

**THE EFFECTS OF THE COMBINATION OF DIETARY FLAXSEED OIL OR
FISH OIL WITH CYCLOSPORINE IN A RAT CARDIAC ALLOGRAFT MODEL**

By

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ABSTRACT

The discovery of new immunosuppressive drugs has resulted in an improvement of short-term graft survival. Despite this achievement, long-term cardiac allograft survival has not been correspondingly improved. Cyclosporine A (CsA), an effective immunosuppressive drug, has been shown to increase the risk of hyperlipidemia, hypertension, kidney injuries and chronic rejection despite its extensive use in the clinical setting. Therefore, these side-effects of CsA, may further contribute to graft failure over long-term. Early studies have shown that fish oil may reduce side-effects of CsA. These beneficial effects of fish oil may be related to n-3 fatty acids (n-3 FAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Flaxseed oil is another major source of an n-3 FA, namely α -linolenic acid (ALA). However, its impact on heart transplantation has not been fully explored.

The current study aimed to investigate whether dietary flaxseed oil and fish oil reduce post-transplant complications and prolong graft function in a rat cardiac allograft model. Male Fischer and Lewis rats were used as donors and recipients, respectively, to generate a heterotopic cardiac allograft model. After transplant, animals were randomly assigned into 3 groups and fed a diet supplemented with: a) 5% w/w safflower oil (control n=7), b) 5% w/w flaxseed oil (n=8) or c) 2% w/w fish oil (n=7) and an intraperitoneal injection of cyclosporine A (CsA) (1.5 mg/kg/d) over 12 weeks. Body weight, blood pressure (BP), plasma levels of lipids, CsA, and select cytokines, fatty acid profile of hearts (native and graft) and liver tissues as well as graft function and chronic rejection features

were assessed. Body weight and blood CsA levels were similar among the groups. As compared to controls, both diet treated groups demonstrated a significantly lower systolic blood pressure (SBP) ($p < 0.001$), diastolic blood pressure (pressure (DBP) ($p < 0.001$), mean arterial pressure (MAP) ($p < 0.001$), heart rate ($p < 0.05$), abdominal fat ($p < 0.05$) and plasma levels of macrophage chemoattractant protein-1 (MCP-1) ($p < 0.05$). Moreover, the fish oil group had significantly ($p < 0.05$) lower plasma levels of triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL), as compared to the control group. High-density lipoprotein cholesterol (HDL) concentrations were significantly higher ($P < 0.05$) in the flaxseed oil-treated group as compared to the other two groups.

Data of this study suggest that both flaxseed oil and fish oil may provide similar biochemical, hemodynamic and inflammatory improvements after heart transplantation; however, these apparent beneficial changes were not accompanied with significant reductions in chronic rejection states or apparent histological evidence of cyclosporine-induced nephrotoxicity in this model.

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DEDICATION

I dedicate this thesis to my dear father and mother, who have offered me unconditional love and support over all these years. I also dedicate this thesis to my husband for his love, support and patient over the years. Finally, I also would like to dedicate this thesis to my son "Qutaibah".

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ACE	Angiotensin-converting enzyme
ALA	Alpha-linolenic acid
BH4	Tetrahydrobiopterin
BP	Blood pressure
CAD	Coronary artery disease
Cho	Cholesterol
CMV	Cytomegalovirus
CRP	C-reactive protein
CsA	Cyclosporine A
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DHA	Docosahexaenoic acid
EDTA	Ethylenediamine tetra-acetic acid
EPA	Eicosapentaenoic acid
G	Graft
GC	Gas chromatography
GFR	Glomerular filtration rate
H&E	Hematoxylin and eosin
HDL	High-density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl Coenzyme A
HPLC	High performance liquid chromatography
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
INF- γ	Interferon- γ
LA	Linoleic acid
LDL	Low-density lipoprotein
LP-a	Lipoprotein-a
LPS	Lipopolysaccharide
LT	Leukotriene
MAP	Mean arterial blood pressure
MCP-1	Macrophage chemoattractant protein-1
MG	Monoglyceride
MHC	Major histocompatibility complex
N	Native
NF-kB	Nuclear factor-kB
NO	Nitric oxide
NOS	Nitric oxide synthase
PAS	Periodic acid schiff
PG	Prostaglandin
PPARs	Peroxisome proliferator-activated receptors
PRA	Panel reactive antibody
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species

SBP	Systolic blood pressure
SD	Standard deviation
SDG	Secoisolariciresinol diglycoside
ISHLT	International society for heart and lung
SMC	Smooth muscle cell
TC	Total cholesterol
TCA	Transplant coronary atherosclerosis
TG	Triglyceride
TLC	Thin-layer chromatography
TNF- α	Tumor necrosis factor- α
TX	Thromboxane
VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very low density lipoprotein

1.0 INTRODUCTION

Over the last decade, dramatic clinical developments have been made in the heart transplantation. Post-transplant complications such as transplant coronary arteriosclerosis (TCA), infection, and other adverse effects of immunosuppressive drugs continue to remain largely unsolved problems. Chronic rejection remains one of the major problems in solid organ transplantation despite the successful use of immunosuppressive agents (Kahn et al., 1971). Long-term use of cyclosporine A (CsA) may increase chronic rejection through increasing risk of hyperlipidemia (Bilchick et al., 2004), hypertension (Patel and Kobashigawa, 2004) and kidney injuries (Ventura et al., 1997a, b).

Epidemiological and experimental studies have shown health benefits of fish oil. These benefits may be related to marine n-3 fatty acids (n-3 FAs) including eicosapentaenoic acid (20:5, EPA), and docosahexaenoic acid (22:6, DHA). Incorporation of these fatty acids (FAs) into the cell membrane may result in a number of changes in the cell function, including reductions in blood triglycerides (TG) (Harris, 2005), blood pressure (BP) and pro-atherogenic cytokines, and improvement of endothelium function (Mori, 2006; Mori and Woodman, 2006), and modulation of the eicosanoid system toward vasodilatation and less pro-inflammation, anti-arrhythmic effects (O'Keefe et al., 2006; Van de Werf et al., 2003), anti-aggregatory effects (Kristensen et al., 2001), and anti-atherosclerotic properties (Connor, 2000). n-3 derived-eicosanoids enhance vasodilatory properties of nitric oxide (NO) and decrease

free radical production by leukocytes; thereby reducing leukocyte binding to the endothelium. Furthermore, they increase prostacyclin PG₁₃, which furthers vasodilatation, decreases platelets aggregation, and inhibits the synthesis of thromboxane A₂ (TXA₂), which advances platelet aggregation and vasoconstriction. These eicosanoids have emerged as potential candidates for primary and secondary cardiovascular prevention (Kris-Etherton et al., 2002; Smith et al., 2006), and treatment of post-myocardial infarction (Van der Werf et al., 2003).

Early Studies have shown that fish oil may offset side-effects of CsA by improving blood lipid profile (Jevnikar et al., 1988), inflammatory cytokine (Grimm et al., 1995), BP (Holm et al., 2001) and kidney function (Homan van der Heide et al., 1993). The current international recommendations for long chain n-3 FAs range from 200 mg up to 1 g/d (Gebauer et al., 2006). However, the public is still slow to adopt these guidelines as some concerns about toxins, taste preferences, and cost in fish grow (Guallar et al., 2002).

Flaxseed oil is another major source of an n-3 FA, namely alpha-linolenic acid (ALA, 18:3 n-3). ALA is converted in the body to EPA and DHA *via* desaturation and elongation processes; however, this conversion in humans and animals seems to be less efficient (Li et al., 1999). The ability of ALA and its metabolites to incorporate in to the cell membrane has led researchers to a flurry of research examining its beneficial effects on plasma lipids (Vijaimohan et al., 2006), platelets function (Allman et al., 1995), inflammation (Rallidis et al., 2003), endothelial cell function (Vogel et al., 2000), and visceral fat (Takei et al., 2001).

These clinical and experimental studies suggest that ALA may favorably reduce plasma lipids, improve platelets function, lower pro-inflammatory cytokine, diminish visceral fat as well as improve endothelial function. The mechanisms underlying these beneficial properties of flaxseed may be mediated through its beneficial effects on lipid metabolism, platelet function, inflammation, endothelial cell function, or arrhythmia (Mozaffarian, 2005). This biochemical, hemodynamic and inflammatory benefits may also ameliorate post-transplant complications.

On the basis of our current stage of knowledge, we sought to investigate whether dietary flaxseed oil would reduce post-transplant complications and prolong graft function in a rat cardiac allograft model.

2.0 REVIEW OF THE LITERATURE

2.1 TRANSPLANT CORONARY ARTERIOSCLEROSIS

2.1.1 Definitions, History, and Incidence

Heart transplantation remains an acceptable prevailing treatment of choice for many patients with heart failure. The indications for heart transplantation may be heart muscle disease, ischemic cardiomyopathy, valvular diseases, congenital heart disease, chronic rejection, and graft dysfunction (Valente et al., 2006). It has now been 40 years since Christiaan Barnard performed the first human heart transplantation in 1967 (Haller and Cerruti, 1968).

A 2005 report by the International Society for Heart and Lung (ISHLT) estimated that more than 4000 heart transplants have been performed annually worldwide over the last ten years (Taylor et al., 2005). This indicates that the number of potential recipients has continued to grow indicating the need for heart transplantation. Approximately, 80-85%, 60-70%, and 40-50% are the estimated survival rates after one year, five years, and ten years of heart transplantation, respectively (Hosenpud et al., 1999).

Chronic rejection is the main obstacle to long-term survival of cardiac transplant recipients. Development of transplant coronary arteriosclerosis (TCA) leads to graft rejection (Yamani et al., 2004). TCA is an accelerated form of coronary artery disease (CAD) characterized by a diffuse, progressive thickening of the epicardial and intramyocardial arteries of the transplanted heart (Baron et al., 2004). The process is a concentric fibrous intimal hyperplasia that occurs

along the whole length of the affected arteries (Baron et al., 2004). The atherosclerotic changes range from a diffused incorporation of lipids to the development of classical focal plaques later in the disease process. Production of neointima rich in vascular smooth muscle cells (SMC) and thickening of the vessel due to infiltration by inflammatory cells as response to alloimmune stimuli, progressively lead to lumen obstruction (Baron et al., 2004; Beranek, 2005). TCA can affect arteries, arterioles, capillaries and occasionally veins (Ramzy et al., 2005). Furthermore, several influences, including hyperlipidemia, hypertension, possible drug toxicity (CsA and steroids), insulin resistance, ischemia-reperfusion injury, and cytomegalovirus infection are thought to be involved in the development of TCA (Soukiasian et al., 2004).

Not until the late 1960s was TCA first noted at Stanford among the original heart transplant recipients (Cupples et al., 2002). The incidence of TCA is unlikely to increase in the first year post-transplant but it has not declined despite the great advances achieved in controlling rejection episodes (Cupples et al., 2002). The incidence of TCA is reported to be 5% to 25% at 1 year, 27% to 61% at 5 years, and 45% to 80% at 8 to 10 years after heart transplantation (Alexander et al., 2005). According to ISHLT, TCA was reported to be the leading cause of death between 1 and 3 years after heart transplantation (Hertz et al., 2002). Furthermore, without donor angiography, TCA can be transmitted inadvertently by means of transplantation in about 7% of patients (Grauhan et al., 2003).

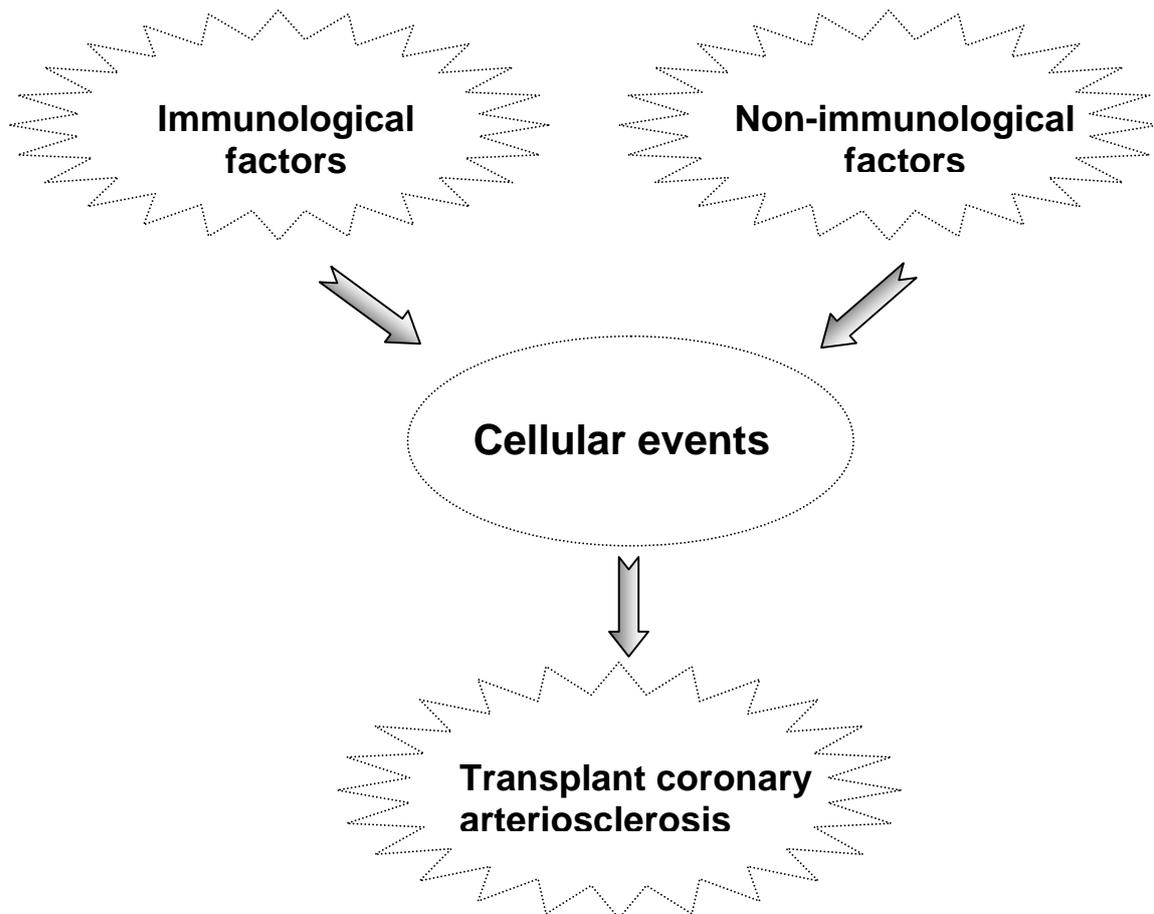
2.1.2 Pathogenesis of Transplant Coronary Arteriosclerosis

Although the exact pathogenesis of TCA is unknown, data from clinical and experimental studies suggest that its development is a combination of immunological and non-immunological factors (von Scheidt, 2000); accounting for endothelial dysfunction, intimal hyperplasia, thickening and lipid core plaque formation (**Figure 1**). This process is accompanied with calcification, luminal narrowing, ischemia, ventricular dysfunction and finally grafts failure (von Scheidt, 2000). In histology, the usual lesions of TCA appear to be concentric and diffuse intimal thickening composed mainly of modified vascular SMC. The lesions also contain lymphocytic and macrophage infiltrate. After advanced intimal proliferation and narrowing of the coronary artery lumen, the vessel becomes obstructed causing cardiac ischemia and infarction (Pietra and Boucek, 2003).

2.1.2.1 Immunological Factors

Many researchers believe that immunological mechanisms may play a significant role in the pathophysiological processes underlying TCA. Pathophysiologically, the presentation of all antigens by antigen presenting donor cells and by recipient cells leads to a concerted response pattern (Deng et al., 2000). This pattern includes antigen specific T-cell proliferation, CD4 T-cell proliferation with a differentiation into a T^{-helper1} and a T^{-helper2} profile, as well as nonantigen-specific proliferation of inflammatory cell responses (Deng et al., 2000; Bundy et al., 2000).

Figure 1. Proposed criteria for the pathogenesis of transplant coronary arteriosclerosis. The precise mechanism of transplant coronary arteriosclerosis (TCA) pathogenesis is unknown; however, the available knowledge from the clinical and experimental studies suggests that TCA development is a conjunction of immunological and non-immunological factors along with subsequent cellular events, accounting for graft dysfunction.



Recipient inflammatory cells invade the donor coronary endothelium and liberate specific cytokines (e.g., interleukin (IL)-2, IL-4, IL-5, IL-6, and tumor necrosis factor- α (TNF- α), which in turn result in migration of medial SMC into the intimal layer, initiating the intimal hyperplasia. Moreover, the continuous endothelial damage may lead to an alteration in growth mechanisms in the vascular wall resulting in acceleration of the atherosclerotic development (Bundy et al., 2000).

2.1.2.2 Non-immunological Factors

2.1.2.2.1 Donor-related Factors

Cardiac allografts obtained from older donors have shown poorer survival rates and an earlier onset of TCA (Costanzo et al., 1998). Donor events pre-transplantation such as the type of brain damage, and explosive brain injury were highly correlated with the incidence of TCA (Bundy et al., 2000). In kidney and aorta transplant models in rats, prolonged ischemic time induced the development of TCA in isografts (Wanders et al., 1995; Kouwenhoven et al., 1999). Furthermore, the severity of vascular lesions has shown a correlation with the duration of the ischemic period (Schmid et al., 1997). The accelerated healing and ongoing stimulation of the immune system could have a significant role in the incidence of TCA (Granger, 1999); however, the precise mechanisms remain to be elucidated.

2.1.2.2.2 Recipient –related Factors

2.1.2.2.2.1 Gender and Race

Male recipients are more susceptible to TCA compared to female recipients (Costanzo et al., 1998). This gender influence could reflect a function for oestrogen. In light of this, oestradiol efficiently prevented TCA after allogeneic aorta transplantation in rats (Saito et al., 1997). Long-term survival of cardiac graft seems also to be related to race (Costanzo et al., 1998). Recently, according to donor race, recipient mortality rate was estimated to be high among African American (23.1%) as compared to Asian (11.1%), Caucasian (18.7%) or Hispanic (14.6%) (Cohen et al., 2007).

2.1.2.2.2.2 Metabolic Factors

The role of hyperlipidemia and hypertension is well-established in the etiology of native atherosclerosis. Thus, it is believed that these metabolic imbalances may accelerate TCA after heart transplantation (Barbir et al., 1992b; Radovancevic et al., 1990). Obesity and immunosuppressive agents are thought to play an essential role in post-transplantation hyperlipidemia (Winters et al., 1990; Becker et al., 1988). Approximately 80% of heart-transplant patients were found to have hypercholesterolemia, hypertriglyceridemia, and low high-density lipoprotein cholesterol (HDL) levels (Deng et al., 2000). In addition, intimal thickening and a higher incidence of CAD were correlated with both greater levels of low-density lipoprotein to HDL- cholesterol ratios (LDL /HDL) and high TG to HDL-cholesterol ratios (TG/HDL) (Deng et al., 2000). A link between high

serum lipoprotein-a (LP-a) concentrations, and increased risk of CAD in heart transplant recipients was also observed (Barbir et al., 1992a). Systemic hypertension appeared to be associated with TCA in cardiac transplant recipients (Radovancevic et al., 1990; Costanzo et al., 1998).

2.1.2.2.2.3 Genetic Factors

The immune response to an allograft varies from one person to another. This individual variation is due to genetic variation in the regulation of cytokine gene expression (Hutchinson IV et al., 1999). It is thought that polymorphic variation in specific genes may lead to development of TCA. Polymorphism was shown in the angiotensin-converting enzyme (ACE) gene of the renin-angiotensin system, and was associated with a variety of cardiovascular diseases (CVD) (Benza et al., 1998).

