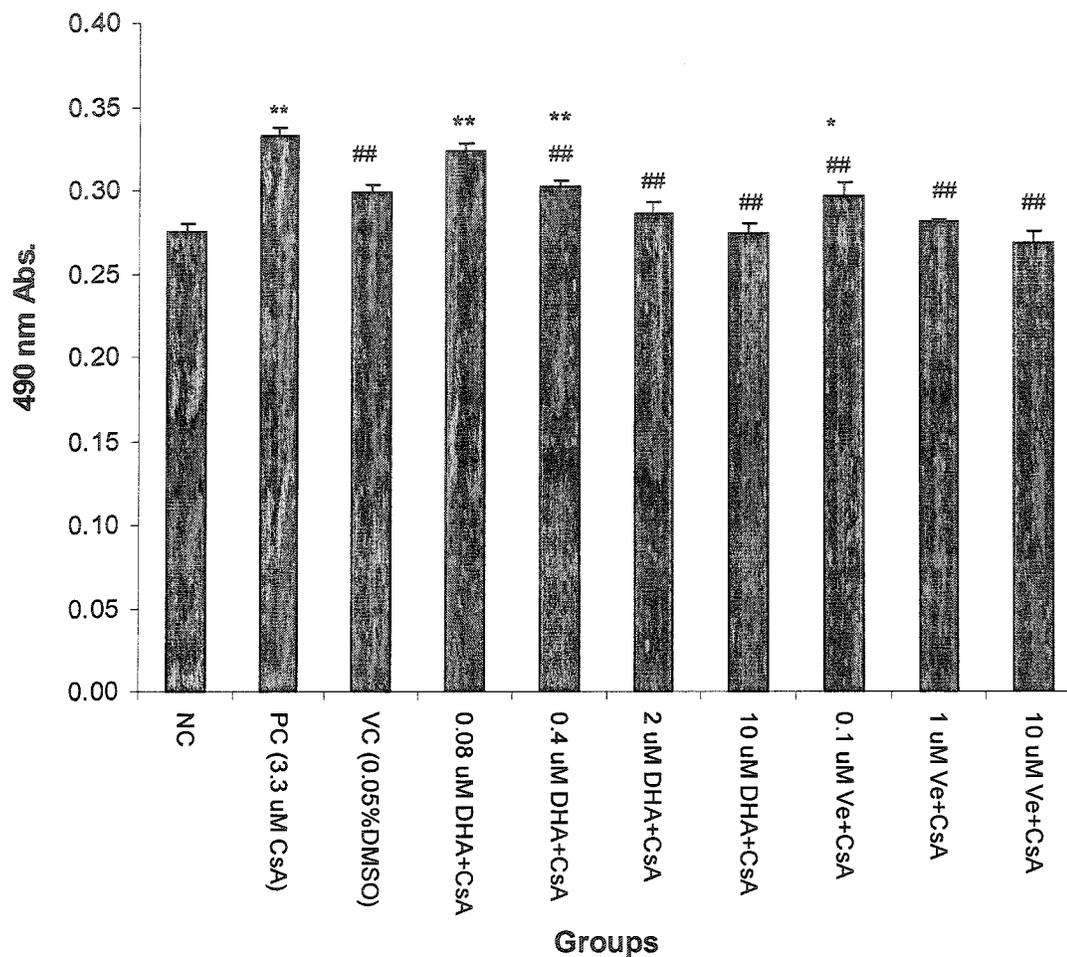


Figure 3.23. LDH levels in the supernatant of LLC-PK1 cells treated with 3.3  $\mu\text{M}$  CsA for 24 h, in combination with various concentrations of DHA (0.08-10  $\mu\text{M}$ ) or V<sub>E</sub> (0.1-10  $\mu\text{M}$ ). The LDH levels were presented as values of absorbance at 490 nm. Data shown are mean  $\pm$  SD (n=5-8).

\* $P < 0.05$ ; \*\*  $P < 0.01$  *v.s.* NC

#  $P < 0.05$ ; ##  $P < 0.01$  *v.s.* PC

NC: normal control; PC: positive control; VC: vehicle control; Ve: vitamin E

### 3.3.7. MDA content in LLC-PK1 cells treated with CsA in combination with DHA, EPA, or V<sub>E</sub>

LLC-PK1 cells were first incubated with various concentrations of EPA (0.01, 0.1, 1  $\mu$ M), DHA (0.01, 0.1, 1, 10  $\mu$ M), or V<sub>E</sub> (0.1, 1, 10  $\mu$ M), respectively, for 4 h, followed by addition of 3.3 or 6.7  $\mu$ M CsA (final concentration). The cells were returned to the incubator for another 24 h. lipid peroxidation was assessed by measuring MDA content in the cell lysate using TBARS assay. The results are presented in Table 3.6.

It can be seen that the level of MDA in cells was generally low. It was hard to draw any conclusion on the relationship between MDA level and CsA concentration.

Table 3.6. MDA concentrations in LLC-PK1 cells after being incubated with 3.3 or 6.7  $\mu\text{M}$

CsA for 24 h in the presence of various concentrations of EPA, DHA or  $V_E$

	Conc. ( $\mu\text{M}$ )	CsA Conc. ( $\mu\text{M}$ )	MDA Conc. (nmol /mg protein)
Control		0	0.053
		3.3	0.018
		6.7	0.049
EPA	0.01	3.3	0.138
		6.7	0.056
	0.1	3.3	0.105
		6.7	0.093
	1.0	3.3	0.079
		6.7	0.122
DHA	0.01	3.3	0.061
		6.7	0.108
	0.1	3.3	0.220
		6.7	0.063
	1.0	3.3	0.029
		6.7	0.201
	10	3.3	0.038
		6.7	0.055
$V_E$	0.1	3.3	0.206
		6.7	0.046
	1.0	3.3	0.022
		6.7	0.049
	10	3.3	0.095
		6.7	0.102

\* Data presented are average of 2 repetitions

## Chapter 4. Discussion

Since its first use in human in 1978 [Caline *et al.*, 1978], CsA has become the hallmark of immunosuppressive therapy in organ transplantation. Today, CsA remains to be the first line drug in clinical immunosuppressive regimens in organ and tissue transplantation. Moreover, CsA has increasingly contributed to effective treatment of several autoimmune diseases, such as psoriasis and rheumatoid arthritis, in recent years.

The major clinical limitations of CsA are its toxicities, especially nephrotoxicity, and unpredicted pharmacokinetics following oral administration, which is thought to be partially responsible for the toxicities. CsA, a highly lipophilic, poorly water-soluble cyclic peptide, initially had been supplied as an olive oil or other vegetable oil solution for clinical use. At present, the widely used commercial formulations of CsA, Sandimmune<sup>®</sup> and Neoral<sup>®</sup>, are microemulsions. Although Neoral<sup>®</sup> provides improved pharmacokinetics, toxicities still remain to be a problem. Many studies have been directed towards optimizing the immunosuppressive efficacy of CsA while minimizing its toxicities. In this project, we studied the potential of substituting the conventionally used vegetable oil with seal oil in the formulation of CsA. Positive results might lead to development of a less toxic formulation by substituting the vegetable oil used in the currently available products with seal oil as the lipophilic vehicle.

### 4.1. CsA in seal oil and corn oil emulsions

Emulsions are heterogeneous, fine-dispersion system composed of two immiscible liquids, *i.e.*, oil and water. Surfactants are included in the formulation of emulsions for

stability reasons. As dispersed systems, emulsions are thermodynamically unstable. However, when the particle size of the dispersed phase is less than 150 nm, the emulsions are optical clear and become thermodynamically stable. They are known as microemulsions. Microemulsion can serve as a drug delivery system [Lundberg, 1991].

CsA emulsions in seal oil or corn oil in the presence of different amounts of surfactants (Cremophor RH-40: Tween 80 = 2:1, w/w) [Mishra *et al.*, 2001] were prepared. It was found that the particle size varied with the amount of surfactants present. 1.5% of surfactants resulted in particle size of around 340 nm and further increase of the amount of surfactant did not further reduce particle size. Both CsA in seal oil and CsA in corn oil emulsions were prepared using 1.5% surfactants and they were found to be stable for at least four weeks, which was the duration of the first *in vivo* study.

#### **4.2. The effect of concomitant administration (*p.o.* or *i.p.*) of seal oil with CsA on the nephrotoxicity induced by CsA**

The predominant function of kidney is filtration of blood to remove the metabolic waste through urine, and reabsorption of filtered nutrients, salts and water in appropriate proportions to maintain fluid and electrolyte balance in the body. The kidneys filter up to 180 liters per day, with 98-99% filtrate reabsorbed and the remainder as waste material removed. When kidney is in the state of impairment caused by toxic chemicals or other interventions, its waste eliminating function will be affected, characterised as elevated BUN and serum creatinine levels, and decreased urinary creatinine excretion. BUN and Clcr are therefore used clinically in monitoring kidney function.

Increased BUN and decreased Clcr levels were observed in rats following administration of CsA in seal oil or corn oil emulsion at 50 mg/kg/day via *p.o.* or 25 mg/kg/day via *i.p.* for 28 days, respectively, indicating kidney functional toxicities. However, the functional impairments were more apparent in rats given CsA in corn oil emulsion via *p.o.* route as compared to their counterparts given CsA in seal oil emulsion.

NAG, a lysosomal enzyme originating from the brush border of renal proximal tubular cells, is released upon cell injury. Urinary NAG is, therefore, a sensitive indicator of kidney tubular injury [Flynn FV, et al. 1992]. Increased urinary NAG levels were observed in rats given both CsA in seal oil and CsA in corn oil emulsions via *p.o.* and *i.p.* routes, suggesting direct renal tubular damage occurred. However, the urinary NAG levels in rats given CsA in seal oil emulsion were lower than those given CsA in corn oil emulsion, suggesting less renal tubular toxicity when seal oil was simultaneously administered with CsA.

In addition, elevated SBP in rats given both CsA in seal oil and CsA in corn oil emulsions via *p.o.* and *i.p.* routes were observed. However, the levels of SBP increased less in rats given CsA in seal oil emulsion than those given CsA in corn oil emulsion following both administration routes, suggesting less hemodynamic toxicity associated with the administration of CsA in seal oil emulsion.

According to all the parameters monitored, it was found that rats given CsA via *p.o.* route at 50 mg/kg/day in corn oil emulsion showed the most severe kidney toxicity among the four CsA given groups; whereas those given CsA in seal oil emulsion via *i.p.* at 25 mg/kg/day showed the least toxicity.

Between the two groups given CsA in seal oil and CsA in corn oil emulsions via *i.p.* at 25 mg/kg/day, there was no apparent difference found. This may be due to the poor absorption of oil following intraperitoneal administration, as fat accumulation in the abdominal cavity was observed when animals were dissected at the end of the experiment. The benefit of seal oil was therefore not fully materialized.

Between the two groups given CsA emulsions via *p.o.* at 50 mg/kg/day, the rats given CsA in seal oil emulsion demonstrated less kidney toxicity and hemodynamic change in comparison with the rats given CsA in corn oil emulsion. The CsA concentrations in blood were found to be comparable suggesting that the difference in kidney toxicity was not related to the CsA concentrations in blood. The attenuated kidney functional impairments and lower SBP observed in rats given CsA in seal oil emulsion over those given CsA in corn oil emulsion was likely due to the difference between seal oil and corn oil used in the formulation, *i.e.*, seal oil has a beneficial effect in reducing kidney toxicity and elevated SBP induced by CsA.

However, no statistically significant reduction of CsA-induced nephrotoxicity by concomitantly administered seal oil can be claimed from this study, although the parameters measured suggested a beneficial effect of seal oil in reducing CsA-related nephrotoxicity when given orally together with CsA in comparison with using corn oil in CsA emulsion.

It was also found that the CsA concentrations in the blood following respective oral administration of CsA in seal oil or corn oil emulsions varied from 5108 to 2725  $\mu\text{g/L}$  within the groups. This was in agreement with the clinical observations [Friman,

Backman, 1996] that the oral bioavailability of CsA is associated with great inter- and intra-individual variations. CsA is known to be extensively metabolized by hepatic and intestinal P450 3A4 mixed function oxidation enzyme. Furthermore, the absorbed CsA molecules are actively pumped back to the intestinal lumen by P-gp along the intestinal wall. These may be responsible for the low and varying bioavailability of CsA following oral administration. Therefore, intravenous administration of CsA directly to the systemic circulation was chosen for the next experiment. The effect of seal oil on kidney toxicities was evaluated by feeding the animal with seal oil enriched diet.