2.1.2.2.2.4 Cytomegalovirus Infection

Solid organ transplants recipients are at high risk for cytomegalovirus (CMV) infection due to seropositivity, a marker for latent infection in organ donors and recipients. When CMV seronegative patients receiving an organ from a CMV seropositive donor (D+/R-), the risk of CMV infection increases 20 times higher than the (D/R-) Combination. These patients may also get CMV infection due to generalized immunosuppressive therapy, blood transfusion, social contacts or inaccurate serologic testing (Fishman et al., 2007). Several studies suggest that CMV infection-mediated events may contribute to both native atherosclerosis and

TCA (Paavonen et al., 1993). Moreover, evidence has accumulated showing that CMV infection of vascular cells could enhance endothelial activation, accentuate endothelial–leukocyte interactions, and direct growth effects in human coronary SMC (Paavonen et al., 1993). This mechanism depends on the similarity between specific antigens of the CMV and allograft cells, which leads to activation of the host defense mechanism with intimal hyperplasia (Deng et al., 2000). Using rat cytomegalovirus in cardiac rat transplant models has indicated that viral infection accelerates the development of TCA (Lemström et al., 1995). In human, CMV infections impair the nitric oxide synthase (NOS) pathway to cause endothelial dysfunction (Weis et al., 2004).

2.1.3 Management of the Transplant Coronary Arteriosclerosis

Treatment of TCA is most difficult; however, the focus remains on prevention of TCA *via* reducing adverse immunologic and non-immunologic reactions including treatment of hyperlipidemia, hypertension, obesity, hyperhomocysteinemia. Preventing endothelial injury at brain death and reducing cold ischemic time and subsequent tissue damage before transplantation may reduce risk of TCA (Ramzy et al., 2005).

2.1.3.1 Lipid-Lowering Agents

The 3-hydroxy-3- methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or statins, are the most commonly used to reduce TC, LDL and very

low-density lipoprotein cholesterol (VLDL), and increasing HDL-cholesterol (Kobashigawa et al., 1995). Statins have been shown to improve vascular function and reduce endothelial dysfunction by increasing production of NO, inhibiting the coagulation cascade and limiting oxidized- LDL-mediated damage to the endothelium (Farmer, 2000; Koh, 2000; Li et al., 2001).

2.1.3.2 Antihypertensive agents

Antihypertensive agents including calcium channel blockers and ACE inhibitors have disease-modifying effects that influence intimal proliferation beyond simple vasodilatation for BP control. Angiographic data revealed that calcium channel blockers or ACE inhibitors seem to mitigate the incidence of transplant CAD in adults (Schroeder et al., 1993; Kobashigawa, 2000). This beneficial effect may mediate intimal and SMC proliferation, and involve stabilization of endothelial function and platelet aggregation inhibition (Schroeder et al., 1993; Kobashigawa, 2000).

2.1.3.3 Antiproliferative agents

Antiproliferative agents such as angiopeptin, a somatostatin analog, have been used to suppress SMC proliferation *via* inhibiting the release of insulin-like growth factor. In addition, heparin with low molecular weight could diminish severity of TCA due to its anticoagulation effect (Kobashigawa, 2000).

2.1.3.4 Monoclonal Antibodies

The chance of graft vasculopathy occurrence intensifies as the number of human leukocyte antigen (HLA) mismatches increases (Valantine, 2004). Most patients perform a panel reactive antibodies (PRA) test before transplantation to identify the higher risk for graft rejection. It was shown that recipients with PRA greater than 10% had a 2- fold higher risk for cardiac graft vasculopathy (Kerman et al., 1998). Reducing PRA by an intravenous use of immunoglobulin, cyclophosphamide, mycophenylate mofetil, and azathioprine may reduce acute rejection and limit the development of cardiac graft vasculopathy (Ramzy et al., 2005).

2.1.3.5 Nutritional Supplements and Vitamin Therapy

Hyperhomocystinemia is associated with endothelial dysfunction in both the general population and transplant recipients (Pietra and Boucek, 2003). Homocystine is thought to cause a premature breakdown in the arterial elastic fibers by activation of a serine protease in SMC, causing elastolytic activities (Pietra and Boucek, 2003). In cardiac transplant, folic acid, cyanocobalamin (vitamin B12), and pyridoxine (vitamin B6) supplements significantly reduced homocysteine levels and rate of coronary restenosis in adult atherosclerotic disease (Schnyder et al., 2001).

2.2 Immunosuppressive Drugs

Immunosuppressive therapies have led to fewer rejections and significant improvement in survival after heart transplantation. Immunosuppressive agents are used to prevent rejection of the transplanted heart and the occurrence or progression of cardiac graft vasculopathy. Of all immunosuppressive agents, CsA is one of the most commonly used calcineurin inhibitors in the transplantation since 1980s (Moien-Afshari et al., 2003).

2.2.1 CYCLOSPORINE A (NEORAL[®])

Cyclosporine A (CsA) is a metabolite of the fungus *Beauveria nivea* consisting of 11 amino acids. Since the early 1980s, CsA has been routinely used in solid organ transplantation to prevent rejection (Moien-Afshari et al., 2003). CsA uniquely acts on the immune system, particularly T-lymphocytes, by inhibiting IL-2-mediated cell proliferation through its binding to cyclophilins, cytoplasmic proteins, in T-lymphocytes (Moien-Afshari et al., 2003). CsA bioavailability is low (30%) in humans as well as rats (Fahr, 1993). CsA can be found in blood: 58% of CsA is in erythrocytes, 9% in leukocytes, and 4% in plasma water, while 21% is bound to lipoproteins (LDL, HDL, VLDL, and other lipoproteins) and 8% is bound to other plasma proteins (Fahr, 1993).

2.2.1.1 Cyclosporine and Transplant Coronary Arteriosclerosis

The general impact of CsA in the clinical studies appears to be protective; however, a huge debate has been issued since CsA treatment was highly

associated with the increased risk of chronic rejection among heart transplant recipients (Valantine, 2004); this may be due to accelerating the incidence of hyperlipidemia, hypertension, and renal dysfunction.

2.2.1.2 Cyclosporine and Hyperlipidemia

Persistent dyslipidemia is commonly seen in the solid organ transplantation recipients affecting 60% to 80% of heart transplant recipients; due to the influence of immunosuppressive drugs (Bilchick et al., 2004). Thus, CsA could indirectly contribute to the development of TCA. The proposed mechanisms of CsA to induce hyperlipidemia may be mediated through the inhibition of 26-hydroxylase, an enzyme that is important in bile acid synthesis from cholesterol. Inhibition of this enzyme leads to impaired bile acid formation, elevated hepatic cholesterol levels, and down-regulation of LDL receptor expression (Bilchick et al., 2004; Patel and Kobashigawa, 2004).

CsA binds to LDL receptors, increasing serum levels of LDL-cholesterol and interfering with LDL-cholesterol clearance. Decreasing hepatic lipase activity with CsA, results in increased LDL and VLDL-cholesterol levels (Patel and Kobashigawa, 2004). Blood CsA levels were significantly correlated with plasma levels of TC, LDL-cholesterol, and TC/HDL-cholesterol ratio in renal transplant recipients (Kuster et al., 1994). CsA was also shown to impair prednisolone clearance, which could be related to its cholesterol-raising effects (Kuster et al., 1994). With CsA therapy, plasma HDL cholesterol levels were inversely correlated with blood CsA concentrations (Kirklin et al., 2002).

2.2.1.3 Cyclosporine and Hypertension

High blood pressure (BP), both systolic and diastolic, is commonly seen in CsA immunosuppressive therapy in heart transplantation. The primary mechanisms of CsA-induced hypertension have not been completely explained. However, many hypotheses have been thought to explain such mechanisms; stimulating the synthesis of vasoconstrictor, endothelin, in cultured human endothelial cells (Ventura et al., 1997a). This produces SMC proliferation and contraction by an influx of extracellular calcium, leading to a direct vascular toxicity (Ventura et al. 1997b). Furthermore, CsA was reported to activate renin-angiotensin and sympathetic nervous system (Patel and Kobashigawa, 2004) and impair NO production. The latter observation is uncertain; however, the proposed mechanism may involve the production of superoxide ($O_2^{\cdot-}$), which will react with NO to yield peroxynitrite. The destruction of NO can lead to vasoconstriction and hypertension (Calò et al. 2000).

CsA-induced free radical production may degrade tetrahydrobiopterin (BH4), a cofactor necessary for NOS activity. This degradation may result in decreased NO production; however, this mechanism is somewhat controversial since CsA is claimed to enhance the synthesis of BH4 (Hattori and Nakanishi, 1995). In addition, CsA may increase renal tubular reabsorption of sodium, modulate intracellular calcium-ion regulation, and enhance production of prostaglandins (PG) (Patel and Kobashigawa, 2004; Olson and Rodeheffer, 1992).

2.2.1.4 Cyclosporine and Kidney Injuries

Treatment with CsA in heart transplant patients is associated to some extent with renal dysfunction. Although the mechanism by which CsA causes renal toxicity remains doubtful, studies have suggested that CsA-induced renal dysfunction comes from an alteration of renal hemodynamics, with an initial dose-dependent reversible decline in glomerular filtration rate (GFR) followed by a subsequent increase in BP (Ventura et al., 1997a,b). The hemodynamic changes observed in the kidneys are likely to be a result of vasoconstriction of the afferent arterioles leading to CsA-induced changes in the PG synthesis. CsA therapy may increase the production of TXA₂ (Ventura et al., 1997a). CsA-induced renal vasospasm may initially cause reversible kidney dysfunction. Long-term use of CsA may cause chronic renal ischemia with irreversible changes involving glomerulosclerosis and interstitial fibrosis (Patel and Kobashigawa, 2004).

Another possible mechanism for CsA-induced renal hypoperfusion may involve decreased production of prostacyclin, an important regulator of renal blood flow (Patel and Kobashigawa, 2004). CsA nephrotoxicity can be identified as a decline in creatinine clearance with elevation of creatinine and blood urea nitrogen, potassium, hypertension, hyperuricemia, and hyperkalemic, hyperchloremic renal tubular acidosis with preserved urine volume and sodium resorption (Patel and Kobashigawa, 2004; Bloom and Doyle, 2006). Proteinuria may also be another indication of advanced renal dysfunction due to CsA (Hartmann et al., 1996). Besides its effect on kidney, CsA altered morphological

structures of pancreatic islet cells and reduced insulin secretion, β cell density, and insulin synthesis (Subramanian and Trence, 2007). Furthermore, a biopsy from pancreatic islet transplants showed notable changes in islet cells morphology, including cytoplasmic vacuolization, immunohistochemical and ultrastructural loss of secretory granules (Drachenberg et al., 1999). This was further confirmed by *in vitro* studies showing the inhibitory effect of CsA on insulin secretion from human pancreatic islet cells (Nielsen et al., 1986 reviewed in Subramanian and Trence, 2007).

2.3 n-3 FATTY ACIDS

n-3 fatty acids (n-3 FAs) have been a subject of considerable research over the past two decades, and may represent an exciting new horizon in the science of fatty acid and human health. The discovery of numerous positive physiological effects of n-3 FAs has generated a great interest worldwide.

2.3.1 Definition and Historical Review

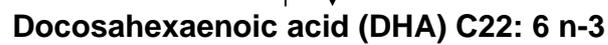
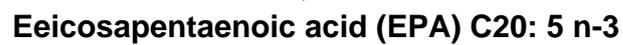
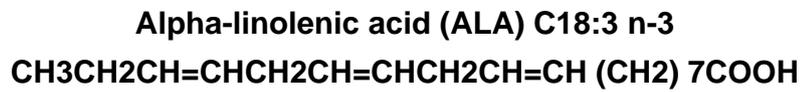
A diet rich in fatty fish was associated with low incidence of CVD in Greenland's Eskimos (Dyerberg et al., 1978). These early, remarkable scientific observations led to an immense interest to extensively study the health-related impacts of n-3 FAs. Such beneficial effects have been provided by epidemiologic studies (Hu et al., 2002) as well as by experimental studies (Kris-Etherton et al., 2002).

A growing body of research has led to an astonishing increase in investigations examining n-3 FAs' beneficial effect on blood lipids, hypertension, inflammatory mechanisms, immune function, atherosclerosis, thrombosis, and depression (Engler et al., 2003; Browning, 2003; Simopoulos, 2002; Hu, 2001; Li et al., 1999). Other studies support the positive effect of n-3 FAs on the risk of strokes, asthma, the survival time of individuals with cancer, systemic lupus erythematosus, as well as multiple sclerosis (Nagakura et al., 2000; Iso et al., 2001; Boudreau et al., 2001; Das, 1994; Nordvik et al., 2002). Furthermore, n-3 FAs have been found to have beneficial effects on hypertension (Andreassen et al., 1997, Holm et al., 2001), coronary endothelial function (Fleischhauer et al., 1993) and hypertriglyceridemia (Durrington et al., 2001) among heart transplant recipients.

2.3.2 Chemical Structure

n-3 FAs are a group of polyunsaturated fatty acids (PUFA) found in marine sources, such as EPA and DHA or in some leafy vegetables, nuts and oils, such as ALA. These three polyunsaturates have three, five, or six double bonds in a carbon chain of 18, 20, or 22 carbon atoms, respectively (**Figure 2**). In fact, clinical studies have shown that human hearts with n-3 FAs in their cell membranes are healthier and less probable to have disease than rigid and stiff cell membranes (Grodner et al., 2000).

Figure 2. Chemical structures of alpha-linolenic acid, eicosapentaenoic acid and docosahexaenoic acid.



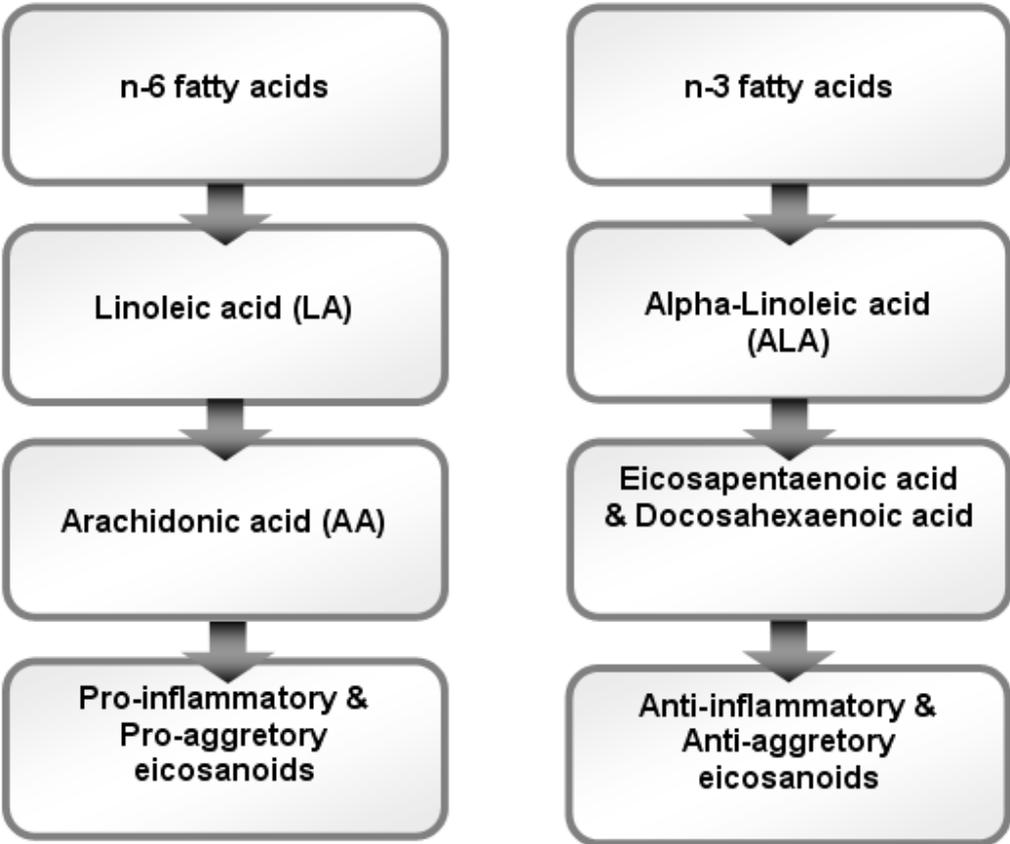
2.3.3 Fish Oil

Much of the interest in fish and fish oil comes from the earliest studies in the Alaskan population, which had a low rate of ischemic heart disease despite their high fat diets (Dyerberg et al., 1978; Kromann and Green, 1980). Much literature however, has solid compelling evidence emphasizing the prevention of CVD by consuming such oil. The most widely available sources of EPA and DHA are tuna, sardines, salmon, mackerel, and herring. In addition, recently fungal and algal sources have been considered a better source of DHA. Using fish oil supplements can also provide these beneficial n-3 FAs in an easy-to-take form (Appleton and Ackerson, 1998). Shifting the diets toward more n-6 FAs and less n-3 FAs may significantly affect a person's health because of their different metabolic pathways. Metabolism of n-3 and n-6 FAs result in eicosanoid production such as PG, leukotriene (LT), and thromboxane (TX). Eicosanoids derived from n-6 FAs are generally pro-inflammatory and pro-aggregatory, while those derived from n-3 FAs are mainly anti-inflammatory and anti-aggregatory **(Figure 3)** (Simopoulos, 1999). n-3 and n-6 FAs compete for conversion into these important metabolites, which in turn, may influence cardiovascular events (DeFilippis and Sperling, 2006).

2.3.3.1 Fish Oil and Dyslipidemia

Clear evidence exists showing the ability of n-3 FAs to favourably affect plasma lipids resulting in a significant decrease in CVD risk. In human trials, daily supplementation with 4 g of EPA decreased TG levels by 23% in mildly

Figure 3. Proposed scheme for metabolic pathway of n-6 and n-3 fatty acids.



hyperlipidemic subjects (Mori et al., 2000) and by 12% in healthy subjects (Grimsgaard et al., 1997). On the other hand, a daily low dose of 1.8 g of EPA for 16 weeks failed to cause any reductions in TG levels in patients with angina and hypertriglyceridemia (Yamamoto et al., 1995). EPA supplementation caused a small but significant decrease in plasma TC levels (Beilin and Mori, 2003).

DHA supplementation decreased TG levels by 17-33% in hypertriglyceridemic subjects (Mori et al., 2000). Using purified DHA showed an 8% increase in LDL particle size (Mori et al., 2000). DHA increased HDL2 cholesterol by 37% in dyslipidemic patients (Mori et al., 2000) and by 12% in diabetic patients (Woodman et al., 2002). It seems that DHA has a different effect on the HDL sub-fractions promoting anti-atherogenic shift in HDL particle size. DHA has also revealed a significant increase in LDL particle size (Mori et al., 2000). In this regards, Contacos et al., (1993) observed an increase of 1nm in the diameter of LDL particles after consumption of fish oil. This could partially explain the increased LDL-cholesterol levels found in clinical trials (Mori et al., 2000; Normén et al., 2004). Furthermore, increased LDL particle size after fish oil may be attributable to its ability to reduce TG content of LDL particles, which in turn reduces lipase hydrolysis of LDL into smaller particles (Suzukawa et al., 1995).

Barbir et al. (1992b) investigated the impact of fish oil and hypolipidemic drugs on heart transplant recipients who had elevated levels of TC, TG, or both. After 3 months of treatment, MaxEPA (EPA + DHA, 10 g/d) reduced TG levels. Likewise, 3.4 g of n-3 FAs induced a significant decrease in TG levels and

tended to increase HDL-cholesterol levels in heart transplant recipients (Holm et al., 2001). Correspondingly, Yun et al., (1991a) assessed the dose response of fish oil on TCA using 10 Dutch-Belted-to-New Zealand White rabbits. The rabbits were pretreated with a high dose of fish oil (1.5 mL/kg/d) for 3 weeks before heart transplantation and were maintained on the same treatment for 6 weeks after operation. Their results demonstrated that fish oil showed a trend toward greater TCA, and lower levels of plasma TG, TC, VLDL and LDL-cholesterol. These seemingly contradictory outcomes could be attributed to interspecies differences; this would include differences in lipid metabolism and histopathologic aspects of TCA lesions as well as method assessment of graft vessel disease.