#### **4.3. The effect of dietary supplementation of seal oil on the nephrotoxicity induced by CsA**

Based on the results of the first *in vivo* experiment, in the second experiment seal oil was given to the animals for four weeks prior to the *i.v.* administration of CsA and during the course of administration (four weeks). It has been reported that lipids are well absorbed [Sizer, Whitney, 2003]. Thus, seal oil was incorporated into the diet fed to the animals. Sunflower oil incorporated diet was used as control.

##### **4.3.1. Rats' diets**

The two diets used were modifications of the formula of AIN-93 Purified Diets for laboratory rodents suggested by the American Institute of Nutrition Ad Hoc Writing Committee [Reeves *et al.*, 1993]. AIN-93G diet with 7% fat and 20% protein is recommended for young to support growth, pregnancy and lactational phases, while AIN-

93M diet with lower protein and fat content is recommended for mature rats. Rats of 250 g and lighter are considered as young and those above 250 g are considered mature. Diets were supposed to be adjusted according to their body weight. However, since protein content in diet influences BUN level, the diet for growing rats (AIN-93G) were used throughout the whole study to minimize variation.

Sunflower oil is commonly consumed in human diets, primarily as cooking oil. It contains predominantly oleic acid (C18:1n9) (49%) and linoleic acid (C18:2n6) (31%) in the form of TAG. Because mammals and humans are unable to synthesize fatty acid with double bonds more distal from the terminal carbon of the fatty acid than the ninth carbon atom, linoleic acid, an essential fatty acid, must be ingested. Therefore, in order to meet the requirement for essential fatty acids, even the seal oil enriched diet (referred to seal oil diet) contained small amount of sunflower oil with 15% of the total lipid being sunflower oil and the rest being seal oil. The amount of  $\omega$ -6 PUFAs in the seal oil diet was found to be 7.67% and that of the  $\omega$ -3 PUFAs was 17.34%, including EPA, DPA and DHA; while the amount of  $\omega$ -6 PUFAs in the diet containing 100% of sunflower oil (referred to sunflower oil diet) was 31.31% and that of the  $\omega$ -3 PUFAs was not detectable.

Because oils containing multiple double bonds easily undergo oxidation, *tert*-butylhydroquinone, an antioxidant, was used in the diets.

Male SD rats of 3-week old were maintained on seal oil diet or sunflower oil diet for four weeks prior to and during the four-week *i.v.* administration of NS or CsA (15 mg/kg/day). The rats fed on sunflower oil diet and administered with NS served as the

normal control. Rats fed on seal oil diet and administered with NS were found to exhibit all parameters comparable to those of the normal control except for urinary excretion of eicosanoids, indicating dietary supplementation of seal oil itself had no influence on kidney function of rats.

#### 4.3.2. Kidney functional alterations

As stated previously, increased BUN and decreased Clcr levels indicated impaired kidney function. One week following the start of the four-week *i.v.* administration of CsA, markedly increased BUN and decreased Clcr levels were observed in rats given CsA, indicating CsA-induced nephrotoxicity. It was also noted that during the last two weeks of CsA administration, the renal waste eliminating function of rats maintained on seal oil diet was not as severely impaired as those of rats kept on sunflower oil diet as suggested by the levels of BUN and Clcr.

The proximal tubule of kidney reabsorbs approximately 50-60% of the glomerular filtrate. Essential nutrients including sugars and amino acids, macromolecules (albumin and other proteins), and bicarbonate are extensively reabsorbed along the initial length of the proximal tubule [Hook, Goldstein, 1993]. In our study, urine volume collected over a 20-h period was measured once a week and it was found that the *i.v.* administration of CsA (15 mg/kg/day) dramatically increased the urine volume, suggesting a serious interruption of water reabsorption. One of the rats kept on sunflower oil diet even excreted 70 mL of urine within 20 h after four weeks of CsA administration. In contrast, rats maintained on seal oil diet with CsA dosing had significantly lower urine volume

during the last three weeks of CsA administration. Moreover, the amount of protein excreted in urine collected over a 20-h period following four weeks of CsA administration was also lower in rats on seal oil diet than those on sunflower oil diet, suggesting more filtered proteins (mainly albumin) were reabsorbed by proximal tubule. Both urine volume and the amount of protein excreted in urine suggested that concomitant dietary supplementation of seal oil with CsA administration was beneficial in preserving the renal tubular reabsorption function.

Because of the large volume of fluid entering and being reabsorbed from the proximal tubule, the proximal tubule is a target for a wide variety of toxicants including CsA. As stated previously, NAG is an enzyme found in the lysosomes of renal proximal tubular cells. The molecular weight of NAG is large enough to preclude its passage through the normal glomerular basement membrane. Increased excretion of NAG has been demonstrated to be more specific for renal tubular pathology [Flynn *et al.*, 1992]. Markedly increased urinary NAG levels were observed in the rats on the sunflower oil diet following the start of CsA administration in a time-dependent manner, indicating serious proximal tubular injury induced by CsA. However, rats maintained on the seal oil diet showed a lower level of NAG following the administration of CsA, suggesting a beneficial effect of dietary seal oil against the toxic action of CsA to the proximal tubular cells.

All the parameters related to kidney function measured in the *in vivo* studies including BUN, Clcr, the volume of 20-h urine collection and the amount of protein excreted in the 20-h urine collection suggested that seal oil appeared to decrease the renal

toxicity induced by CsA. The less functional impairment in rats on seal oil diet was also accompanied with fewer morphological changes when compared with their counterparts on sunflower oil diet.

However, there have been conflicting results from the literature [Barcelli *et al.*, 1982; Hansen *et al.*, 1995; Kooijmans-Coutinho *et al.*, 1996] with respect to the degree of benefit of diets with a high content of  $\omega$ -3 PUFAs on renal function. The discrepancies between these reports and the present study are likely due to different experimental protocols, such as the quality and quantity of oils given to animals and the length of experiments, and high individual variability of the disease process.

#### **4.3.3. Hemodynamic alteration**

It was reported that use of CsA based immunosuppressive regimens could lead to increased incidence of hypertension among solid organ transplantation recipients, especially among the patients who have undergone heart transplantation, and the incidence of hypertension was found to rise from 20% prior to the clinical introduction of CsA to 90% after the use of CsA [Olivari *et al.*, 1989; Ventura *et al.*, 1992]. In this study, it was shown that one week of intravenous administration of CsA (15 mg/kg/day) significantly elevated SBP in SD rats maintained on the sunflower oil diet. This is in agreement with previous reports that administration of CsA (Sandimmune, Neoral) in rats at 10-30 mg/kg/day for three days or seven weeks was found to have induced hypertension [Shimizu *et al.*, 2001; Tavares *et al.*, 2002]. However, rats maintained on the seal oil diet showed lower SBP values than those maintained on the sunflower oil diet

following the administration of CsA, suggesting less hemodynamic alterations induced by CsA when large portion of seal oil as dietary lipid was simultaneously consumed.

The decrease in renal toxicity and hypertension induced by CsA in rats maintained on the seal oil diet did not appear to be associated with the bioavailability of CsA. It was shown that *i.v.* administration of CsA (15 mg/kg/day) to rats resulted in comparable CsA levels in blood ( $4416 \pm 1003$ ,  $4231 \pm 664$   $\mu\text{g/L}$  in rats on the seal oil diet and the sunflower oil diet, respectively). The only difference between the two groups is the lipid composition in their diets: one is composed of 85% seal oil and 15% sunflower oil, and the other is composed of 100% sunflower oil. Our results suggested that concomitant supplementation of seal oil with CsA administration is beneficial in reducing the renal and hemodynamic toxicities induced by CsA, and the beneficial effects might be attributed to the  $\omega$ -3 long chain PUFAs in seal oil.

There have been many reports suggesting that  $\omega$ -3 PUFAs exert favorable actions against the toxicities associated with CsA in experimental mammals and humans. In those studies  $\omega$ -3 PUFAs were either provided through dietary supplementation of marine oils [Alexander *et al.*, 1998] or using fish oils as the vehicle for CsA administration [Elzinga *et al.*, 1987a; Elzinga *et al.*, 1987b]. Attenuation of CsA-induced hypertension by  $\omega$ -3 PUFAs supplementation has also been reported both in experimental animal models [Mills *et al.*, 1992] and clinical trials with organ transplantation patients [Ventura *et al.*, 1993; Maachi *et al.*, 1995].

#### 4.3.4. CsA arteriopathy

The pathogenesis of nephrotoxicity and hypertension associated with CsA is largely unknown, but a number of studies suggest that CsA alters renal hemodynamics after the first dose with an immediate, prolonged but reversible vasoconstriction of glomerular afferent arteriole [Myers *et al.*, 1986]. This leads to a reduction in glomerular capillary perfusion, which may consequently result in a decrease in transmembrane pressure and GFR, and decreased renal plasma flow (RPF) with Na<sup>+</sup> retention [Dieperink *et al.*, 1986]. CsA nephrotoxicity is characterized by a decrease in GFR, which has been observed in both clinical and animal studies [Thomson *et al.*, 1989; Sabbatini *et al.*, 1988]. In our *in vivo* studies, the Clcr levels in rats significantly decreased following the administration of CsA, suggesting the elevation of renal vascular resistance and the consequently decreased blood flow and GFR evoked by CsA. However, the lower levels of Clcr in rats supplemented with seal oil than those supplemented with vegetable oil implied a protective role of seal oil against the CsA-induced renal arteriopathy.

The underlying mechanism of CsA arteriopathy is still unclear. It had been reported that CsA could enhance *in vitro* platelet aggregation and subsequent TXA<sub>2</sub> release [Kawaguchi *et al.*, 1985]. CsA was also found to increase platelet aggregation by reduced PGI<sub>2</sub> production by the vessel wall [Langman, Yatscoff, 1994]. These findings suggest the implication of CsA in vascular abnormality and increased risk of progression of atherosclerosis. This CsA arteriopathy affects renal arterioles and glomerular capillaries. It has been observed glomerular capillary thrombosis in biopsy specimens taken 1 h after surgery from renal transplant patients pretreated with CsA [Parmar *et al.*,

1994]. There are also numerous reports of thromboembolic complications occurring later in the posttransplant period in CsA-treated renal allograft patients [Vanrenterghem *et al.*, 1985; Randhawa *et al.*, 1996]. Thus, the renal dysfunction seems to be originated from vascular constriction caused by CsA-induced augmentation of thromboxane production.

Increased platelet aggregation and thromboxane release have been shown in patients and normal volunteers treated with CsA [Grace *et al.*, 1987], suggesting that the potential interruption by CsA to vascular tone may occur not only in pathological but also in normal conditions. Both systemic and renal vasoconstrictions of CsA may lead to the development of hypertension [Bennett, Porter, 1988; Scherrer *et al.*, 1990].

Based on these reports, CsA associated kidney dysfunction to some extent is probably related to kidney ischemia induced by vasoconstriction of afferent arteriole and glomerular capillary. Therefore, the basic and common pathological pathway of both kidney dysfunction and hypertension associated with CsA administration seems to be sustained arteriole vasoconstriction which is at least partially caused by interrupted production of eicosanoids with CsA treatment, although other mechanisms have also been suggested. In view of the similarity of pathogenesis between CsA-induced disorders and ischemic cardiovascular diseases, strategies used for prevention and treatment of cardiovascular disease may be applied for CsA-induced abnormalities. Marine oil supplementation has been proved to exhibit beneficial effects to ischemic cardiovascular diseases such as atherosclerosis. Thus, seal oil, a superior source of  $\omega$ -3 PUFAs, might favorably affect CsA-related vascular pathological changes.

#### 4.3.5. Alteration of eicosanoids production in CsA administration

Among the postulated mechanisms involved in the vasoconstriction associated with CsA treatment, accumulating evidences revealed an important role of eicosanoids which may be influenced by dietary fatty acids intake. Therefore, our attention was focused on eicosanoids when studying the effects of seal oil rich in  $\omega$ -3 PUFAs on CsA related toxicities.