2.3.3.2 Fish Oil and Inflammation

Inflammation plays a significant role in the pathogenesis of atherosclerosis. n-3 FAs may represent a potential therapeutic agent for inflammatory and autoimmune diseases (Mori and Beilin, 2004). Chan et al., (2002) have reported a reduction in C-reactive protein (CRP) and IL-6 levels in obese individuals taking 4 g of EPA + DHA and atorvastatin (40 mg), a lipid-lowering drug, but not with fish oil supplements alone. Similarly, no reported reductions in CRP, IL-6 or TNF- α levels were observed among obese men taking 1.35 g EPA + DHA for 6 weeks (Jellema et al., 2004).

Administration of EPA and DHA reduced *ex-vivo* production of TNF- α and IL-6 following lipopolysaccharide (LPS) stimulation of monocytes and lymphocytes (Mori and beilin, 2004). In light of this, *in-vitro* studies have revealed

that DHA decreased expression of pro-inflammatory cytokines, cell-adhesion molecules, and monocyte adhesion to endothelial cells (De Caterina et al., 2000). In particular, DHA was stronger than EPA in inhibiting expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin. The effects of DHA on VCAM-I expression were accompanied by reductions in VCAM-I mRNA (De Caterina et al., 2000).

Daily supplements of 1.1 g of EPA and DHA, was found to decrease circulating concentration of soluble VCAM-I in elderly subjects (Miles et al., 2001). Additionally, n-3 FAs reduced monocyte synthesis of TNF- α and IL-1 β in healthy subjects (Caughey et al., 1996). On the contrary, neither EPA nor DHA given at (4 g/d) for 6 weeks significantly decreased IL-6 or CRP, but both FAs remarkably reduced TNF- α by 25% in type II diabetic individuals (Mori et al., 2003). Moreover, dietary docosahexaenoate supplement decreased exercise-induced inflammation by reducing CRP and IL-6 in healthy subjects (Phillips et al., 2003).

These anti-inflammatory effects of n-3 FAs may be direct, such as their impacts on transcription factors to influence gene expression or mediated through a shift to synthesize eicosanoids (Browning, 2003). The eicosanoid formed is particular to the fatty acid from which it is derived. For instance, EPA and DHA form PG of the 3 series and LT of the 5 series, which are less inflammatory precursors, Whereas, the n-6 fatty acid arachidonic acid (AA) form 2 series PG and 4 series LT, which are more inflammatory precursors (Browning, 2003). Therefore, a diet with a higher n-3 to n-6 FAs ratio diet has better

modulation in the overall inflammatory environment. In addition, n-3 FAs may reduce adhesion molecule levels by inhibiting the activation of nuclear factor-kB (NF-kB) system of transcription factors. Since the activation of NF-kB needs the presence of reactive oxygen species (ROS), a possible mechanism would entail decreased production of ROS by n-3 FAs (De Caterina et al., 2000).

2.3.3.3 Fish Oil and Blood Pressure

n-3 FAs may play a role in regulation of BP; this effect may be mediated through a shift in the balance between vasoconstrictive PG (diminishing production of vasoconstrictive TX and increasing vasodilatory prostacyclin). n-3 FAs lessened BP, particularly in hypertensive patients (Beilin and Mori, 2003). DHA had a greater dose-response effect than EPA on BP (Agren et al., 1996).

n-3 FAs caused significant reductions in systolic blood pressure (SBP) and diastolic blood pressure (DBP) in overweight subjects (Mori et al., 1999). These changes may be mediated by improvements in endothelial and smooth muscle function (Mori, 2006). n-3 FAs could have favorable effects against CsA-induced hypertension after heart transplantation (Ventura et al., 1993). In renal transplanted patients, a daily dose of 6 g of fish oil for 3 months significantly lowered mean arterial pressure (MAP) in relative to control group (Homan van der Heide et al., 1993). Likewise, n-3 FAs decreased SBP in heart transplant patients (Andreassen et al., 1997). This decline in SBP was inversely correlated with increases in serum concentrations of EPA and DHA.

2.3.3.4 Fish Oil and Heart Rates

n-3 FAs are suggested to decrease heart rates and susceptibility to fatal arrhythmias. In a crossover design, O'Keefe et al., (2006) recruited 18 men with a history of myocardial infarction to receive either placebo or n-3 FAs (585 mg of DHA and 225 mg of EPA) for a period of 4 months. Although BP, plasma lipids, and inflammatory markers remained comparable between the two groups, n-3 FAs significantly reduced high rates and accelerated return to normal heart rate after standing and exercising. The reductions in high rate are proposed to be results of an increase in vagal activity (O'Keefe et al., 2006). Animal and preliminary human studies have also shown that intravenous infusion of n-3 FAs results in an immediate improvement in vulnerability to dysrhythmias (Billman et al., 1999; Schrepf et al., 2004). Recently, Harris et al., (2006) have suggested that n-3 FAs may influence high rate independent of vagal input after investigating the impact of n-3 FAs supplementation on high rate in patients with denervated hearts after orthotopic heart transplantation.

2.3.3.5 Fish Oil and Nephrotoxicity

The changes in renal haemodynamics are considered an outcome of CsA-induced imbalance between the production of vasoconstrictors and vasodilators, as well as direct toxic effects to vascular endothelia, resulting in vasoconstriction of the afferent arterioles (Santos et al., 2000). n-3 FAs may increase TXA3 formation, coinciding with a fall in TXA2 and a significant increase in total prostacyclin levels (Santos et al., 2000). n-3 FAs may alter the production of

vasoactive eicosanoids by competing with AA; thus, it may reduce CsA-induced arteriolar vasoconstriction. Homan van der Heide et al., (1990) showed that supplementing the diets of 11 recipients of renal transplants with 6 g of fish oil for 3 months caused significant increases in GFR and decreases in MAP. Co-administration of CsA with EPA at dose of 600 mg/kg/d in hereditary hypertriglyceridemic rats prevented the elevation in the serum levels of creatinine and decreased the rise in the serum urea levels (Bohdanecká et al., 1999).

2.3.3.6 Fish Oil and Obesity

Dietary intake of n-3 FAs could be beneficial in body weight management. In a long-term intervention study among overweight patients with impaired glucose tolerance, the dietary n-3 FAs significantly turned down cumulative diabetes incidence (Woodman et al., 2003). Studies suggest that overweight people who follow a weight loss program including exercise tend to achieve better control over their blood glucose and cholesterol levels when they consume fatty fish (Woodman et al., 2003). Consuming n-3 FAs -enriched diet may help lower TG and raise HDL-cholesterol levels; therefore, people with diabetes may benefit from eating seafoods or taking DHA and EPA supplements (Morris et al., 1993).

2.3.3.7 Adverse Effect of Fish Oil Supplements

There have been some concerns about the fish contamination with heavy metals. Fish meat is more likely to contain some methyl-mercury contaminants

than fish oil. Despite the fact that there is no solid evidence indicating harmful effects of maternal fish oil supplementation, eating some types of fish (e.g., shark, swordfish, king mackerel, or tilefish) was reported to be cautioned in young children and pregnant or breastfeeding women because they contain high levels of mercury (Helland et al., 2001). An intake of 3 g/d or more of n-3 FAs might augment the risk of bleeding although there are no recorded cases of abnormal bleeding after fish oil supplementation.

Very large intakes of fish oil/ n-3 FAs ("Eskimo" amounts) may intensify the risk of hemorrhagic stroke (Harris, 2004). High doses of n-3 FAs may result in an increased lipid peroxide generation along with a reduction in the circulating levels of vitamin E (Harris, 2004). Many clinical trials have reported small attenuations in BP with n-3 FAs intake (Beilin and Mori, 2003); thus, caution is warranted in hypotensive patients or in those who are taking hypotensive drugs (Morris et al., 1993). Elevations in LDL-cholesterol by 5-10% are shown with intake of marine n-3 FAs; thereby, caution is warranted to those with high levels of LDL-cholesterol despite vagueness of the clinical relevance of this finding (Harris, 1997). Regular use of fish oil supplements is usually accompanied with nausea and fishy burps. In addition, diarrhea can occur particularly with extremely high doses (Glaum et al., 1990).

2.3.4 Flaxseed Oil

Flaxseed or linseed (*Linum usitatissimum*) is an ancient crop that dates back to around 9000-8000 B.C. (Hall et al., 2006). Flaxseed contains 45% of its

mass as oil of which 51–55% is ALA (18:3 n-3 FAs). Flaxseed is also a good quality source of dietary fiber and lignans (Vijaimohan et al., 2006). To a limited extent, the body converts ALA by enzymatic desaturation and elongation processes into EPA, which is a precursor of the series-3 PG, series-5 LT and series-3 TX. These eicosanoids have anti-inflammatory and anti-atherogenic properties. Incorporation of ALA and its metabolites in cell membranes can influence membrane fluidity and may play a role in anti-inflammatory activity, hypolipidemic effects and inhibition of platelet aggregation. In addition, flaxseed oil contains small amounts of lignan, secoisolariciresinol diglycoside (SDG) which is believed to be good antioxidants (James et al., 2000).

2.3.4.1 Historical View

Flaxseed cultivation dates back to 6000 BC when flax fiber was first recognized in Eastern Turkey to make cloth primarily linen (Judd, 1995) and Later on, the oil, known as linseed oil, was used as a drying agent in paint, and flaxseed meal was used as an ingredient in animal feed (Judd, 1995).

Flaxseed oil has been a coveted source of health for millennia. The attention on the potential benefits of flaxseed oil began in the 1950s when German biochemist, Johanna Budwig, recommended flaxseed oil as a treatment for her cancer patients (an article can be found at www.budwigflax.com; accessed January 09, 2008). These preliminary observations have been a great focus for animal and human studies, which greatly suggest beneficial effects of flaxseed oil on a variety of conditions, including hyperlipidemia, cardiovascular

thrombotic infarction, anxiety and constipation. In addition, its lignan content may play a role in cancer prevention (Judd, 1995).

2.3.4.2 Chemistry and Dietary Sources

ALA is composed of carboxylic acid with an 18-carbon chain and three *cis* double bonds; the first double bond is located at the third carbon from the omega end (Harris, 2004). The chemical structure of ALA is depicted in **Figure 2**. ALA is found in various oils, namely flax oil 8.5 g/tbsp, flaxseeds 2.2 g/tbsp, canola (rapeseed) oil 1.3 g/tbsp, soybean oil 0.9 g/tbsp, and walnut oil 0.7 g/tbsp (Roche and Gibney, 2000). Flaxseed meal or flaxseed oil can easily be incorporated into common dietary items such as breads, muffins, margarines, and salad dressings. The FDA does commonly regard ALA as safe for public consumption in doses up to 3 g/d (Harper et al., 2006).

2.3.4.3 Conversion of Alpha-linolenic Acid to Eicosapentaenoic Acid and Docosahexaenoic Acid

There has been a matter of debate about the efficiency of conversion of ALA to EPA and DHA in humans (Gerster, 1998; De Deckere et al., 1998). The evidence available indicates that conversion of ALA to EPA takes place in most people, but that mechanism is still slow and less efficient than the direct incorporation of dietary EPA and DHA into tissues (Li et al., 1999). One of the main reasons is that ALA is so scarcely converted to longer-chain EPA and DHA because it is mostly used for energy, while EPA and DHA are not (Harris, 2004).

However, it is important to mention that about 46% of radioactivity associated with an ALA dose was found 48 hrs later in skin and fur lipids of guinea pigs, whereas lesser than 0.1% was found in brain (Fu and Sinclair, 2000). This finding show that a considerable amount of ALA is transported to the skin; however more research has to be conducted before establishing this observation in humans.

ALA supplementation significantly increases ALA and EPA levels in plasma and cells; however, it does not increase DHA (Roche, 1999). The extent of incorporation of n-3 FAs from the diet can fluctuate depending on the total fat and types of FAs consumed. High dietary content of linoleic acid (LA) may impede the incorporation of n-3 FAs into tissue pools and slow down the conversion rate of ALA to EPA and DHA (reviewed in Sinclair et al., 2000). Therefore, from a practical point of view, the way to increase the n-3 FAs content of tissue is to decrease dietary LA at the same time as increase n-3 FAs content (Cleland et al., 1992). Furthermore, evidence from recent trials suggests that consumption of marine n-3 FAs may decrease the conversion of ALA to EPA and DHA because of down-regulation of desaturase enzymes (Sanderson et al., 2002). Recently, it is roughly estimated that the degree of ALA conversion to EPA and DHA is 5–10% and 2–5%, respectively (Davis and Kris-Etherton, 2003). This conversion may also vary by gender. In light of this, Bakewell et al., (2006) showed that conversion of ALA to DHA was higher in women but not men; this may be due to the regulating effects of oestrogen.

2.3.4.4 Physiological Effects of Flaxseed Oil

2.3.4.4.1 Flaxseed Oil and Dyslipidemia

A number of studies have examined the impact of ALA on plasma lipids. A high ALA flaxseed diet was associated with reduced levels of TG (Craig, 1999), TC (Cunnane et al., 1993), LDL-cholesterol, and LDL/HDL-cholesterol in humans (Caughey et al., 1996). In animal studies, in rats fed a high fat diet, ALA supplementation significantly lowered the increased levels of plasma TC, TG, LDL-cholesterol, LDL/HDL-cholesterol, and TC/HDL-cholesterol (Vijaimohan et al., 2006). Similarly, feeding rats a diet supplemented with 10% perilla oil, a source of ALA, for 4 weeks showed significant decreases in post-prandial plasma TC and TG levels (Wiesenfeld et al., 2003). Yet, no reported alterations in serum lipids were observed in rabbits fed a diet containing 5 % flaxseed oil (Lee and Prasad, 2003).

Favorable effects of ALA on plasma lipid and lipoproteins seem to be still more controversial. In fact, human studies in this area have reported many conflicting results. As ALA intake at 0.81 and 0.69 g/d in men and women significantly reduced plasma TG levels, respectively (Djoussé et al., 2003), no significant changes in plasma lipids of normolipidaemic men after dietary replacement of LA (15g) with ALA (15g) were reported (Pang et al., 1998). The data suggests that flaxseed oil or flaxseed may reduce hepatic lipids due to the conversion of ALA to EPA and possibly DHA or to its lignan content.

2.3.4.4.2 Flaxseed Oil and Inflammatory Markers

There is preliminary clinical evidence suggesting that flaxseed oil can decrease inflammation. Caughey et al., (1996) showed that 14 g of ALA from a diet containing flaxseed oil reduced *in vitro* synthesis of TNF- α and IL-1 β after LPS stimulation in healthy subjects. ALA did not affect cytokine production in healthy adults (Wallace et al., 2003). Failure of ALA to reduce the levels of inflammatory cytokines may be attributed to its limited conversion to EPA and DHA.

2.3.4.4.3 Flaxseed Oil and Blood Pressure

The evidence for the hypotensive effect of flaxseed or its components is somewhat unconvincing. A human observational study has shown that dietary and tissue ALA were correlated with lowered BP (Bemelmans et al., 2000). Despite limited animal evidence, no human clinical trials have reported the hypotensive effect of flaxseed or its components (Singer et al., 1984 reviewed in Bloedon and Szapary, 2004). Consumption of 9.2 g of ALA from flaxseed oil to 3.4 g of EPA and DHA from fish oil for 6 weeks, only the fish oil supplemented diet decreased SBP by 5 mm Hg (Kestin et al., 1990).

2.3.4.4.4 Flaxseed Oil and Obesity

Human studies on the effect of flaxseed oil on body weight reported mixed results. In human, ALA containing diet along with calorie-restricted diets showed a significant reduction in body fat after 16 weeks (Takei et al., 2001). In mice,

ALA administration showed a reduction in body weight and visceral fat (Meyer et al., 2001; Hase et al., 2001). These studies suggested that the reduction in visceral fat after ALA treatment could be a result of reduced triacylglycerol synthesis and increased fatty acids oxidation.

2.3.4.4.5 Adverse Effect of Flaxseed-Containing Products

There is long-standing historical use of flaxseed products without many reports of side effects. Overall, flaxseed and flaxseed oil supplements appear to be well tolerated in the available research. Human studies using up to 50 g/d of flaxseed for up to one month revealed no adverse effects, however, an increase in bowel movements was reported (Cunnane et al., 1995). In addition, flaxseed hypersensitivity may result in anaphylactic episodes (Gall, 2000).

There is little information in humans and animal studies regarding its use in pregnancy (Tou et al., 1998). In addition, it should be cautiously used in women with hormone sensitive conditions because of the possible estrogen-like effects of flaxseed “lignans”. Consumption of raw flaxseed or flaxseed plant may enhance blood levels of cyanide; however, the data thus far suggest that high doses of baked flaxseed powder do not increase urinary thiocyanate levels (Cunnane et al., 1993 reviewed in Bloedon and Szapary, 2004).

3.0 RATIONALE FOR THE STUDY

Heart transplantation is one of the most common strategies of life saving in patients with end stage heart failure. In order to prevent graft rejection, the recipients must be treated with immunosuppressive drugs like CsA. However, CsA-induced side effects are still the major limiting factors. CsA increases blood lipid levels, BP and kidney injuries, which altogether could jeopardize the life of the recipients. n-3 FAs have been shown to beneficially modify plasma lipids, blood pressure and immune function (Bemelmans et al., 2000; Bloedon and Szapary, 2004).

Fish oil contains n-3 FAs EPA and DHA and flaxseed oil is a rich source of n-3 fatty acid ALA. Thus, the benefits of n-3 fatty acids seem to be applicable to post-transplant complications.

4.0 STUDY HYPOTHESES AND OBJECTIVES

4.1 HYPOTHESIS

Simultaneous administration of dietary n-3 FAs with cyclosporine will result in attenuation of post-transplant complications, including cyclosporine-induced hyperlipidemia, in a rat cardiac allograft model. The source of dietary FAs is not a major determinant.

4.2 RESEARCH OBJECTIVES

The objectives of this study include:

1. To test whether addition of n-3 FAs reduce post-transplant hyperlipidemia.
2. To test whether n-3 FAs attenuate elevations in BP and improves kidney function after CsA treatment.
3. To investigate whether n-3 FAs prolongs graft function and prevents TCA in rats.
4. To compare the biological effects of fish oil and flaxseed oil on post-heart transplant complications.

5.0 MATERIALS AND METHODS

5.1 EXPERIMENTAL ANIMALS

A reproducible model of cardiac transplant rejection has been developed in rats and widely used to mimic human disease. Heterotopic heart transplant models have proven to be a valuable resource to study chronic rejection since the early 1970's (Corry et al., 1973; Judd and Trentin, 1973; Han et al., 2000).

Abdominal heart transplantation model is performed by the insertion a donor heart into the abdomen of the recipient. The anastomosis is performed between the aorta of the donor and the abdominal aorta of the recipient rat and between pulmonary artery of the donor heart and the inferior vena cava of the recipient rat (Alexis et al., 2003). This allograft model, linked with the use of minor and major histocompatibility-mismatched rat strains, has been used to study the pathogenesis and therapy for TCA (Ono and Lindsey, 1969; Suzuki et al., 2002).

To study chronic allograft rejection, models have been developed that cross only minor histocompatibility barriers. Lewis and F344 rats differ slightly in their major histocompatibility class II antigens, and have been used as common transplant models (Günther and Walter, 2001).