The prostanoids including PGs and TXs are cyclic eicosanoids [Darlametsos, Varonos, 2001]. PGI<sub>2</sub> and PGE<sub>2</sub> are vasodilators and potent inhibitors of platelet aggregation. Their biological properties are opposite to the effects of TXA<sub>2</sub>. Within the kidney, TXA<sub>2</sub> can be synthesized by messangial and epithelial cells. It has significant effect on renal hemodynamics, and its pathogenic role in some chemical compounds induced toxicity is well-known. A physiological balance between the activities of vasodilatory PGs and vasoconstrictor TXA<sub>2</sub> is important in maintaining a healthy vascular tone.

However, following CsA administration, increased production of TXA<sub>2</sub> by platelets has been reported [Perico *et al.*, 1986; Parra *et al.*, 1998]. On the other hand, concomitant administration of a specific TX synthase inhibitor with CsA results in attenuation of CsA associated nephrotoxicity without compromising the immunosuppressive activity of CsA [Petric *et al.*, 1990; Gladue, Newborg. 1991]. There has also been a conflicting report that Fisher rats given CsA developed nephrotoxicity with no change in urinary excretion of TXB<sub>2</sub> observed [Schnabel *et al.*, 1991].

PGs are well recognized as potential modulators of renal hemodynamics [Scharschmidt *et al.*, 1986]. CsA has been reported to reduce the synthesis of vasodilatory PGs. Concomitant administration of a vasodilatory PGE<sub>1</sub> analogue, misoprostol, to rats given CsA not only reduced acute nephrotoxicity but also resulted in a reduction of systemic blood pressure [Paller, 1988], suggesting a possible link between CsA associated renal and cardiovascular pathogenesis. Moreover, administration of nonsteroidal anti-inflammatory drugs inhibiting production of PGs led to augmented renal vasoconstriction induced by CsA [Sturrock *et al.*, 1994], suggesting inhibition of PGs synthesis may be an important operative mechanism in CsA-induced nephrotoxicity. These studies revealed an enhanced production of vasoconstrictor TXs and suppressed production of favorable vasodilator PGs associated with the administration of CsA.

As stated previously, dietary lipids are involved in eicosanoids metabolic pathway, and ingested oils containing different classes of PUFAs play an important role in the production of eicosanoids. Supplementation of seal oil rich in  $\omega$ -3 PUFAs (EPA, DHA, and DPA) can change the fatty acids composition in tissue. Since  $\omega$ -3 PUFAs have a higher affinity for COX than the  $\omega$ -6 class, COX would preferentially interact with them. Upon biological stimuli, more  $\omega$ -3 PUFAs were released to compete with AA (20:4n6), through COX pathway producing reduced 2-series but increased 3-series prostanoids (see Figure 4.1.). The 3-series PGs are also vasodilator and anti-aggregatory agents, whereas TXA<sub>3</sub> is inactive [Needleman *et al.*, 1979]. Furthermore, it was found that EPA in marine oils decreased TXA<sub>2</sub> but increased PGI<sub>2</sub>, PGE<sub>2</sub> production

[Abeywardena *et al.*, 1989]. Thus, seal oil supplementation would lead the eicosanoids production which favors vasodilation.

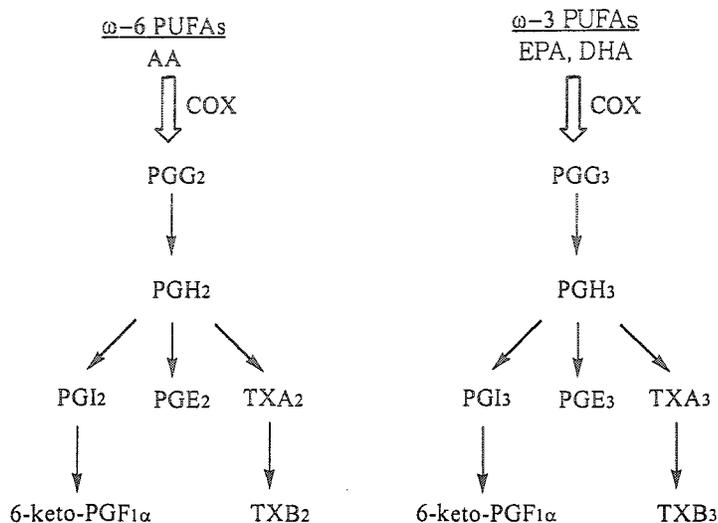


Figure 4.1. Metabolism of prostanoids derived from  $\omega$ -3 and  $\omega$ -6 PUFAs.

PGI<sub>2</sub> is unstable with a half-life of approximately 3 min *in vitro* [Rais *et al.*, 1979], and it undergoes spontaneous hydrolysis to 6-keto-PGF<sub>1 $\alpha$</sub> . TXA<sub>2</sub> has a half-life of about 30 sec and is rapidly hydrolyzed to its stable, biologically inactive metabolite, TXB<sub>2</sub>. Due to the quick spontaneous hydrolysis of PGI<sub>2</sub> and TXA<sub>2</sub>, the quantitative determination of 6-keto-PGF<sub>1 $\alpha$</sub>  and TXB<sub>2</sub> are accepted as a measure of PGI<sub>2</sub> and TXA<sub>2</sub> production, respectively [McCann *et al.*, 1981; Fitzpatrick *et al.*, 1977]. Our data showed that rats maintained on seal oil diet and given NS exhibited higher value of urinary 6-keto-PGF<sub>1 $\alpha$</sub> /TXB<sub>2</sub> than their counterparts on sunflower oil diet, indicating dietary supplementation of seal oil resulted in a higher ratio of vasodilator PGs to vasoconstrictor TXs production. In this study, administration of CsA significantly reduced this ratio in rats maintained on either seal oil diet or sunflower oil diet in comparison with the rats

administered NS, which was consistent with a previous report [Morphake *et al.*, 1994]; However, between the two groups given CsA, the rats maintained on seal oil diet showed a higher value of 6-keto-PGF<sub>1α</sub>/TXB<sub>2</sub> than those maintained on sunflower oil diet, suggesting dietary supplementation of seal oil to some extent counteracted CsA-induced decline of the ratio of 6-keto-PGF<sub>1α</sub>/TXB<sub>2</sub>.

It should also be pointed out that the levels of 6-keto-PGF<sub>1α</sub> and TXB<sub>2</sub> measured in this study are actually a mixture of 6-keto-PGF<sub>1α</sub> metabolized from PGI<sub>2</sub> and PGI<sub>3</sub>, and a mixture of TXB<sub>2</sub> from active TXA<sub>2</sub> plus TXB<sub>3</sub> from inactive TXA<sub>3</sub>, respectively. Due to the cross activity, even the monoclonal antibodies used for the determination of PGs and TXs by enzymeimmunoassay, do not separate the 2- from the 1- and 3-series of PGs and TXs.

Taking into account that the extent of decreased renal functions was parallel to the degree of lowered value of urinary 6-keto-PGF<sub>1α</sub>/TXB<sub>2</sub>, it can be inferred that the ratio of 6-keto-PGF<sub>1α</sub>/TXB<sub>2</sub> is closely related to renal function. Concomitant supplementation of seal oil with CsA administration resulted in partially reserved ratio of urinary 6-keto-PGF<sub>1α</sub>/TXB<sub>2</sub> and amelioration of the impaired kidney functions induced by CsA. Therefore, the beneficial effects of seal oil against CsA-induced kidney toxicity might be exerted by counteracting the imbalanced production of vasoactive prostanoids.

#### **4.3.6. Influence of dietary oils on fatty acids composition of phospholipid**

There were significant changes of phospholipid fatty acid composition in erythrocyte membrane in rats maintained on seal oil diet for eight weeks in comparison

with those in rats maintained on sunflower oil diet. A major difference was seen in the type of phospholipid fatty acids found in the membrane. The total  $\omega$ -6 PUFAs (including LA (18:2n6), AA (20:4n6) and 22:4n6) in erythrocyte membrane phospholipid in rats kept on sunflower oil diet was two times of that in rats kept on seal oil diet; whereas the latter had six times higher total  $\omega$ -3 PUFAs (including EPA (20:5n3), DPA (22:5n3), and DHA (22:6n3)) content with a concurrent decrease in  $\omega$ -6 PUFAs than the former. This was probably resulted from the competition between the  $\omega$ -6 and  $\omega$ -3 PUFAs for the desaturation system. In both animals and human, the pathway for biosynthesis of AA (20:4n6) and DHA (22:6n3) involves desaturation and elongation of the essential fatty acids LA (18:2n6) and ALA (18:3n3), respectively [Innis *et al.*, 1995]. The  $\omega$ -6 and  $\omega$ -3 PUFAs pathways are believed to use the same desaturase enzymes, and desaturation is subjected to substrates competition and product inhibition [Innis, 1991]. Thus, if ALA, EPA and/or DHA are ingested, desaturation of LA to yield AA can be inhibited. This effect had also been found in liver, kidney, and brain [Innis *et al.*, 1995]. Our results of fatty acid compositions of the phospholipids extracted from erythrocyte membrane showed an obvious shift of  $\omega$ -6 to  $\omega$ -3 class when seal oil, rich in EPA, DPA and DHA, were the main dietary lipid for eight weeks.

These changes were in agreement with previous reports that diets rich in  $\omega$ -3 fatty acids lead to a displacement of  $\omega$ -6 fatty acids in erythrocyte membrane [Kim *et al.*, 1992]. More importantly, it has been found that the changes in phospholipid fatty acid composition of erythrocyte membrane are reflective of the changes that take place in cell membranes of other tissues or organs including kidney [Gibson *et al.*, 1984]. Therefore,

analysis of the phospholipid fatty acid compositions in RBCs is considered a practical and valid method of estimating the changes in other tissues, and was adopted in our study. Moreover, dietary supplementation of fish oil altering fatty acid composition in various organs including kidney has been reported [Aukema *et al.*, 1992].

The biological effects of dietary fatty acid are the consequences of the incorporation of these fatty acids into the cellular structure of different tissues and organs. Of all the fatty acids, the  $\omega$ -3 PUFAs were the most influenced by diet, especially EPA and DHA [Soriguer *et al.*, 2000]. In our study, of the interest is the fact that the alteration of fatty acid composition of phospholipids is in conformity with the favorable increase of urinary 6-keto-PGF<sub>1 $\alpha$</sub> /TXB<sub>2</sub> ratio, which might have resulted from an increased EPA/AA ratio of fatty acid composition in renal cell membranes. This may be the original cause of the attenuation of the CsA-induced toxicities in the rats supplemented with seal oil.

#### **4.3.7. Lipid peroxidation**

Besides the contraction effect of CsA on renal arterioles, a number of other mechanisms and mediators have been proposed to account for the decreased renal function associated with CsA administration. There are experimental observations reported that CsA caused a time- and concentration-dependent increase of lipid peroxidation in hepatic and renal microsomes *in vitro* [Inselmann *et al.*, 1990]; administration of CsA *in vivo* reduced antioxidant glutathione levels, and glutathione depletion increases the susceptibility to cyclosporine-induced liver and kidney injury [Inselmann *et al.*, 1994]. Wang *et al.* used an antioxidant lazaroid to reduce CsA-induced

lipid peroxidation both *in vitro* and *in vivo* [Wang, Salahudeen, 1994]. These experimental data suggested a possible role of reactive oxygen species (ROS) in CsA related toxicity.

The relationship between DHA levels in tissue and oxidative stress is a controversial topic [Yavin *et al.*, 2002]. Someone argued that the multiplicity of double bonds in the structure of DHA or EPA makes it easily be attacked by ROS during oxidative stress [Halliwell, Chirico, 1993]. A DHA-enriched diet increased peroxidation products in plasma and several tissues [Song *et al.*, 2000], whereas rats fed on a diet lacks DHA precursors showed less retinal injury after exposure to light [Organisciak *et al.*, 1999]. However, it was also reported that DHA-supplemented human lymphocytes are less vulnerable to hydrogen-peroxide induced oxidative damage [Bechoua *et al.*, 1999]. A recent study investigated the dose-dependent effect of DHA on the redox status of human platelets *in vitro*, and the results showed a biphasic effect of DHA with antioxidant effects at low; whereas prooxidant effects at high concentrations [Vericel *et al.*, 2003]. In another study, each of the  $\omega$ -3 PUFAs ALA, EPA and DHA in combination with dietary vitamin E were fed to rats for 22 days to compare their peroxidation effects. The results showed that in rats fed either the EPA diet or the DHA diet with an adequate level of vitamin E, the extent of lipid peroxidation in liver, kidney and testis were less than expected from the relative peroxidizability index. But the suppression of lipid peroxidation below the relative peroxidizability index was not with ALA, which has three double bonds [Saito, Kubo, 2003]. These latest data suggested potential antioxidative effects of DHA and EPA when supplied together with adequate amount of vitamin E.