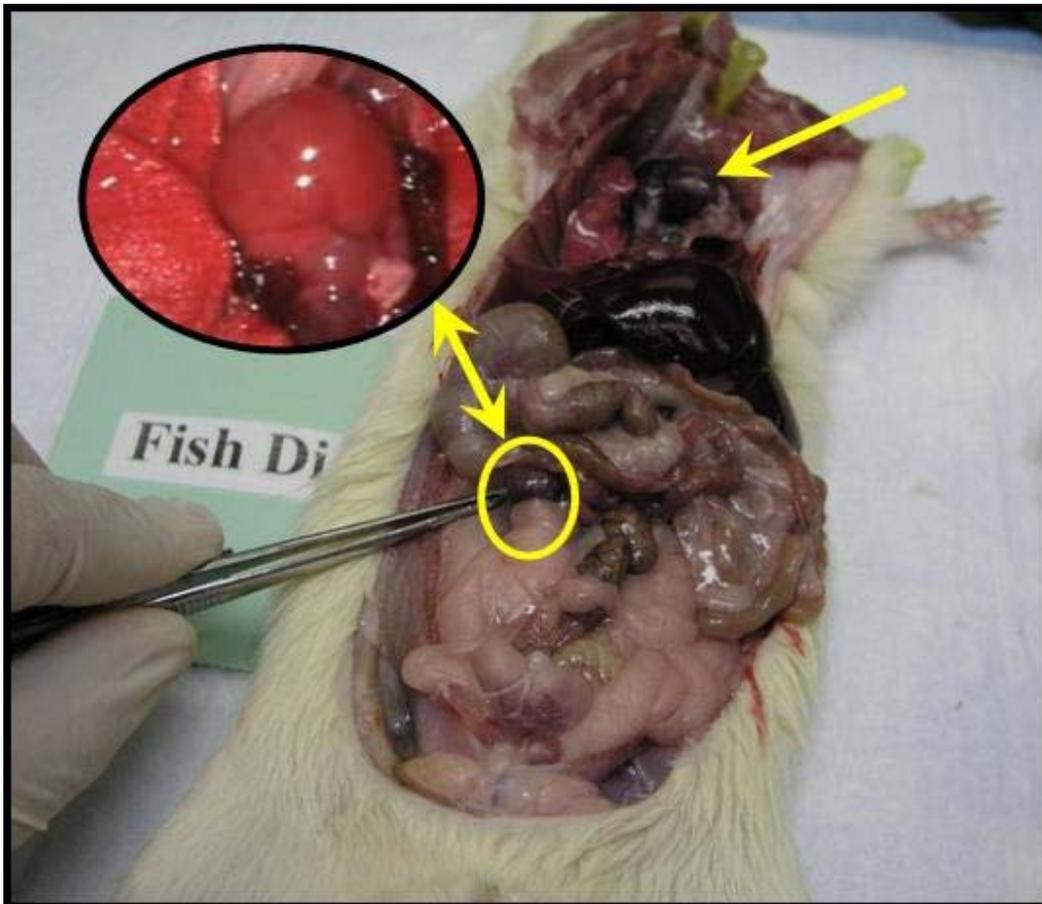
In the current study, forty-four, five week old Fischer-344, and Lewis rats – with a body weight (150 to 200 g) – were purchased from Charles River Laboratories (Montreal, PQ, Canada). Rats were housed in stainless steel cages at an ambient temperature of 22-24°C and a 12:12-h light-dark cycle in an environmentally monitored room. For 7 days of acclimation period, all rats had an

ad libitum access to chow and water at the Animal Facility of St. Boniface Research Centre (Winnipeg, MB, Canada). Thereafter, standard abdominal heterotopic cardiac transplantation procedures were performed to generate 22 allografts using Lewis rats as recipients and Fischer-344 rats as donors. Lewis rats have strong cellular immune response and associated polarized production of T-^{helper1} cytokines including IL-2, IF- γ (Groen et al., 1993).

5.2 HETEROTOPIC HEART TRANSPLANTATION PROCEDURES

As previously described (Ono and Lindsey, 1969), standard heterotopic abdominal heart transplant procedures were performed between Fischer (donors) and Lewis (recipients). Anesthesia was induced with 5% isoflurane and maintained on isoflurane by mask at 2%. The recipients' abdominal aorta and inferior vena cava were located and clamped and implantation was conducted by anastomosis the donor's aorta and pulmonary artery to the recipient's abdominal aorta and inferior vena cava, respectively, in an end-to-side manner. A representative photograph of the native heart and graft is shown in **Figure 4**.

Figure 4. Representative photograph of a rat at sacrifice shows native heart (arrow) and graft (double-headed arrow).



5.3 EXPERIMENTAL DESIGN AND DIETS

The experimental design of the study is summarized in **Figure 5**. After complete recovery of surgery, the rats were randomly assigned into three treatment groups and fed PicoLab rat diet (Ren's feed and supply Ltd) supplemented with: a) 5% (wt/wt) safflower oil (control group, n=7); b) 5% (wt/wt) flaxseed oil (flaxseed oil treated group, n=8); and c) 2% (wt/wt) fish oil (fish oil treated group, n=8). The diets composition are indicated in the **Table 1**; the diets were prepared with the oils were added to a regular rat chow, water was then added to produce dough and pellets were made. Thereafter, they were put in an oven to dry at approximately 40 to 45 °C overnight. To prevent oxidation, all diets were stored at 4°C until use. Body weight of the animals was recorded weekly.

5.4 DIETARY OILS AND MEDICATIONS

Fish and flaxseed oils were used in the fish and flaxseed diets, respectively. Safflower oil, on the other hand has a low proportion of n-3 FAs, was used in the control diet. Flaxseed and safflower oils were supplied from DYETS Inc; Bethlehem, PA, USA while fish oil (EPAX 5500 TG) was a generous gift from Pronova Biocare, Sandefjord, Norway. CsA (Neoral®, Novartis) was purchased from the pharmacy at St. Boniface General Hospital, Winnipeg, Manitoba, Canada and intraperitoneally administered at 1.5 mg/kg/d.

Figure 5. Experimental Design. i.p.; intraperitoneal; CsA;cyclosporine.

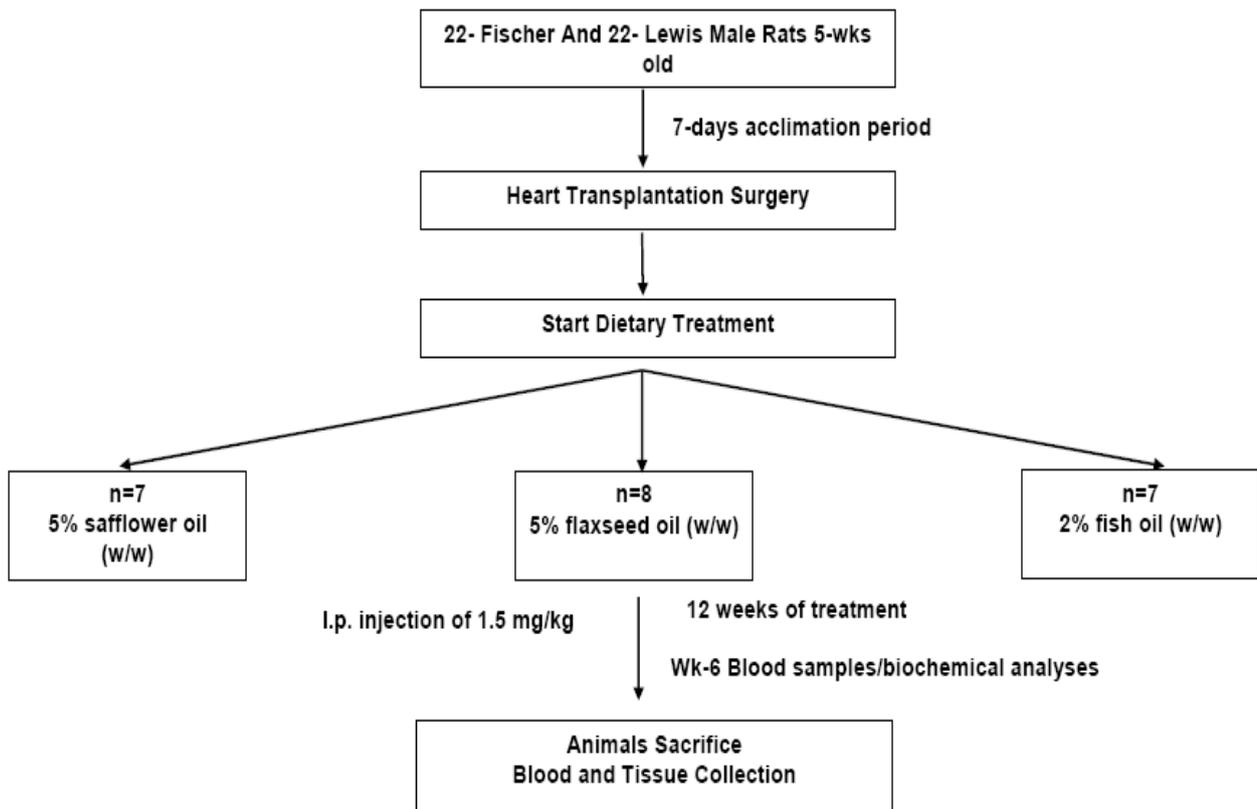


Table 1. Nutrient contents of the experimental diets per 100g.*

Macronutrient (grams)	Control diet	Flax diet	Fish Diet
Protein	19.5	19.5	20.1
Carbohydrate	50.35	50.35	51.94
Fat	13.55	13.55	10.82
Cholesterol (mg/Kg)	285	285	285
Ash and vitamin	4.56	4.56	4.70
Fibre	2.57	2.57	2.64
Moisture	9.5	9.5	9.8
Total	100.00	100.00	100.00

**Chaw diet was mixed with either safflower oil at 5%(w/w) as a control diet, flaxseed oil at 5% (w/w) as a flaxseed diet or fish oil at 2% (w/w) as a fish diet.*

5.5 DATA COLLECTION

5.5.1 Blood collection

The night before blood sampling, food was removed from animals' cages at 8:30 PM, and blood was drawn the following morning starting from 8:30 AM. Blood samples at wk 6 were taken from jugular vein (under light anesthesia induced by 1-2% isoflurane using heparinized syringes, and centrifuged at 4°C (5000 rpm for 10 minutes) using an Eppendorf Centrifuge. Aliquots of the plasma were used for analyses. At wk 12 (the end of the study), each rat was injected with 100 i.u of heparin intraperitoneally 10 minutes before sacrifice. Thereafter, the rats were euthanized using CO₂, final blood samples were collected through cardiac puncture using heparinized syringes, and centrifuged at 4°C (5000 rpm for 10 minutes).

5.5.2 Lipids Analysis

Plasma TC and TG levels at week 6 were quantified using a standard enzymatic kit (Diagnostic Chemicals Limited, Charlottetown, Canada) and a microplate reader (Moghadasian et al., 1997). All experimental samples were analyzed in duplicate and mean values were calculated. Plasma lipoprotein fractions in final blood samples were quantified by high performance liquid chromatography method (HPLC) as previously described (Usui et al., 2002; Okazaki et al., 2005). In brief, 5 µL whole plasma sample was injected into 2 connected columns (300X7.8 mm) of TSKgel LipopropakXL (Tosoh) and extracted by TSKeluent Lp-1 (Tosoh). The effluent from the columns was continuously monitored at 550 nm after an online enzymatic reaction with a

commercial kit. The cholesterol concentration in major lipoproteins and their subclasses was calculated by a computer program, which was intended to process complex chromatograms with the modified Gaussian curve fitting for determining the overlapping peaks by mathematical treatment.

The number, position, and width of each Gaussian component peak were determined for subclass analysis to perform a sufficient curve fitting analysis of various samples under the constant condition in which the peak width and position of each Gaussian curve remained unchanged. The conversion of elution time to particle diameter was carried out using a column calibration curve. The none-HDL-cholesterol was calculated by subtraction of HDL-cholesterol levels from TC levels. Similarly, TC/HDL and LDL/HDL ratio was calculated.

5.5.3 Blood Pressure and Heart Rates

BP was measured at wk 6 and at the end of the study using the tail-cuff method in conscious state as previously reported (Bunag, 1973; Donnelly et al., 1995). To minimize the effects of stress and its impact on BP, the rats were accustomed to training sessions every day for 3 to 4 days before BP measurement. These procedures were performed in a quiet, dark place and at the same time of day in order to avoid the influence of the circadian rhythm. The following instruments were used during the measurement: 1) a tail cuff, 2) an inflation device and pressure readout, 3) a pulse sensor (highly sensitive pressure transducer which discerns pulsations in a small rubber bulb; the bulb is taped to tail distal to the cuff), 4) a readout for the pulse sensor, MP100WS

Workstation; which includes acquisition hardware, AcqKnowledge software, cables, and manuals, 5) one amplifier for the pulse transducer and one amplifier to record pressure and 6) a low-cost disposable pressure transducer.

BP was measured in rat tails non-invasively in a way similar to that used to measure BP in humans. A cuff was put around the tail, and inflated above the systolic pressure, which then results in pulsations at a more distal pulse sensor to halt. When the cuff was gradually deflated, the reappearance of pulsations was noticed, and the cuff pressure at this time was recorded as the SBP (mmHg) in the tail. DBP was detected by noting the occlusion cuff pressure immediately after the signal detected by the sensing cuff begins to diminish. To determine the point, an average signal level was calculated and the envelop slop was extended. The point of intersection was then extended upward to the occlusion cuff pressure tracing to determine DBP. Mean arterial pressure (MAP) was calculated from the following formula: $MAP = DBP + 1/3 (SBP - DBP)$. Theoretically, MAP is the effective BP that directs blood to peripheral regions. Heart rate is defined as a number of heartbeats per minute (BPM). To determine BPM, the interval of each pulse was calculated and the average of the intervals was taken.

5.5.4 Cytokines

At wk 6, fasting blood samples were collected from the jugular veins of lightly anesthetized animals, centrifuged and plasma was obtained. For this analysis, 2 to 3 plasma samples of each experimental group were pooled to

generate 3 samples for each experimental group, and assayed with the RayBio™ Rat Cytokine Array system I & 1.1 map (RayBiotech Inc., Norcross, GA, USA).

Rat cytokine array membranes coated with 19 specific cytokine antibodies were probed with isolated protein samples. The membranes were blocked by incubation with the blocking buffer at room temperature for 30 minutes and incubated with sample at room temperature for 1 hour. Membranes were washed three times with Wash Buffer I and two times with Wash Buffer II at room temperature for 5 minutes per wash and incubated with biotin-conjugated antibodies at 4 °C overnight. Finally, the membranes were washed, incubated with HRP-conjugated streptavidin at room temperature for 1 hour and with detection buffer for 1 minute, and exposed to X-ray film for 40 seconds. The exposed films were digitized and the relative cytokine levels were compared after densitometry analysis. The RayBio™ cytokine array identified profiles of multiple cytokines in plasma samples as previously reported (Watanabe et al., 2005). Cytokine concentrations were expressed in optical density.

5.5.5 Whole Blood Cyclosporine

At week 11, blood samples (1mL) were collected in a non-fasting state, between 9:00–10:30 AM after the last CsA dose. The collected samples were sent to the Winnipeg Health Science Centre for determination of blood CsA levels using standard clinical laboratory methods.

5.5.6 Creatinine and Urea

At the end of the study, plasma samples were sent to the clinical lab at St. Boniface General Hospital for measuring creatinine and urea levels using standard methods.

5.5.7 Graft Function

Graft function was assessed by an independent individual weekly by palpation and rated on a scale of 0 to 4, with 4 indicating vigorous normal beats and 0 denoting absence of beats (Grimm et al., 1998). In addition, echocardiography techniques were also performed using a 13-MHz probe (Vivid 7, GE Medical Systems) to assess the graft functionality.

5.5.8 Histological Examination

At the time of sacrifice, both native hearts and graft tissues as well as the kidneys were collected and placed in fresh formalin for 48 hours before embedding in paraffin for sectioning. Serial sections for native heart and graft were cut and stained with hematoxylin and eosin (H&E) and Masson's trichrome for histological and morphometrical examinations (Moghadasian et al., 1999). The status of graft rejection was scored using the Billingham rejection grading system, including 0 (no rejection) to 3 (severe rejection) (Stewart et al., 2005). Similarly, cross sections of the kidneys were stained with H&E, Masson's trichrome and PAS for evaluation of evidence for chronic cyclosporine-induced

nephrotoxicity (alterations in arteriols, glomeruli and tubulointerstitium) as described previously (Sis et al. 2006).

5.5.9 Tissue Lipid Extraction and Fatty Acid Analysis

Lipid extraction of native heart, graft, and liver tissues was carried out according to Folch et al., (1957). Total tissue weight, lipid weight, and percentage of lipid of total tissue weight were calculated. The tissue was homogenized with 0.05% calcium chloride; chloroform: methanol (2:1 v/v) was added and vortexed to extract the lipid. The solvent (lower) phase was removed and dried down to obtain total lipid weight, which was then resuspended in chloroform/methanol (2:1 v/v). Thin-layer chromatography (TLC) with G-silica gel was used to separate neutral lipids: phospholipids, cholesterol esters, free fatty acids, and TG. The developing solvent system was composed of petroleum ether, diethyl ether, and acetic acid (80:20:1 v/v/v).

After allowing the plate to run to completion, visualizing with 0.01 % (wt/vol) aniline-naphthalene-sulfonic acid (ANSA) in water, and then quantifying by using the Fluorchem FC digital imaging system (Alpha Innotech San Leandro, CA, USA), the respective neutral and phospholipid bands were scraped for each sample. The TLC methods used were according to Suh et al., (1994). Each individual lipid class was saponified (only cholesteryl ester, TG) and then methylated with BF₃. The fatty acid methyl esters were dried down under nitrogen gas and resuspended with hexane to prepare samples for fatty acid analysis with gas chromatography (GC). At the end, the proportion of TC and TG

of the tissues was analyzed with standard enzymatic assays and quantified spectrometrically.

5.6 ETHICS

Animal care and all experimental procedures of this study received ethic approval from the Animal Care Committee on the use of Animals in Research, at the University of Manitoba, Winnipeg, MB, Canada.

5.7 SAMPLE SIZE AND STATISTICAL ANALYSIS

Data were analyzed using SPSS 11.5 statistical software for Windows (SPSS Inc., Chicago, IL, USA). Results were expressed as mean \pm one standard deviation (SD). The level of statistical significance was set at $p < 0.05$. One-way analysis of variance (ANOVA) was selected to determine differences in outcome measurements between dietary treatment groups. Post-hoc analysis was performed using the *Tukey* multiple group comparison procedure to determine significant differences among various experimental groups. For heart lipids, two - way ANOVA was used to identify the main effect of graft and diet. For liver phospholipids, *t*-test was used to detect differences between fish and flax groups. *Chi*-square test was used to determine the effect of dietary treatments on the distribution of rejection grades. For the sample size, we estimated 7 to 8 rats per group to provide sufficient statistical power of $>85\%$ for detecting difference of $\frac{1}{2}$ SD as significant at the level of $p < 0.05$.

6.0 RESULTS

6.1 BODY WEIGHT

Final body weights of rats were not significantly different among the experimental groups ($P=0.858$). **Figure 6** demonstrates that rats in all the experimental groups gained weight during the 12-wk study. The extent of mean weight gain was comparable among all of the experimental groups, which may have indicated that the diets were well-tolerated and animals were healthy during the course study.

6.2 TISSUE WEIGHTS

Table 2 exhibits tissue weights in all of the 3 experimental groups. At the end of study, all the tissues including heart, graft, liver, abdominal fat, right -and left -kidney and spleen were harvested and weights were presented in grams. Interestingly, the weight of abdominal fat was significantly different between the groups ($P=0.003$). Flaxseed and fish oil-treated rats had significant decreases (-21% and -31%; $P=0.03$, and $P=0.002$ respectively) in the weight of abdominal fat in relative to their counterparts in the control group despite their comparable body weights.

Figure 6. Mean body weights at baseline and 3-wk intervals. All the experimental groups showed a consistent weight gain.

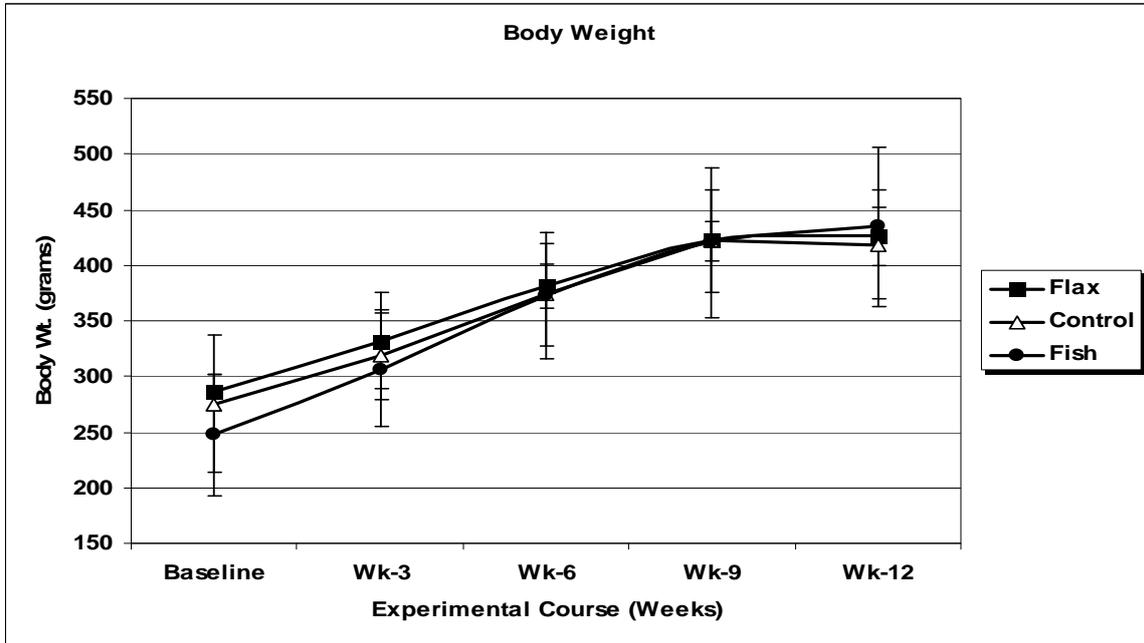


Table 2. Tissue weights in grams. Data are mean \pm SD; L, left; R, right.

Groups	Control	Flax	Fish
Heart	1.4 \pm 0.2	1.2 \pm 0.1	1.4 \pm 0.2
Graft	0.4 \pm 0.1	0.6 \pm 0.3	0.4 \pm 0.2
Liver	11.6 \pm 2.3	13. 1.6	12.9 \pm 1.2
Abdominal Fat	17.1 \pm 2.4 ^a	14.2 \pm 3.7 ^b	12.3 \pm 2.7 ^b
R-Kidney	1.5 \pm 0.2	1.5 \pm 0.1	1.5 \pm 0.1
L-Kidney	1.4 \pm 0.2	1.5 \pm 0.1	1.5 \pm 0.1
Spleen	0.7 \pm 0.1	0.7 \pm 0.1	0.7 \pm 0.1

Values with different superscript letter in a row are significantly different, P<0.05.

6.3 PLASMA LIPIDS CONCENTRATIONS

Data on plasma lipids are shown in **Table 3**. Fish group showed significant ($P<0.05$) reductions (-35% and -45%) in plasma TG concentrations at wk 6 as compared to the control and flax groups, respectively. Similarly, at wk 6, there was a significant ($P<0.001$) difference in TC levels among groups. TC levels were significantly decreased in the fish-treated group as compared to the flax and control groups (-19% and -23%; $P<0.01$ and $P<0.001$, respectively). At wk 12, TC plasma concentrations remained significantly lower in the fish group relative to the flax and control groups (-18% and -15%; $P<0.001$; $P<0.01$, respectively).

The clinical importance of the cholesterol levels within lipoprotein subclasses has not been fully established; nonetheless, it is been reported that increased cholesterol levels in small LDL particles might predict the risk of coronary heart disease in the healthy populations (Arsenault et al., 2007). In the current study, plasma LDL-cholesterol levels were significantly ($P<0.001$) different among groups at wk 12. Significant reductions in plasma LDL-cholesterol levels were observed in fish-treated group relative to flaxseed oil-treated and control groups (-25% and -34%; $P=0.02$ and $P=0.001$, respectively). While large, medium, and small LDL subclasses cholesterol levels remained comparable among the experimental groups, fish group had significantly reduced very small LDL-cholesterol levels as compared to the flaxseed and control groups ($P=0.001$ and $P<0.001$, respectively).

Table 3. Plasma lipids concentrations.

Measurement	Control n=7	Flax n=8	Fish n=7
TC (mg/dL)			
wk6	64.7±6.9 ^a	61.5±4.3 ^a	49.8±3.7 ^b
Wk12	65.6±4.1 ^a	68.8±4.6 ^a	56.1±2.3 ^b
TG(mg/dL) wk 6	70.4± 2 ^a	84.2±22.5 ^a	45.9±11.9 ^b
VLDL-C (mg/dL)			
Large VLDL	3.4±1.9	3.0±2.5	2.0±1.7
Medium VLDL	0.2±0.1	0.3±0.4	0.1±0.1
Small VLDL	1.3±0.8	1.1±0.9	0.8±0.8
Very Small VLDL	1.5±0.8	1.1±0.7	1.0±0.7
LDL-C (mg/dL)			
Large LDL	16.5±2.4 ^a	14.5±1.6 ^a	10.9±1.7 ^b
Medium LDL	2.3±1.0	1.8±0.7	1.52±0.7
Small LDL	2.8±0.9	2.4±0.5	2.00±0.6
Very Small LDL	2.3±0.8	2.4±0.4	1.87±0.3
HDL-C (mg/dL)			
Very Large HDL	9.0±1.4 ^a	7.9 ±1.2 ^a	5.46±0.3 ^b
Large HDL	46.2±5.0 ^{ab}	51.2±5.0 ^a	43.2±1.8 ^b
Medium HDL	12.6±2.3	11.7±4.0	8.7±1.0
Small HDL	17.4±2.1 ^a	20.1±2.0 ^b	17.4±0.8 ^{ac}
Very Small HDL	10.1±1.2 ^a	12. 2±2.0 ^b	10.8±1.0 ^{ab}
N-HDL-C (mg/dL)	3.5±0.4 ^a	4.3±0.7 ^b	3.7±0.3 ^{ab}
TC/HDL-C ratio	2.6±0.3 ^a	3.0±0.4 ^b	2.6±0.2 ^{ab}
LDL-C/HDL-C ratio	19.9±3.8 ^a	17.6±3.2 ^{ab}	12.9±3.1 ^b
LDL-C/HDL-C ratio	1.4±0.1	1.4±0.1	1.3±0.1
LDL-C/HDL-C ratio	0.4±0.1 ^a	0.3±0.0 ^b	0.3±0.1 ^b

Data are mean ± SD; TC, total cholesterol; TG, triglyceride; VLDL-C, very low density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; N-HDL-C, non-high density lipoprotein cholesterol. Values with different superscript letter in a row are significantly different, P<0.05.

In addition, there was a tendency for VLDL subclasses cholesterol levels to be reduced in the fish group but not significantly different compared to the flax and control groups. Flaxseed oil-treated rats had a significant increase in HDL-cholesterol levels (+19%; $P=0.009$) as compared to the fish-treated animals but was not significantly different from the control. Moreover, flaxseed oil-treated rats were associated with significant ($P<0.05$) increases in large HDL-cholesterol as compared to the fish and control groups, and in medium, small and very small HDL-cholesterol relative to control. Non-HDL-cholesterol, on the other hand, was significantly lower in fish group (-35%; $P=0.005$) as compared to control group. LDL/HDL-cholesterol ratios were significantly reduced in the fish group (-31%; $P=0.005$) as compared to those in the control group. Similarly, flax group had a significant decrease (-22%; $P<0.05$) in LDL/HDL-cholesterol ratio relative to the control; this may be due to a significant increase (+19%) in HDL-cholesterol levels in the flaxseed group.

6.4 LIPOPROTEIN PARTICLE SIZE

After 12 weeks of dietary treatments, VLDL, LDL, and HDL particle size remained comparable among the groups. Data on lipoprotein particle size is shown in **Table 4**.

6.5 BLOOD PRESSURE AND HEART RATES

Data on blood pressure and heart rates are shown **Table 5**. Dietary supplementation with either flaxseed oil or fish oil caused significant reductions in

Table 4. Lipoprotein particle size calculated by cholesterol and triglyceride plots (nm).

Groups	Particle size calculated by Cho Plot (nm)			Particle size calculated by TG Plot (nm)		
	Control n=7	Flax n=8	Fish n=7	Control n=7	Flax n=8	Fish n=7
VLDL (30-80nm)	36.1±0.6	36.7±2.1	35.4±0.4	36.8±0.9	38.1±2.4	36.1±0.7
LDL (16-30nm)	21.2±0.8	21.5±0.7	21.7±0.6	24.9±0.4	24.3±0.6	24.8±0.3
HDL (8-16nm)	12.0±0.1	11.9±0.3	11.8±0.1	10.5±0.4	10.7±0.9	10.4±0.4

Data are mean ± SD; Cho, cholesterol; TG, triglyceride; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

Table 5. Blood pressure and heart rates.

Measurement	Control n=7	Flax n=8	Fish n=7
SBP (mmgH)			
wk6	150±19 ^a	122± 9 ^b	109±15 ^b
Wk12	149±16 ^a	118 ±7 ^b	118 ± 7 ^b
SDP (mmgH)			
wk6	114±3 ^a	89±7 ^b	87± 8 ^b
wk12	101±7 ^a	87±3 ^b	82± 7 ^b
MAP(mmgH)			
wk6	126±7 ^a	100±6 ^b	92±10 ^b
wk12	117±9 ^a	97±3 ^b	94± 6 ^b
Heart rate (BPM)			
wk6	416±19	403±45	373±38
wk12	478±57 ^a	384±20 ^b	394±33 ^b

Data are mean ± SD; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial blood pressure; BPM, beat per minute. Values with different superscript letter in a row are significantly different, P<0.05.

BP. At wk 6, both flaxseed and fish oil-treated rats exhibited significant reductions (-19% and -27%) in SBP as compared to control (122 ± 9 and 109 ± 15 vs. 150 ± 19 mmHg; $P=0.004$ and $P <0.001$ respectively). These decreases in SBP remained equally significant (-21%; $P<0.001$) in both groups as compared to the control at the end of the study. Likewise, at wk 6 significant attenuations (-22% and -26%) in the DBP were observed in the flaxseed and fish-treated animals as compared to those of the control group (89 ± 7 and 84 ± 9 vs. 114 ± 3 mmHg; $P<0.001$, respectively) and remained significantly lower until the end of the study. At wk 6 and wk 12, MAP, which was calculated from the following formula: $MAP = DBP + 1/3(SBP - DBP)$, significantly ($P<0.001$) reduced in both flaxseed and fish oil-treated groups as compared to the control. These changes were accompanied by similar significant ($P<0.01$) reductions in heart rates in both treated groups at wk 12 as compared to control.

6.6 PLASMA CYTOKINES PROFILE

Plasma cytokines were analyzed in pooled samples (three samples each group) and expressed in optical density data by RayBio™ Rat Cytokine Array at wk 6 of the study as previously described (Watanabe et al., 2005). Of interest was the significant ($P<0.05$) decrease in MCP-1 levels in the flaxseed- and fish-treated groups as compared to control group. The levels of pro-inflammatory cytokines (IL1- β , IL1- α , IL-6 and TNF- α) or anti-inflammatory cytokines (IL-4, IL-10, and IFN- γ) remained comparable and no significant differences were found among all the groups. Plasma cytokine levels are summarized in **Table 6**.

Table 6. Optical Density data of plasma cytokine profiles (with background subtraction) in flaxseed, fish and control groups after 6 weeks of study.

Plasma Cytokines	Control	Flax	Fish
IL1-α	4112 \pm 221	4127 \pm 596	4153 \pm 225
IL-1β	11271 \pm 2283	8906 \pm 608	9752 \pm 358
IL-4	3768 \pm 264	3519 \pm 726	3734 \pm 537
IL-6	5750 \pm 1017	5880 \pm 525	6082 \pm 381
IL-10	5437 \pm 759	4823 \pm 18328	5318 \pm 424
Leptin	4467 \pm 725	3781 \pm 542	3975 \pm 469
MCP-1	14770 \pm 597 ^a	12499 \pm 583 ^b	12489 \pm 1092 ^b
TNF-α	9060 \pm 1646	10103 \pm 596	8919 \pm 855
IFN-γ	7325 \pm 314	7678 \pm 1335	6903 \pm 496

Data are mean \pm SD; IL1- α , interleukin 1 alpha; IL1- β interleukin 1 beta; MCP-1, macrophage chemotactic protein-1; TNF- α , tumor necrosis factor alpha; IFN- γ interferon gamma. Values with different superscript letter in a row are significantly different, $P < 0.05$.

6.7 WHOLE BLOOD CYCLOSPORINE LEVELS

Whole blood CsA levels are shown in **Figure 7**. CsA was intraperitoneally administered at (1.5 mg/kg body wt/d) for 12 wk. The whole blood CsA concentrations were measured at wk 11 of the study. There were no statistically significant differences in the blood CsA levels among all the experimental groups.

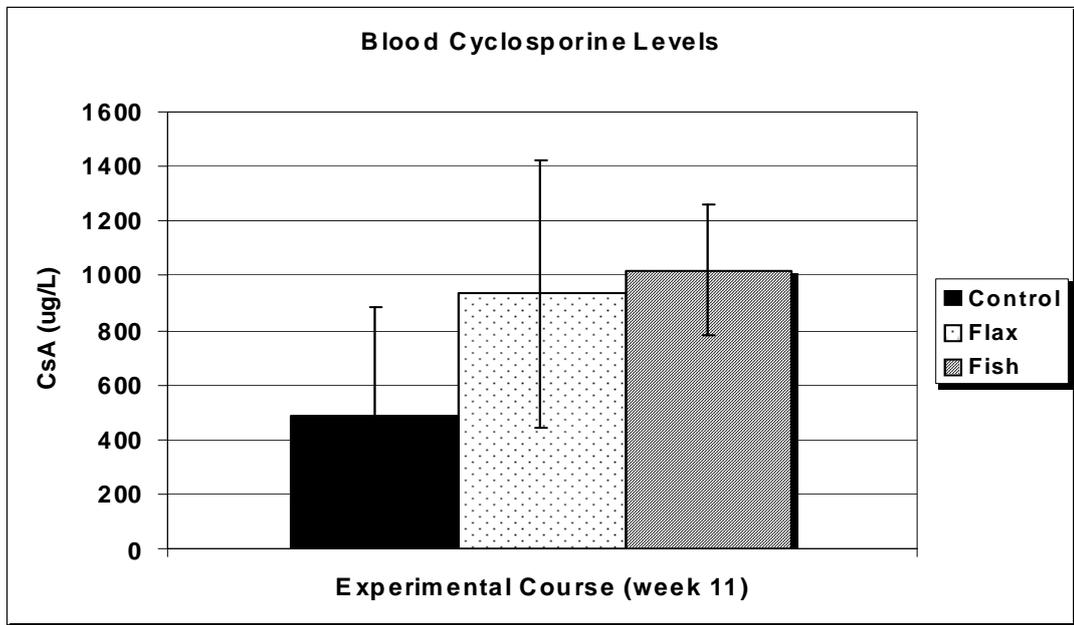
6.8 PLASMA CREATININE AND UREA LEVELS

CsA has been shown to increase nephrotoxicity after heart transplantation *via* elevations in the production of endothelin, TXB2 and the reduction in production of prostacyclin (PGI2) and PGE2, alteration in intracellular calcium regulation, activation of sympathetic system and alteration of eicosanoids production (Ventura et al. 1997a). In the flaxseed and fish-treated animals, plasma creatinine and urea levels were comparable with those of control group (3.3 ± 1.2 , 4 ± 1.6 vs. 3 ± 1.1 $\mu\text{mol/L}$) and (6 ± 1.0 , 6 ± 0.8 vs. 6 ± 1.0 mmol/L), respectively. Plasma creatinine and urea levels are shown in **Figure 8-A** and **8-B**, respectively.

6.9 HISTOLOGY AND REJECTION GRADES

Histological examinations of the sections obtained from the graft tissues showed variations among the experimental groups in regard to features of chronic rejection. These included presence of inflammatory cells, accumulation of fibrotic tissues and destruction of myocardium. These features were the basis of

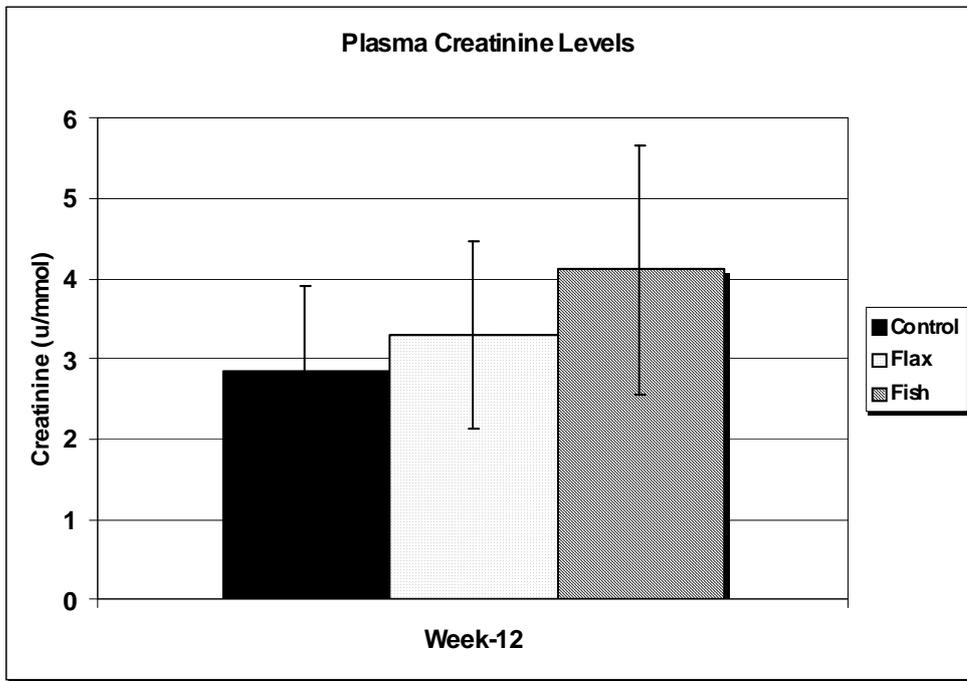
Figure 7. Whole blood cyclosporine levels in the experimental groups.



All the experimental groups showed comparable levels of whole blood cyclosporine; n=(7-8).

Figure 8. Plasma creatinine and urea levels of all treated groups after 12 weeks of study. No significant differences were observed among different protocols;(n=7-8).