Our *in vivo* experiment with rats showed a significant increase of MDA content, the lipid peroxidation product, tested by TBARS colorimetric assay in kidney tissue in rats given CsA, indicating the enhancement of lipid peroxidation by CsA, which was consistent with the literatures. When seal oil was used as the main lipid source in the diet, the rats maintained on a seal oil diet with CsA administration showed lower levels of lipid peroxidation in comparison with their counterparts on a sunflower oil diet, although the MDA content in kidney tissues were still much higher than that in rats given NS. Our results agreed with the report that EPA and DHA can suppress lipid peroxidation when supplied with adequate vitamin E. Sufficient amount of vitamin mixture was given to the rats as a necessary component in our specific rats' diets.

#### **4.3.8. Growth suppression**

Suppression of body weight gain in rats given CsA appeared one week following the start of CsA administration. The difference of body weight between rats given CsA and NS became significant at two weeks following CsA administration. Besides suppressed body weight gain, there were no other signs of illness during the whole observation. The high dose of CsA might have been a factor. Others also reported growth suppression by CsA even with a lower dose administered to rats [Ferguson *et al.*, 1993].

It should be pointed out that a high dose of CsA was administered to the rat model employed in this study to induce toxicity over a relative short term. The relevance of using a large dose of CsA for this study could be criticized, since the value of trough CsA concentrations in blood were seven to ten times above the upper limit of clinical

therapeutic levels. However, our purpose was to examine renal function with reduced GFR mimicking clinical patients who rely on CsA for the rest of their lives after organ transplantation. We reasoned that relative preservation of kidney function under this condition would argue against a direct toxic action of CsA to the kidney. Moreover, the amount of seal oil used in this study is hard to achieve in clinical practice. Further studies are needed before applying seal oil to clinical setting.

#### 4.4. *In vitro* study

To further investigate the effective components and mechanism of the effects of seal oil on CsA-induced nephrotoxicity at the cellular level, *in vitro* studies were also conducted. As suggested by previous reports, CsA toxicity occurs primarily in the glomerulus and epithelial cells lining the proximal tubes of kidney [Mihatsch *et al.*, 1995]. LLC-PK1 is a continuous, porcine kidney cell line expressing many characteristics of proximal tubule epithelia [Hull *et al.*, 1976; Gstraunthaler *et al.*, 1985]. It is often used as a model of the proximal tubule for the study of renal toxicity. It was reported that in the kidney epithelial cell line LLC-PK1, CsA has been shown to cause a loss of cell contacts, reduction in brush border integrity, and a disruption of cell-cell adhesion [Zimmerhackl *et al.*, 1997]. CsA also inhibited DNA and protein synthesis of LLC-PK1 cells [Healy *et al.*, 1998]. Therefore, in our study LLC-PK1 cell line was chosen as a renal proximal tubular model to assess effects of seal oil and various fatty acids on CsA cytotoxicity.

Knowledge about the growth state of a certain cultured cell line and its kinetic parameters, is important in the design of *in vitro* experiments using cultured cells. The same cell lines may have different characteristics when they are in the lag phase, the log phase (period of exponential growth), and the stationary phase. Cells that have entered the stationary phase grow slowly, and have different morphology. They generally tend to secrete more extracellular matrix and become more difficult to be trypsinized. Cell cultures in the log phase are commonly most consistent and uniform. Therefore, it is important to determine which growth state the cells are in.

It is also important to consider the effects of the duration of an experiment on the transition from one state to another. Adding a drug at different growth state may give quite different results, due to altered properties [Freshney, 2000]. Therefore, first of all, the growth pattern of LLC-PK1 cells was determined. Our results showed LLC-PK1 is a relatively fast growing cell line with a cell population doubling time of 29 h. From the second day after seeding at a density of  $1 \times 10^4$  cells/mL, the cells entered a stable exponential growth period, lasting for four to five days.

The conversion of yellow-colored MTT dye to the purple-colored formazan depends on the activity of mitochondrial dehydrogenase, which is NADH and DADPH dependent, thus, the amount of MTT conversion is an indicator of cell metabolic activity. The absorbance of the purple color of formazan is only linearly proportional to the number of metabolic active cells in a certain range when cells were cultured in multiwell microtiter plate. Therefore, the prerequisite of using MTT assay is the number of cells at the endpoint of experiment still staying in the linear range of cell number to the

absorbance of the purple color of formazan. Under our experimental condition, it was found when cells seeded at  $2.5 \times 10^3$  -  $4 \times 10^4$  cells/ mL, there was a linear relationship between absorbance of the converted MTT dye at 570 nm and cell numbers following 72 h incubation, which was the designed duration of this *in vitro* study.

The results of both cell viability and LDH release from LLC-PK1 cells following incubation with various concentrations of CsA ranging from 0.63 to 10  $\mu$ M for 24 h showed concentration-dependent cytotoxicity of CsA. These were in agreement with a previous report which showed direct toxic effects of CsA on established LLC-PK1 cell model of renal tubules [Zimmerhackl *et al.*, 1997]. The cellular mechanisms of CsA-induced toxicity in renal proximal tubular cells are still unclear; however, the beneficial effects of antioxidative substances suggested the role of ROS in CsA-induced LLC-PK1 cell injury [Wang, Salahudeen, 1994]. CsA was shown to induce oxidative injury probably by disrupting membrane integrity and altering energy status [Massicot *et al.*, 1994]. Furthermore, CsA has been reported to enhance the generation of hydrogen peroxide in cultured hepatocytes [Wolf *et al.*, 1994] and mesangial cells [Pérez de Lema *et al.*, 1997].

Since our *in vivo* studies indicated that supplementation of seal oil is beneficial in reducing nephrotoxicity of CsA, seal oil emulsion was tested in renal epithelial LLC-PK1 cells. It was found that higher than 0.00016% of seal oil emulsion was toxic to the cells. However, in the presence of 0.0001% of seal oil emulsion, which was not toxic to the LLC-PK1 cells (as demonstrated by MTT assay), enhanced cytotoxicity of CsA in the cells was observed after 24 h incubation with various concentrations of CsA, as

evidenced by decreased IC<sub>50</sub> values of CsA and increased LDH release. Lack of beneficial effects of seal oil in the growth of LLC-PK1 cells and against the cytotoxicity of CsA were likely due to the lack of lipase in the cultured cells which are essential to release PUFAs from their TAGs.