A



B

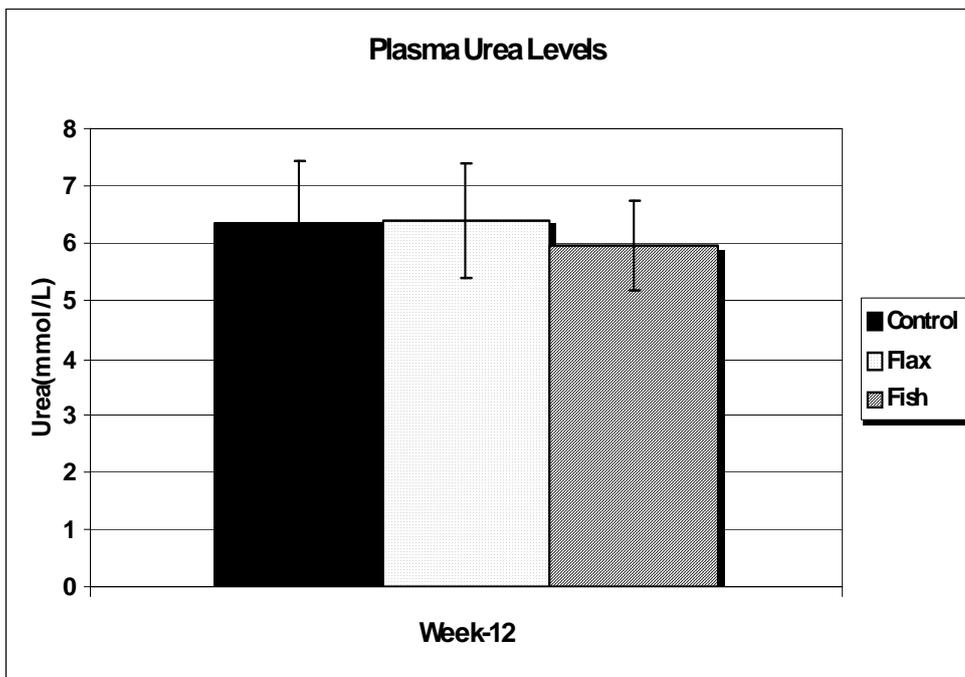


Figure 9 illustrates representative sections with grade 0 (Panel A), grade 1 (Panel B), grade 2 (Panel C) and grade 3 (Panel D). While Panel A shows almost-normal-looking architecture of cardiac morphology, Panel 4 presents multi-focal injuries including massive inflammation, complete destruction of myocardium and extensive extracellular matrix. Four animals in the flax group and 4 animals in the control group showed grade 3 rejection; neither control nor flax group had grade 0 rejection. On the other hand, none of the animals in the fish group showed grade 3 and 2 animals showed grade 0. However, chi-square test did not detect statistically significant differences in the distribution of rejection grades among the groups ($P>0.05$). All of the native hearts regardless to the experimental treatments showed normal cardiac morphology. The distribution of chronic rejection grades among the experimental groups is displayed in **Table 7**. **Figure 10** demonstrates representative photomicrographs of sections from the kidneys highlighting apparent cyclosporine-induced nephrotoxicity. The arrows in **Figure 10**, Panels A, B and C show similar extent of PAS-positive hyaline-thickening of the arteriolar wall at the entry to the glomerulus in all 3 groups.

6.10 HEART AND GRAFT LIPIDS

Lipid distribution was significantly changed in the graft (**Table 8**). Regardless of the dietary treatments, TG and MG were significantly ($P<0.05$) increased while phospholipid was significantly ($P<0.05$) decreased in the graft as compared to the native hearts. The grafts from fish oil-treated group, however,

Figure 9. Representative photomicrographs of sections taken from the grafts with various degrees of rejection. Panel A, Grade 0 rejection showing no evidence of rejection; Panel B, Grade 1 rejection showing one focus of myocyte damage (arrow) and inflammatory infiltrate; Panel C, Grade 2 rejection presenting at least 2 foci of myocyte damage and more inflammatory infiltrate (double-headed arrow); Panel D, Grade 3 rejection presenting diffuse multifocal myocyte damage and prominent perivascular infiltrate (double-headed arrow), major tissue destruction and calcification (arrow) along with increased apparently apoptotic bodies. hematoxylin and eosin, X 400

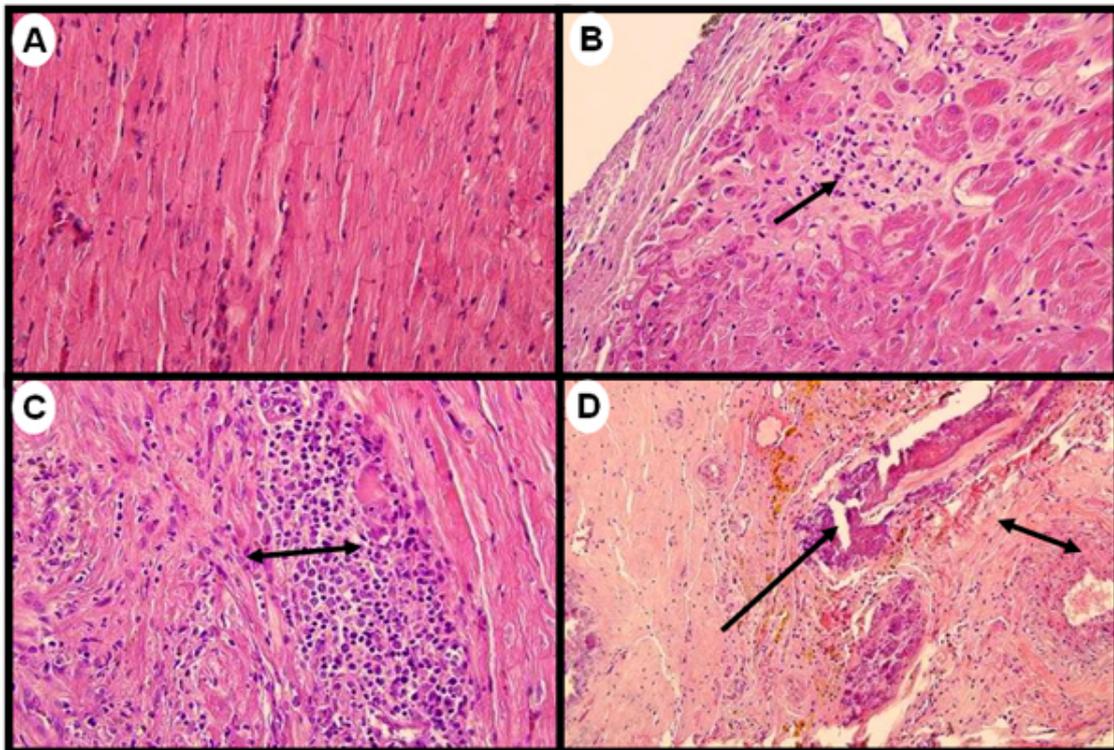


Table 7. Distribution of chronic rejection grades according to Billingham criteria among the experimental groups.

Rejection Grades	Control (n=7)	Flax (n=8)	Fish (n=7)	Total
0	0	0	2	2
1	1	1	1	3
2	3	3	4	10
3	3	4	0	7
Total (n)	7	8	7	22

The distribution of rejection grades are not significantly ($P=0.241$) different among groups according to the chi-square test analysis.

Figure 10. Representative photomicrographs of cross sections taken from the kidneys of Control (A and D), flaxseed-oil-treated (B and E) and fish-oil-treated (C and F) stained with Masson's trichrome (A, B, C, X400) and PAS (D, E, F, X400). A comparable extent of focal interstitial fibrosis and evidence of focal tubular atrophy are illustrated in all of 3 groups of animals (arrows). Similarly, PAS-positive narrowing of the arteriolar wall (arrows) is illustrated to be similar in all of 3 groups of animals.

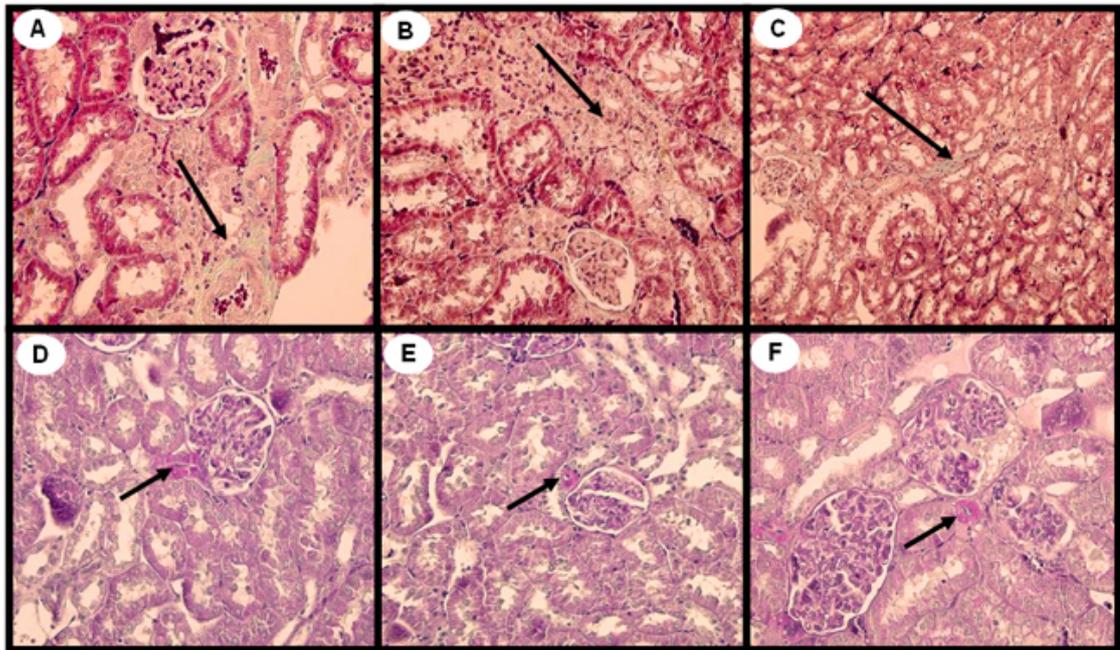


Table 8. Lipid fractions of native hearts and grafts.

	Native heart			Graft		
	Control	Flax	Fish	Control	Flax	Fish
%PL	47.5±9.48 ^a	42.5±8.62 ^{ab}	50.4±9.12 ^a	44.6±6.52 ^{ab}	39.1 ± 5.87 ^{ab}	33.1± 9.28 ^b
%Cho	22.1±3.18	25.1±7.83	22.2±3.28	18.7±7.99	22.3 ± 2.99	18.2 ± 4.07
%FFA	18.0±5.16	18.3±3.82	16.2±4.07	15.5±5.56	16.1 ± 2.26	14.8 ± 4.73
%MG	4.1±0.44 ^c	6.2±2.85 ^{abc}	5.5±1.60 ^b ^c	6.8±2.81 ^{abc}	7.5 ± 1.88 ^{ab}	9.2 ± 2.95 ^a
%TG	8.3±7.86 ^{bc}	7.9±4.46 ^{bc}	5.6±1.81 ^c	14.4±7.71 ^b	15.0 ± 5.15 ^b	23.9 ± 4.50 ^a

Values are expressed as percent of lipids in tissues and represented as mean (n= 5-6) ± SD. Values having a different superscripts are significantly different, P<0.02; PL, Phospholipids; Cho, cholesterol; FFA, free fatty acids; MG, monoglyceride; TG, Triglyceride

showed significantly higher TG levels as compared to that in either control or flax oil-treated animals.

6.11 LIVER LIPIDS

As shown in **Figure 11**, percentage lipid of total liver weight data was comparable among groups. Similarly, results for hepatic cholesterol and TG were comparable among groups. Data on TG and cholesterol levels are presented as ratio of total Liver weight and shown in **Figure 12-A and 12-B**, respectively. The fatty acid composition of the liver phospholipid, triglyceride, free fatty acid, cholesterol ester fractions are summarized in **Table 9, Table 10, Table 11, and Table 12**, respectively.

Figure 11. Percentage lipid of total liver weight of all experimental groups. All the experimental groups showed comparable percentages lipid of total liver weight;n=(7-8).

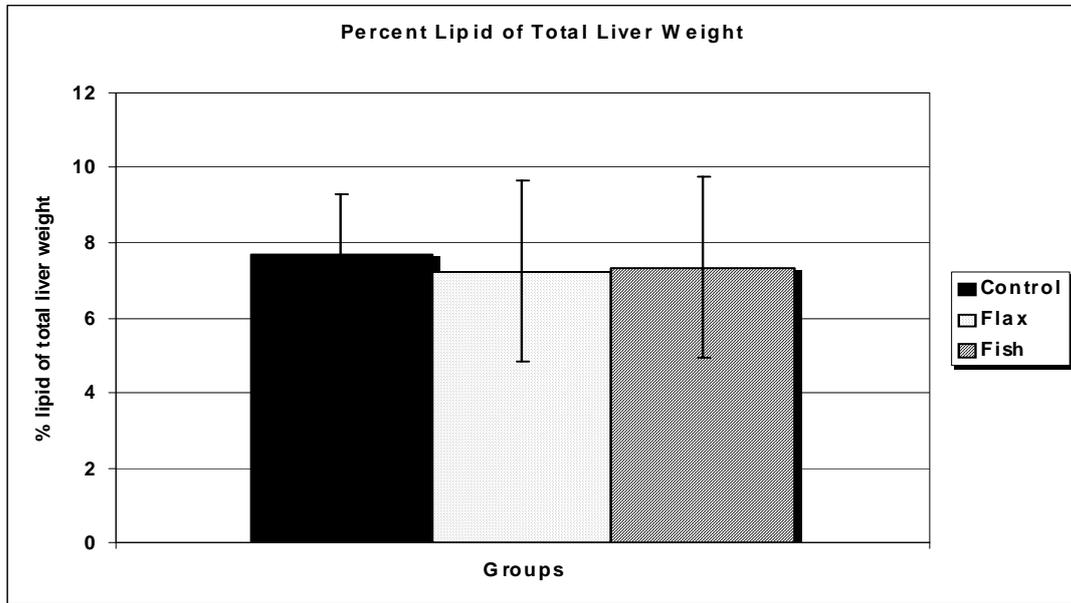
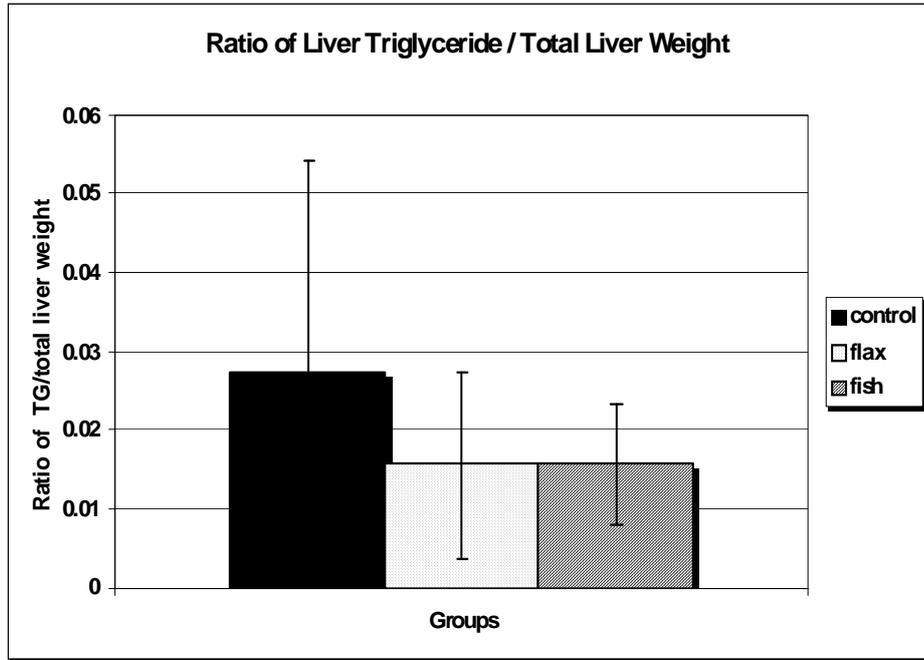


Figure 12. Mean ratio of triglyceride and cholesterol to total liver weight. No significant differences were observed among different protocols;n=(6-8).

A



B

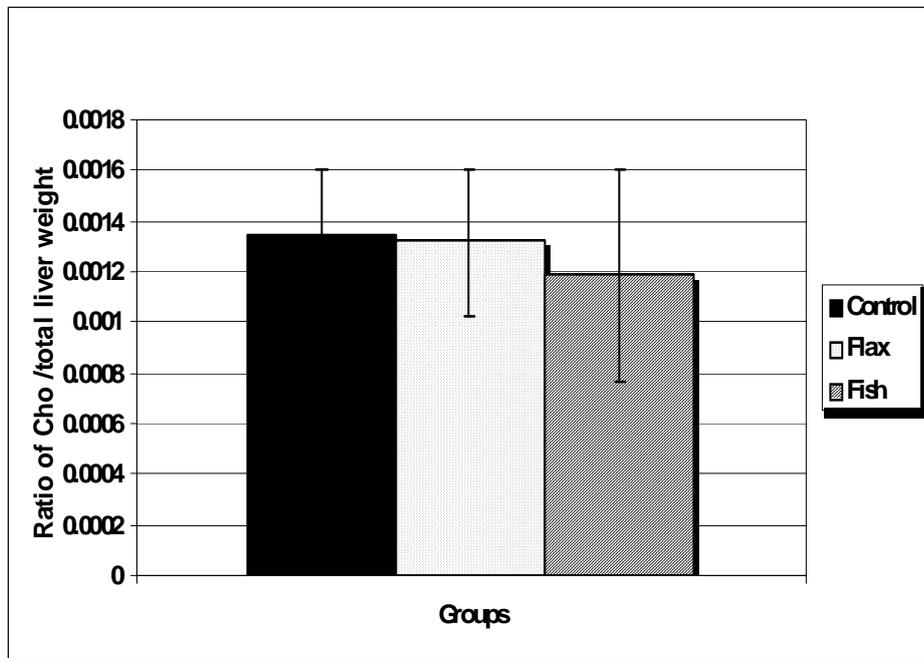


Table 9. Fatty acid composition of liver phospholipids.

Fatty acid (% w/w)	Control (n=2)	Flax (n=6)	Fish (n=3)
11:0	0.02±0.01	0.09±0.17	0.00±0.01
11:1	0.01±0.01	ND	ND
12:0	ND	ND	0.06±0.10
12:1	ND	0.02±0.03	ND
14:0	0.08±0.01	0.11±0.10	0.05±0.04
14:1	0.00±0.01	0.00±0.01	ND
15:0	0.12±0.02	0.10±0.06	0.11±0.01
15:1	ND	0.01±0.01	ND
16:0	18.02±1.78	19.16±3.84	21.24±2.85
16:1 (5 + 7)	0.09±0.08	0.06±0.05	0.08±0.07
17:0	0.55±0.02	0.55±0.10	0.61±0.14
17:1	0.10±0.09	0.10±0.10	0.01±0.01
18:0	28.25±6.90	37.81±10.61	35.08±11.46
18:1 (7 + 9)	4.87±1.18	5.91±1.83	5.43±1.13
18:2 (6)	ND	8.34±6.80	7.09±6.83
20:0	0.08±0.08	0.30±0.14	0.03±0.05*
18:3 (3)	0.08±0.09	0.06±0.08	0.04±0.07
20:2	0.35±0.34	0.17±0.15	0.18±0.16
20:3 (6)	0.00±0.00	0.57±0.54	1.04±0.51
20:4 (6)	34.86±5.33	18.05±7.92	14.90±7.68
22:0	0.21±0.07	0.16±0.13	0.27±0.10
20:5 (3)	0.20±0.12	0.78±0.48	1.84±1.23
22:4 (6)	0.22±0.26	0.25±0.41	0.09±0.08
24:0	0.67±0.27	0.70±0.54	0.53±0.53
24:1	0.38±0.09	0.36±0.18	0.42±0.08
22:5 (6)	ND	ND	0.79±1.37
22:5 (3)	1.27±0.33	1.40±0.89	1.57±1.02
22:6 (3)	9.57±0.82	4.94±3.59	8.55±5.61

*One-way ANOVA with Tukey test could not be conducted due to there only being 2 samples in the control group. t-test was used to detect differences between fish and flax groups; * p<0.05 compared to flax group; ND, Not detected.*

Table 10. Fatty acid composition of liver triglycerides.

Fatty acid(%w/w)	Control (n=4)	Flax (n=4)	Fish (n=4)
14:0	0.03±0.05	0.05±0.04	0.05±0.10
15:0	0.22±0.03	0.27±0.21	0.39±0.15
16:0	62.91±6.56	65.02±4.79	61.10±4.41
16:1 (5 + 7)	0.08±0.09 ^a	0.21±0.14 ^a	0.52±0.17 ^b
17:0	1.58±0.10	1.45±0.11	1.46±0.19
17:1	ND	0.06±0.07	0.04±0.07
18:0	26.52±5.50 ^a	22.84±1.65 ^{ab}	19.82±4.21 ^b
18:1 (9)	3.69±1.10 ^a	6.31±2.41 ^{ab}	7.54±2.69 ^b
18:1 (7)	0.81±0.10 ^a	0.83±0.14 ^a	1.17±0.17 ^b
18:2 (6)	1.74±1.59	0.53±1.05	3.89±3.56
20:0	0.05±0.10	0.28±0.49	0.08±0.16
18:3 (3)	0.17±0.21	0.18±0.14	0.17±0.20
20:1 (5)	ND	0.04±0.07	0.04±0.08
20:4 (6)	0.83±0.45 ^{ab}	0.36±0.58 ^b	1.12±0.21 ^a
20:5 (3)	0.97±1.13	1.03±1.08	1.07±1.38
24:0	ND	ND	ND
24:1	ND	ND	ND
22:5 (6)	ND	0.41±0.81	ND
22:5 (3)	ND	ND	0.22±0.44
22:6 (3)	ND	ND	0.70±0.88

Different letters between groups indicate significant differences, P<0.05; ND, Not detected.

Table 11. Fatty acid composition of liver free fatty acids.

Fatty Acid(% <i>,w/w</i>)	Control (n=4)	Flax (n=5)	Fish (n=3)
13:0	0.10±0.21	ND ¹	ND
14:0	ND	ND	0.29±0.51
14:1	0.41±0.82	ND	0.29±0.51
15:0	ND	0.16±0.15	0.19±0.16
16:0	32.70±10.53	27.80±3.93	23.26±3.88
16:1 (5 + 7)	0.60±0.86	0.18±0.22	1.03±0.90
17:0	0.07±0.14	0.48±0.35	0.34±0.32
17:1	ND	0.02±0.03	0.04±0.08
18:0	8.21±5.20 ^{ab}	14.07±10.17 ^a	2.88±0.59 ^b
18:1 (7 + 9)	24.56±2.09	25.20±11.90	32.93±2.70
18:2 (6)	30.90±14.70	19.04±6.12	22.30±6.15
20:0	ND ^a	3.17±2.08 ^b	1.31±1.39 ^a
18:3 (3)	ND	0.06±0.13	ND
20:1 (5)	0.14±0.29	0.21±0.21	0.47±0.43
20:2	0.21±0.42	0.08±0.18	ND
20:3 (6)	ND	0.23±0.33	0.12±0.21
20:4 (6)	1.50±1.74	4.03±3.67	1.35±1.58
20:3 (3)	ND	0.02±0.04	0.32±0.56
20:5 (3)	ND	0.97±0.64	1.57±1.61
22:4 (6)	ND	ND	ND
24:0	ND	0.04±0.1	ND
24:1	ND	0.02±0.04	ND
22:5 (6)	ND	0.02±0.03	ND
22:5 (3)	0.21±0.43 ^a	1.04±1.04 ^a	2.73±1.15 ^b
22:6 (3)	0.37±0.75 ^a	1.61±1.25 ^a	5.89±2.21 ^b

Different letters between groups indicate significant differences, P<0.05; ND, Not detected.

Table 12. Fatty acid composition liver cholesteryl esters.

Fatty Acid(%w/w)	Control (n=6)	Flax (n=5)	Fish (n=5)
14:0	0.20±0.22	0.12±0.26	0.13±0.30
14:1	ND ¹	0.10±0.21	ND
15:0	0.11±0.12	0.41±0.34	0.32±0.21
15:1	ND	ND	0.02±0.04
16:0	30.71±3.92	30.14±3.75	31.04±3.82
16:1 (5 + 7)	0.52±0.69	0.78±0.95	0.42±0.17
17:0	0.76±0.41	1.74±1.39	0.98±0.15
17:1	0.01±0.02	ND	ND
18:0	30.40±8.47	32.58±10.27	33.86±5.08
18:1 (9)	7.97±2.35	8.62±2.73	7.42±1.83
18:1 (7)	1.76±0.59	1.30±0.34	1.51±0.27
18:2 (6)	15.41±4.32	11.89±1.57	11.16±3.18
20:0	0.03±0.08 ^a	1.51±0.47 ^b	0.08±0.11 ^a
18:3 (3)	0.07±0.10 ^a	0.34±0.15 ^b	0.25±0.15 ^b
20:1 (5)	0.11±0.13	0.06±0.14	ND
20:2	0.26±0.29	0.05±0.12	ND
20:3 (6)	0.19±0.21	0.24±0.24	ND
20:4 (6)	8.94±2.80 ^a	6.45±1.40 ^a	7.18±1.73 ^b
20:5 (3)	0.64±1.27	0.51±0.49	1.89±0.37
22:4 (6)	0.05±0.13	ND	ND
24:0	0.05±0.07	0.55±1.23	0.11±0.24
24:1	0.01±0.02	ND	0.03±0.07
22:5 (6)	0.44±0.99	0.06±0.14	0.04±0.08
22:5 (3)	0.13±0.16	0.54±0.39	0.54±0.32
22:6 (3)	1.01±0.83 ^a	1.19±0.71 ^b	2.78±0.79 ^b

Different letters between groups indicate significant differences, P<0.05; ND, Not detected.

7.0 DISCUSSION

7.1 EFFECTS OF DIETARY FLAXSEED OIL AND FISH OIL ADMINISTRATION

The primary objective of the present study was to investigate whether dietary flaxseed oil reduces post-transplant complications and prolongs graft function in a rat cardiac allograft model. Herein, we provide the evidence that both dietary flaxseed oil and fish oil similarly attenuated abdominal fat, reduced inflammation and hypertension. As fish oil and flaxseed oil reduced LDL-cholesterol levels and increased HDL-cholesterol, respectively, both of these oils similarly decreased LDL/HDL-cholesterol ratio. Moreover, none of these oils were able to prevent or delay the onset of chronic allograft rejection and cyclosporine-induced nephrotoxicity as evaluated by histological analysis in this cardiac allograft model.

7.1.1 Body Weight and Abdominal Fat

Obesity is believed to play an essential role in the progression of TCA among heart transplant recipients (Grady et al., 1991). Although cardiovascular health benefits of n-3 FAs have been noticed since 1970s, their potential effects on body composition and energy metabolism in humans have received very little attention. Dietary intake of n-3 FAs could result in a beneficial effect on body weight. In animals, significant reductions in body weight and visceral fat in mice were noticed after ALA administration (Hase et al., 2001). This study suggested that the reduction in visceral fat was a result of reduced triacylglycerol synthesis and increased FA oxidation. Of diets supplemented with either 7% of linseed oil,

soybean oil or sunflower oil, only linseed oil-supplemented had a lower body weight, inguinal fat pad weight, and adipocyte size as well as a decreased leptin level in suckling pups' rats (Korotkova et al., 2002). Incorporation of n-3 FAs into adipose tissue phospholipids may modify adipocyte differentiation and active molecules production (Reginato et al., 1998).

Fish oil has been shown to reduce body fat mass (Hill et al., 1993), retroperitoneal adipose tissue weight (Pérez-Matute et al., 2007) and abdominal and epididymal adipose tissue hypertrophy in rodents but did not change body weight (Belzung et al., 1993; Parrish et al., 1990). Dietary fish oil reduced adipocytes cell size in the sucrose-rich diet fed rats (Lombardo et al., 2007) and increased adiponectin secretion in genetically obese ob/ob mice (Itoh et al., 2007). On contrary, no significant changes were displayed in fat mass or body weight of mature rats receiving 14% of their energy intake as fish oil over four weeks (Awad et al., 1990). Treatment with flaxseed oil over 12 weeks resulted in a lower fat mass in male rats compared to those who treated with corn oil (Weiler et al., 2007).

In healthy subjects, fish oil accounted for significant diminutions in body fat mass (Couet et al., 1997). In accordance, n-3 FAs along with exercise was contributed to reduced body fat in controlled study with overweight volunteers (Hill et al. 2007). This study suggested that both n-3 FAs and exercise are involved in fatty acid mobilization and its delivery to muscles for oxidation. Similarly, in a double-blind parallel design, 3 g/d of fish oil for 3 months caused remarkable reduction in total fat mass and subcutaneous adipocyte diameter in

women with type II diabetes mellitus (Kabir et al., 2007). Moreover, a negative correlation was found between the adipocyte size and n-3 FAs content of subcutaneous adipose tissue of overweight/obese patients who had abdominal surgery (Garaulet et al., 2006). In men, significant decreases (-6.3% to -13.4%) in body fat area and increases in the resting metabolism were attributed to ingesting diets containing ALA- diacylglycerol (Katsuragi et al., 2001; Takei et al., 2001).

In agreement with our study, supplementing diets with either fish oil or linseed oil showed a considerable reduction of abdominal fat in chicken (Newman et al., 2002) and broilers (Crespo and Esteve-Garcia, 2002); this effect was further supported by increased fatty acid oxidation (Crespo and Esteve-Garcia, 2002). In the present study, the observed reductions in the abdominal fat mass when the rats consumed diets supplemented with fish oil or flaxseed oil did not potentially result in alterations in body weight. Attenuations in the abdominal fat after dietary n-3 FAs might be due to a decrease in the accumulation of TG in adipose tissue and thereby a decline in adipocyte trophic growth (Parrish et al., 1990). One may speculate that modulation in the body fuel utilization by dietary n-3 FAs was not sufficient to alter body weight in this animal model.

The decrease in fat mass with n-3 FAs may suggest lipid oxidation (Halminski et al., 1991). n-3 FAs may mediate lipid oxidation by up-regulating several nuclear receptors (Davidson, 2006) and increasing the expression of genes involved fatty acid transport and β -oxidation e.g. lipoprotein lipase (Chapman et al., 2000), acetyl-CoA carboxylase-2 (Schrauwen et al., 2002), fatty

acid translocase, carnitine palmitoyl transferase 1 (Flachs et al., 2005; Shirouchi et al. 2007), and mitochondrial uncoupling protein 3 (Baillie et al. 1999).

Furthermore, n-3 FAs may reduce visceral fat by depressing sympathetic nerve activity (Matsumura, 2007) and increasing hormone-stimulated lipolysis (Parrish et al., 1991). Some of the changes in body composition with n-3 FAs supplementation may also be because of improved blood flow that may have increased the delivery of fats to the sites of metabolism e.g. skeletal muscle (Hill et al., 2007).

7.1.2 Plasma Lipids

To assess whether consumption of flaxseed oil and fish oil could alter lipid homeostasis, we determined the effects of dietary treatments on circulating levels of plasma lipids and lipoproteins. Higher concentrations of plasma cholesterol and TG are linked with a higher risk of TCA (Park et al., 1996). n-3 FAs have been shown to reduce plasma lipid levels, particularly TG levels. In animal studies, feeding rats a diet supplemented with 10% perilla oil, the source of ALA, for 4 weeks has shown significant decreases in post-prandial plasma TC and TG levels (Wiesenfeld et al., 2003). Likewise, ALA of flaxseed oil significantly reduced increased plasma levels of TC, TG, LDL, LDL/HDL, and TC/HDL-cholesterol ratios in rats fed high fat diet (Vijaimohan et al., 2006). In contrast, Lee and Prasad et al., (2003) failed to report any alterations in serum lipids in rabbits fed 5 % flaxseed oil containing diet.

In a cross-sectional study, ALA intake at 0.81 and 0.69 g/d in the men and the women, respectively, reduced only plasma TG levels (Djousse et al. 2003). Similarly, consuming ALA containing diet (1.1 energy % ALA) by healthy volunteers for 6 weeks resulted in remarkable decreases in TC levels and TC/HDL- cholesterol ratio but not TG levels (Petra et al., 2005).

Addition of flaxseed oil to functional oil composed of plant sterol and medium chain triglyceride, decreased TC and LDL-cholesterol levels but not TG levels in healthy overweight men after 4 weeks of treatment (St-Onge et al., 2003). Postprandial TG and TC concentrations were attenuated in healthy subjects after consuming diet supplemented with rapeseed oil (Nielsen et al., 2002). On the other hand, no significant alterations in plasma lipids however, observed after dietary replacement of LA (15 g) with ALA (15 g) in normolipidaemic men (Pang et al., 1998).

In the present study, and in agreement with previous reports (Yun et al., 1991a; Harris and Bulchandani, 2006; Goyens and Mensink, 2005), we observed significant reductions in TG, TC, LDL/HDL-cholesterol ratio, non-HDL and LDL-cholesterol levels in the fish group. Furthermore, a significant attenuation (-22%) in LDL/HDL-cholesterol ratio was also observed in the flax group relative to the controls; this may be due to a significant increase (19%) in HDL-cholesterol levels in the flax group. This observation is in agreement with previous reports (Morise et al., 2004). These changes in lipoprotein profile suggest that both oils (fish and flaxseed) may have anti-atherogenic properties regardless of the type of n-3 FAs. Increased atherogenic lipoprotein profile has been shown in both clinical

and experimental models of heart transplantation (Aranda Jr and Hill, 2000; Shi et al., 1997). This could be one of the main factors contributing to chronic rejection of grafts.

Hypolipidemic effect of these FAs may be mediated through reduced endogenous triacylglycerol-rich lipoproteins (TRL) synthesis, enhanced TRL removal, or a combination of both (Roche and Gibney, 2000), and lowered plasma free FAs, the main substrate for hepatic TG synthesis (Singer, 1992). n-3 FAs attenuated the production and export of triacylglycerols by the liver (Harris, 1996); this decline may be due to increased hepatic mitochondrial and peroxisomal oxidation of n-3 FAs.

Interaction of n-3 FAs with transcription factors may mediate at least four families of transcription factors including peroxisome proliferator-activated receptor alpha (PPAR α), liver X receptors, hepatic nuclear factor-4 α and sterol regulatory element-binding protein-1c (reviewed in Jump, 2004; Jump et al., 2005). Upon its activation, these transcription factors influence genes responsible for β -oxidation (Stulnig, 2003) and block also nuclear factor KB (NF-KB) signaling (Sweeney et al., 2005; Mayer et al., 2006).

n-3 FAs are also known for eicosanoids productions (Calder, 2004) which have been shown to influence protein kinase C activity (Takahashi et al., 2005). Together with protein kinase C, eicosanoids modulate transcription factor activity. Indirect effects on gene regulation may result from alterations in lipid raft composition by n-3 FAs (Ma et al., 2004). Such mechanisms influence the

activity of G-linked proteins (Niu et al., 2004) and other membrane-bound receptors (Li et al., 2005).

n-3 FAs may also attenuate VLDL triglyceride synthesis by the following mechanisms: (a) lessened substrate (i.e. FA) availability which could be secondary to an increase in beta-oxidation, decrease in delivery of non-esterified fatty acid to the liver, or decrease in lipogenesis, (b) decreased activity of triglyceride-synthesizing enzymes, such as diacylglycerol acyltransferase or (c) increased phospholipid synthesis which could draw diacylglycerol away from diacylglycerol acyltransferase (Harris and Bulchandani, 2006).

7.1.3 Plasma Cytokines

Several studies suggest that inflammation plays a pivotal role in the pathogenesis of atherosclerosis (Libby, 2002; Willerson and Ridker, 2004; Buono and Lichtman, 2004). For that reason, we sought to explore the relationship between n-3 FAs consumption and inflammatory markers. Marine and plant n-3 FAs have previously been shown to lower pro-inflammatory cytokines in postmenopausal women (Ciubotaru et al., 2003), diabetics patients (Mori et al., 2003), hypercholesterolemic men and women (Zhao et al., 2004; Rallidis et al. 2003), healthy subjects (Caughey et al. 1996) and obese individuals who are on lipid-lowering drug treatment (Chan et al. 2002) but not in obese men (Jellema et al. 2004).

In rat allogenic small intestinal transplantation, fish oil reduced plasma levels of IL-2, IFN- γ , TNF- α and IL-1 β as compared to soybean oil (Ogita et al.,

2003). Similarly, in a rat heterotopic heart transplant model, fish oil exhibited a trend toward reducing levels of IL-6 and the number of circulating T-cells (Grimm et al., 1995). This decline in T-cell recruitment might be explained by the hypothesis that n-3 FAs incorporation into the phospholipid bilayer modifies membrane fluidity and cytokine receptor interaction, which mediates cell emigration from the lymphoreticular system (Kinsella, 1990).

MCP-1 is a chemoattractant cytokine that is involved in the activation and recruitment of monocytes in cardiac grafts (Adams et al., 1993). In the current study, both fish and flaxseed oil equally reduced plasma MCP-1 levels (-12%) as compared to controls. It is suggested that anti-inflammatory actions of these oils may be due to their impacts on transcription factors influencing gene expression (Ntambi and Bene, 2001; Takahashi et al., 2002) and/or synthesis of eicosanoids (Browning, 2003).

7.1.4 Blood Pressure and Heart Rates

Hypertension is a major complication in CsA treated heart transplant recipients. The mechanisms are still unclear; however, the previous reports suggest that progressive CsA-induced nephropathy is the major pathogenic mechanism. Endothelial cell mediated effects through an imbalance between vasoconstrictor and vasodilatory factors may also be contributed (Starling and Cody, 1990; Ventura et al., 1997a). n-3 FAs have potentially favorable effects that may protect against CsA-induced hypertension early after heart

transplantation (Ventura et al., 1993). Herein, we sought to find out whether consumption of n-3 FAs could alter BP, and heart rates.

Results obtained in various clinical trials with respect to the effects of n-3 FAs on BP are controversial. With respect to flax oil, the evidence for the hypotensive effect is yet somewhat unconvincing. Nonetheless, there was a human observational study that reported dietary and tissue ALA were correlated with lowered BP (Bemelmans et al., 2000). In healthy volunteers, no changes in BP were noted after consuming ALA rich diet (Singer et al., 1986 reviewed in Singer, 1992). On contrary, in subjects with mild essential hypertension, a significant decrease in SBP was found after ingesting linseed oil (Singer et al., 1990). In animals, diets high in flax oil have mixed effects on BP (Singer et al., 1884). However, feeding spontaneously hypertensive rats diet containing 2.5 and 5% linseed oil for 15 to 16 weeks reduced the high SBP and DBP of spontaneously hypertensive rats.

Fish oil reduced BP in overweight subjects (Mori et al., 1999), medication-treated hypertensive patients (Bao et al. 1998; Beilin and Mori, 2003), untreated hypertensive subjects (Appel et al., 1993) but not normotensive subjects (Howe, 1997; Grimsgaard et al., 1998). In addition, a meta-analysis of clinical trials showed that DHA had somewhat greater dose-response effect than EPA on BP ($-1.5/ -10.77$ mmHg versus $-0.93/ -0.53$ mmHg/g) (Morris et al., 1993; Mori, 2006).