To test this postulation, PUFAs, DHA and EPA, respectively, were tested in the LLC-PK1 cells. In addition, antioxidant nutrients (*e.g.* Vitamins E and C) were found to inhibit the synthesis of ROS and TXs, and the lipid peroxidation process induced by CsA in kidney structures [Parra *et al.*, 2003]. Therefore, vitamin E was also tested using LLC-PK1 cells treated with CsA. Vitamin E (0.1-10  $\mu$ M) exhibited a beneficial role in a concentration-dependent manner as assessed by cell viability and LDH release. DHA (0.4-10  $\mu$ M) also showed a beneficial effect in a concentration-dependent manner on the cytotoxicity demonstrated by CsA as evaluated by cell viability and LDH release. However, EPA (0.1-10  $\mu$ M) showed no significant effect on the cytotoxicity of CsA.

Vitamin E is a well-known antioxidant that stabilizes cell membranes by interfering with lipid oxidation process [Tappel, 1970]. The reduction of CsA-induced cytotoxicity in LLC-PK1 cells by vitamin E observed in the present study may be attributed to the involvement of vitamin E in the inhibition of lipid peroxidation. Our results seemed in conformity with the concept that ROS was involved in the process of CsA related cytotoxicity. Of the interest is the fact that the action of DHA appeared similar to that of vitamin E, *i.e.* DHA might reduce the cytotoxicity of CsA by the same mechanism of inhibiting lipid peroxidation.

To investigate the possible mechanism by which DHA exhibited its beneficial effect on the cytotoxicity of CsA, the lipid peroxidation product MDA contents in the cell culture supernatant were measured by TBARS assay with a spectrofluorophotometer. However, due to the low content of MDA in the cell culture supernatant and the limited sensitivity of the method, the results were rather inconclusive. More sensitive detection methods are needed, such as confocal laser scanning microscopy, as suggested in the literature [Nishida *et al.*, 2003].

A weak point of this *in vitro* experiment is that CsA (Neoral<sup>®</sup>) used is a commercial product, the exact components are unknown. Therefore, it is impossible to prepare a control to show that any toxicity exhibited by Neoral<sup>®</sup> does not pertain to substances other than CsA in the formulation.

It has been reported that CsA could be prepared as stock solution by dissolving CsA in an organic solvent such as ethanol, DMSO or DMF (around 30 mg/mL), which could be diluted using cell culture media [Nishida *et al.*, 2003; Andres, Cascales, 2002]. In our study, CsA stock solutions in DMSO, DMF and ethanol ranging from 4 - 50 mg/mL were prepared. However, upon more than 50 times dilution with cell culture medium, CsA precipitations were observed under microscope, although the preparations were visually clear. CsA at 50-100  $\mu\text{M}$  was reported to be used for inducing cytotoxicity [Nishida *et al.*, 2003; Zimmerhackl *et al.*, 1997]; however, 20  $\mu\text{M}$  CsA incubated for 24 h was found to result in almost 100% cell death in our study.

#### 4.5. Other potential roles of DHA against CsA toxicities

The protective role of  $\omega$ -3 PUFAs in the renal process of CsA is widely believed to be ascribed to their effects on eicosanoids metabolism. In addition, there have been reports suggesting that marine oils may play a role (in cell membrane) to counteract CsA-induced physical alteration of cell membrane structure.

LeGrue *et al.* [LeGrue *et al.*, 1983] found that there is almost the same binding affinity of CsA to lymphocytes and phospholipids which lack a specific binding site for CsA. This finding suggested that the toxic effect of the lipophilic CsA to vasculature might be exerted because of the physical intercalation into the endothelial cell membrane. A number of studies focusing on the interaction of CsA with the membrane bilayers showed that CsA caused increased permeability of liposomal membrane to  $\text{Na}^+$  and  $\text{Ca}^{2+}$  [Fahr, Seelig, 2001; Wolf *et al.*, 1998], and decreased membrane fluidity [Rossaro *et al.*, 1988]. Another study which studied the effects of CsA on the plasma membrane of peripheral blood lymphocytes found that CsA can decrease membrane fluidity in one to five minutes after addition to cultured cells at doses similar to those found in patient's blood immediately following intravenous administration [Niebylski, Petty, 1991]. These studies revealed that CsA can alter membrane structures by intercalation into the membrane bilayer thereby resulting in altered functions.

On the other hand, when dietary fatty acids are ingested, they can be incorporated into the structure of membrane phospholipids, and then can produce mediators for biological reactions upon stimuli. DHA, abundant in marine oils, was found to be tenaciously retained in tissues [Salem *et al.*, 1986]. When added to membrane bilayers as

either a free fatty acid or as part of a phospholipid, DHA can induce major changes in membrane fluidity [Ehringer *et al.*, 1990; Simopoulos, 1991] and increase phospholipid vesicles and the permeability of cancer cell [Stillwell *et al.*, 1993]. Moreover, DHA was also reported to alter membrane protein and receptor functions [Zerouga *et al.*, 1995], and change the membrane bilayer thickness [Lee *et al.*, 1986]. These alterations rendered by DHA in endothelial cell membrane structure could counteract the membrane changes caused by CsA.

According to the literature, due to its ability to alter membrane physical properties when incorporated, DHA is potentially beneficial to reverse CsA induced cell membrane damage. Thus, EPA and DHA, the main  $\omega$ -3 PUFAs in marine oils, can counteract the toxicities related to CsA from the two different biochemical and physical pathways respectively, making concomitant supplementation of marine oils with CsA administration theoretically reasonable. However, in the current study we have not explored the effects of DHA on cell membranes.

#### 4.6. Summary

Our results suggest that use of seal oil, a superior source of EPA, DPA and DHA, reduced CsA nephrotoxicity *in vivo* and the beneficial effects may be due to its effects on biosynthesis of prostanoids, although other mechanisms, such as suppression of lipid peroxidation, modulation of cell membrane properties and depression of lipoxigenase metabolites, are also possible.

## Chapter 5. References

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