In agreement with our findings, renal transplanted patients who received 6 g /d of fish oil for 3 months had significantly lower MAP in relative to control

group, who consumed 6 g/d of coconut oil (Homan van der Heide et al., 1993). In parallel with our findings, daily treatments with either 3 g or 4 g of n-3 FAs significantly reduced MAP and SBP in heart transplant recipients with (Ventura et al., 1993) or without hypertension (Andreassen et al., 1997). Moreover, the decline in SBP was inversely correlated to increases in serum concentrations of EPA and DHA (Andreassen et al., 1997).

In agreement with the present study, significant increases in SBP were noticed in clinically stable hypertensive heart transplant patients who received four capsules daily of highly concentrated ethyl esters of corn oil (placebo group) for 12 months but not with those who received an equal amount of ethyl esters of n-3 FAs (Holm et al., 2001). In the present study, treatment with flaxseed oil was associated with a significant decline in SBP, DBP and MAP as compared to controls. It is interesting that these effects of flaxseed oil were comparable to those of fish oil in this animal model.

The possible mechanisms of action n-3 FAs may be related to their effect on endothelial and smooth muscle function (Mori and Woodman, 2006), and autonomic nerve function or β -adrenoreceptor activity (Leaf and Kang, 1996). In addition, n-3 FAs may induce vasodilatation due to an improved PG profile (Elzinga et al., 1987a) or endothelial NO production, inhibit ACE activity, reduce angiotensin II production (Mori, 2006), lower transforming growth factor beta levels, known to be elevated in patients with uncontrolled essential (Ismail, 2005). Previous reports indicated that there may also be effects *via* vasoconstrictor response to stressor hormones, and reduced blood viscosity

(Appel et al., 1993). Although release of NO is the main factor affecting flow-mediated dilatation in conduit vessels, n-3 FAs improve vascular function by additional mechanisms including changes in the release of ADP, endothelium-derived hyperpolarizing factor (Beilin and Mori, 2003).

High heart rates at rest have been strongly related to cardiovascular disease events, (Hjalmarson, 1998). Increased n-3 FAs intakes resulted in a significant decrease in heart rates (Mozaffarian et al., 2005; O'Keefe et al., 2006). Higher intake of dietary total ALA was inversely associated with heart rate-adjusted QT and JT intervals in a dose-response manner in both men and women (Djoussé et al., 2005). n-3 FAs, particularly DHA significantly reduced clinic standing and supine heart rates (Woodman et al., 2002) and 24-h, awake and asleep heart rates in humans (Mori et al., 1999) and prevented stress induced significant increases in heart rates in male Wistar rats (Rousseau et al. 1998). In parallel with these findings, the current study did show significant attenuations in heart rate in both treated groups (flaxseed and fish oils) after 12-weeks of dietary treatment.

The reductions in the high rates after n-3 FAs treatments may be due to increased vagal activity, which may explain observed attenuation in the risk of sudden cardiac death after n-3 FAs supplementation (O'Keefe et al., 2006). Furthermore, n-3 FAs may reduce high rate independently of vagal activity. In patients with denervated hearts after orthotopic heart transplantation, n-3 FAs supplementation reduced high rates by influencing electrophysiologic properties of the myocardium itself (Harris et al., 2006).

Antiarrhythmic effect of n-3 FAs is thought to be a result of the release of EPA and DHA from myocardial membrane phospholipids by ischemia-activated phospholipase A2, and the subsequent interaction of the free fatty acids with ion channels (Leaf et al., 2003). n-3 FAs have also been reported to alter the kinetic properties of the fast, voltage-dependent sodium current and the L-type calcium currents in *in-vitro* studies (Leaf et al., 2003). Finally, n-3 FAs may influence heart rates by two possible ways; one neurogenic which refers to changes in cardiac autonomic control, with a shift toward increased vagal tone and the other cardiogenic which refers to changes in the myocardium itself (Harris et al., 2006).

7.1.5 Kidney Function

Nephrotoxicity – impaired renal function and altered morphological structures of the kidney – continues to be a main problem in the clinical use of CsA. The changes in renal haemodynamics are considered an outcome of CsA-induced imbalance between the production of vasoconstrictors and vasodilators, as well as direct toxic effects to vascular endothelia, resulting in vasoconstriction of the afferent arterioles (Santos et al., 2000). The precise mechanism of vasoconstriction is still unclear; however, it seems to be due to a substantial impairment of endothelia cell function, leading to a decline in the production of vasodilators (PG and NO) and a rise in the release of vasoconstrictors (endothelin and TX) (Santos et al., 2000).

High endothelin and TXB2 production and reduced prostacyclin (PGI2) and PGE2 production, alteration in intracellular calcium regulation, and activation of sympathetic system may involve provoking of CsA-induced nephrotoxicity (Morphake et al., 1994; Yang et al., 2005). Some reports have suggested that CsA induces renal microsomal lipid peroxidation and reduces antioxidant glutathione levels others indicated that CsA nephrotoxicity could be decreased if lipid peroxidation diminished by fish oil (Elzinga et al., 1987b; Ventura et al., 1993).

The mechanisms underlying the beneficial effect of n-3 FAs on renal function remain uncertain; however, it is suggested that n-3 FAs may increase TXA3 formation along with a decline in TXA2 and an increase in total prostacyclin levels (Yang et al., 2005). Moreover, beneficial effect of n-3 FAs on kidney function might also be attributed to its antihypertensive effect (Holm et al., 2001). Fish oil reduced CsA-induced nephrotoxicity in rats (Casas et al., 1995) and improved BP and GFR in kidney transplant patients (Bennett et al., 1995). Supplementing the diets of 11 recipients of renal transplants with 6 g/d of fish oil for 3 months improved GFR and MAP (Homan van der heide et al., 1990).

Similarly, 45 clinically stable hypertensive heart transplant recipients were randomly assigned in a double blind to receive either 3.4 g/d of n-3 FAs or placebo for 1 year. As compared to the n-3 FAs group, the placebo group had increased SBP by 8 ± 3 mmHg, systemic vascular resistance by 14 % and plasma creatinine, and decreased GFR after 12 months. In contrast, no increases in BP, or plasma creatinine in the n-3 FAs group were shown (Holm et

al., 2001). In contrast, renal allografts recipients received either (30% EPA and DHA) or coconut oil at 6 g/d for 3 months showed no significant changes in renal function (Kooijmans-Coutinho et al., 1996) but it did show a favorable effect on the BP (Santos et al., 2000).

In the animal studies, fish oil reduced renal cortical content of TXB₂, (Elzinga et al., 1987b) and increase in TXA₂ synthesis, prevented CsA-induced nephrotoxicity (Kelley et al., 1989) and attenuated CsA-induced nephrotoxicity (Yang et al., 2005); this was also further supported by histological data (Morphake et al., 1994). In the present study, the levels of plasma creatinine and urea in all the groups, however, showed comparable levels. Furthermore, neither fish oil nor flaxseed oil was able to prevent apparent cyclosporine-induced nephrotoxicity as evaluated by histological examinations.

7.1.6 Allograft Rejection

Fish oil decreased TCA in Brown-Norway-to-Lewis rat heterotopic cardiac transplant model (Sarris et al., 1989b), dogs implanted with venoarterial autographs (Sarris et al., 1989a), Fischer-to-Lewis rat heterotopic small intestinal model (Ma et al., 2007) but neither in Lewis-to-Brown-Norway rat heterotopic cardiac allografts (Yun et al., 1991b), rabbit heterotopic heart transplants (Yun et al., 1991a), nor nonhuman primates (Boerboom et al., 1997). Consuming 6 g of fish oil daily was associated with a decreased number of rejection episodes (-60%), and an improved 1-year graft survival from 84% to 96% in kidney transplant recipients (Homan van der Heide et al., 1993). In contrast, studies with

similar design failed to show any beneficial effect of fish oil on the graft rejection rate (Hernandez et al., 2002) and the number of rejection episodes, renal histopathological and immunohistochemical parameters (Kooijmans-Coutinho et al., 1996).

In a synergistic manner with arginine, fish oil improved allograft survival in rat heterotopic cardiac allografts (Alexander et al., 1998). Feeding rats with diets containing n-3 FAs for 2 to 4 weeks before transplantation accounted for significant increases in graft survival even without CsA treatment (Otto et al., 1990). In addition, there was a significant and direct correlation between allograft survival and the donor heart phospholipid n-3: n-6 FA ratio (Otto et al., 1990). In rat heterotopic heart transplant model, fish oil decreased the number of infiltrating cells up to 50%; this was further confirmed by immunohistological analysis showing a low number of circulating T-cells with fish treatment (Grimm et al., 1995). Taken together, the contradictory results in the animals studies may be due to the difference in species, differences in the type of fish oil used, dose, and the shorter duration of the studies.

In the present study, neither of the oils statistically significantly prevented chronic rejection. Since the number of animals was limited to 7 to 8 per group, there is a possibility that treatment effect was too subtle to be detected by the statistical power inherent in this study. However, because the data demonstrate that fish oil treatment showed a trend toward reduction in the extent of chronic rejection as compared to the flax and control groups, it seems very possible that

increasing sample size within practical limits would have revealed a treatment effect.

The lower number of rejection grades in the fish group might be because of incorporation of adequate amount of EPA and DHA into phospholipid bilayer of the graft tissue, resulting in modification of membrane fluidity, intracellular signal transduction, production of less harmful eicosanoids, less pro-inflammatory cytokine release, and reducing cytokine receptor interaction. The net outcome of these events will be reduced T-cells recruitment and the extent of inflammation (Kinsella, 1990; Alexander et al., 1998).

Available evidence suggests that the cardioprotective mechanism of n-3 FAs depends on their presence in myocardial cell membranes (Leaf et al., 2003). Since ALA has to be desaturated and elongated in the body by certain enzymes to form EPA and DHA; it is unclear how much ALA is required to generate sufficient cardiovascular protection. Dietary n-3 FAs of flaxseed oil might not be incorporated into the tissue phospholipids at a similar rate to those of fish oil. It is unknown the extent to which ALA will be incorporated into tissue phospholipids. It is also unclear if all n-3 FAs might have beneficial effects or if the benefit is only limited to EPA and DHA. In the present study, it is possible that non-physiologic circulation of heterotopic heart transplant model could have limited the distribution of ALA to the graft tissue and then its subsequent elongation and desaturation. Additionally, it is unknown how rapid ALA is converted to EPA and DHA, and gets incorporated into the graft tissues. Thus, it is possible that the conversion of ALA to EPA and DHA in the graft tissue was insufficient. It is

evident that the extent of reductions in BP and anti-inflammatory state as well as other apparent beneficial effects of flaxseed oil was not adequate to prevent chronic rejection in this model.

7.1.7 Heart, Graft and Liver Lipids

Since higher tissue content of n-3 FAs has been associated with lower risk for CVD, the possibility that incorporation of n-3 FAs into heart phospholipids membrane could have a protective effect against chronic rejection should be rational. The importance of n-3 index, the percentage of EPA and DHA in the phospholipids bilayer membrane as a modifiable risk factor in CVD has been previously emphasized (Schacky, 2000; Schacky and Harris, 2006). n-3 FAs supplementation may improve vascular system (Nishizawa et al., 2006) but their deficiency may jeopardize health and increase risk for CVD (Ristic et al., 2006).

The Mediterranean diet rich in ALA has been reported to attenuate coronary events and cardiac deaths in humans (De Lorgeril et al., 1994). ALA supplementations significantly increased EPA and DPA in red blood cell membrane and plasma phospholipids but not DHA in healthy subjects (Wilkinson et al., 2005; Wallace et al., 2003; Li et al., 1999). This is also consistent with the isotope-tracer studies showing that majority of the ALA supplementation studies provided a limited conversion of ALA to its long-chain n-3 derivatives (Goyens et al., 2005). Nonetheless, failure of ALA supplementation to increase DHA blood compartment concentrations does not indicate that DHA levels do not increase in tissues. In fact, conversion of ALA may be efficient in developing neural tissue,

retina (Brenna, 2002), and brain (Su et al., 2000; Barcelo-Coblijn et al., 2005). Such conversion may also depend on the gender (Bakewell et al., 2006) and the concentration of EPA, DHA and n-6 FAs in the diet (Davis and Kris-Etherton, 2003).

Dietary DHA can fulfill the DHA requirements of several tissues. For instance, in baboons, dietary DHA was seven times more effective for DHA in nervous tissue than dietary ALA (Su et al., 1999). One can speculate that DHA produced from ALA might be incorporated into brain membranes prior cardiac membranes. In the current study, variations in the pattern of lipid distribution in native hearts and grafts may indicate abnormal function of grafts after transplant. n-3 FAs could have favorable impact on cardiovascular diseases by reducing hepatic lipids. In male Sprague-Dawley rats, hepatic TG and total cholesterol levels were reduced by fish oil and perilla oil diets; these reductions were also negatively correlated with EPA and DHA contents, suggesting hypolipidemic effect of n-3 FAs could be due to the increase of EPA and DHA in hepatic membrane (Kim and Choi, 2001).

In the current study both flaxseed oil and fish oil did not modify total liver lipids and cholesterol levels in our rats and did not reduce hepatic TG ratio significantly despite a trend for TG to be lower than in control animals. Herein, fish oil feeding resulted in a differential incorporation of n-3 FAs into liver lipid fractions. In the current study, the significantly higher incorporation of DHA from fish diet into liver free fatty acid and cholesteryl ester fractions was not accompanied with higher incorporation of these fatty acids into liver

phospholipids or TG fractions. Incorporation of DPA and DHA into liver free fatty acids fraction was significantly greater in fish oil group as compared to the other groups. Of interest was that flaxseed oil was similar to fish oil in term of significantly increasing DHA contents in liver cholesteryl esters fraction as compared to control group but not in liver phospholipids, TG, or free fatty acids fractions. Similarly, both of these oils significantly increased incorporation of ALA into liver cholesteryl ester fractions as compared to control group.

Lack of ALA incorporation into hepatic phospholipids, TG, or free fatty acids fractions could suggest a rapid metabolism or oxidation of this fatty acid in the liver. The extent of ALA partitioning towards β -oxidation might determine the availability of ALA for conversion to longer chain FAs, which in turn may rely on the physiological and nutritional state (Burdge, 2006). It is been suggested that the higher susceptibility of ALA for β -oxidation could be due to the greater affinity of carnitine: palmitoyl transferase-1, the rate limiting enzyme in mitochondrial fatty acid β -oxidation, for ALA compared to other unsaturated fatty acids (e.g., LA) (Burdge, 2006).

Furthermore, ALA metabolites could also be used for *de-novo* synthesis of saturated, monounsaturated FAs and cholesterol (Brenna, 2002) or transferred to skin (Fu and Sinclair, 2000) escaping desaturations and elongations pathways. It is a worthy notice that neither of these lipid fractions reflect hepatic ALA conversion exactly. Furthermore, both fish and flaxseed diets did not significantly increase EPA contents in hepatic lipids fractions as compared to DHA. This observation could be because of the fact that EPA is incorporated more into

circulatory pools while DHA is selectively concentrated in extracirculatory pools (Grimsgaard et al., 1997). In disagreement with the current findings, Vidgren et al., (1997) have reported that DHA was preferentially incorporated into phospholipids and triacylglycerol in the plasma lipids, but was very little concentrated in cholesterol esters, while EPA was in favor of phospholipids and cholesterol esters fractions incorporation. Taken together, the current findings conclude that the limited incorporation of dietary ALA into the liver lipids contributes to the low hepatic conversion of dietary ALA into DHA in hepatic phospholipids, TG, or free fatty acids fractions but not in cholesteryl esters fraction.

7.2 A SUMMARY OF MAJOR FINDINGS OF THE STUDY AND CONCLUSIONS

7.2.1 Study Major Findings

1. Administration of flaxseed oil or fish oil with CsA of 1.5 mg/kg body weight/d was well-tolerated in a rat cardiac allograft model over 12 weeks.
2. Flaxseed oil and fish oil significantly attenuated abdominal fat in 12 weeks when compared to control.
3. The attenuation in abdominal fat mass by the flaxseed and fish oil - treatment was not accompanied with significant reductions in the body weight.

4. Dietary fish oil protocol significantly reduced plasma TG, TC, LDL/HDL ratio, non-HDL and LDL-cholesterol levels.
5. Flaxseed oil dietary protocol significantly increased HDL-cholesterol levels and significantly attenuated LDL/HDL-cholesterol ratio as compared to controls.
6. No significant differences in VLDL, LDL, and HDL particle size were observed among the different dietary protocols over 12 weeks.
7. Both fish and flaxseed oil dietary protocols significantly reduced plasma levels of MCP-1 as early as 6 weeks in relative to controls.
8. Flaxseed- and fish oil administration significantly reduced SBP, DBP, and MAP as early as 6 weeks relative to the controls.
9. Fish and flaxseed oil dietary protocols significantly reduced heart rates after 12 weeks relative to controls.
10. No significant differences in plasma urea and creatinine levels were observed among the different dietary protocols over 12 weeks.
11. Lipid distribution was significantly changed in the graft. Regardless the dietary protocols, all grafts had significantly higher levels of TG and MG in comparison to native hearts. These increases were much higher in animals fed the fish diet.
12. There was a lack of association between graft TG contents and graft chronic rejection in this model. The fish oil-treated animals showed a high amount of graft TG and a low grade of chronic rejection, while this was opposite in the other two groups of animals.

13. Relative to the native hearts, phospholipid was significantly decreased in the grafts regardless of diet treatment.
14. Fish oil significantly increased DPA and DHA contents in liver free fatty acid and ALA and DHA levels in cholesteryl ester fractions but not in liver phospholipids or TG fractions compared to the other two groups of animals.
15. As comparable to fish oil, flaxseed oil significantly increased DHA content in liver cholesteryl ester fractions but not in liver phospholipids, TG or free fatty acids fractions.

7.2.2 Study Conclusions

This study reports that both dietary flaxseed oil and fish oil may reduce the extent of lipid, hemodynamic and inflammatory abnormalities after heart transplantation in rats. We found no evidence in this study that either dietary flaxseed oil or fish-oil supplementation significantly reduced chronic rejection states or cyclosporine-induced nephrotoxicity as assessed by histological analysis. However, substantially more work is required to understand whether the extent of incorporation of long-chain n-3 FAs in graft tissues is important for prevention of chronic rejection.

7.3 STRENGTHS AND LIMITATIONS OF THE STUDY

7.3.1 Strengths

We selected Fischer and Lewis rats as a cardiac allograft model for this study because this combination is one of the best models for studying TCA and chronic rejection (Corry et al. 1973; Judd and Trentin 1973). These models develop TCA and chronic rejection in a remarkably resemblance to those seen in human beings. This would mimic the current pattern of heart transplant recipients and would, in turn, suggest better reflection of the impact of our protocol if to be applied to human subjects.

Study design and length are among strengths of our study. We carried out the study for a relatively long period – 12 weeks – because chronic allograft rejection is a chronic condition that has been reported to increase with time. Moreover, this study is related to solid organ transplantation and well-being. We believe that research on the effects of nutraceuticals (i.e. n-3 FAs) in heart transplant recipients, and understanding the biochemical and molecular mechanisms by which food influences post-transplant status is an important area for research. We determined blood concentrations of CsA in treated animals in the presence of dietary n-3 FAs; this may help understand pharmacodynamic interactions between immunosuppressive drugs and dietary agents.

7.3.2 Limitations

The present study, like most others, has several limitations. The first limitations of this study is that heterotopic working cardiac allografts may be different from a working orthotopic transplant model in terms of circulation. This model is not entirely physiologic and does not totally mimic clinical transplant procedures. Another limitation of this study is that we did not measure animal food intake during the experimental period. The fact is that, the animals in all of the experimental groups were housed in a group of 3 to 4 rat per cage. Thus, it was not feasible to measure each animal food intake; however, based on literature we consider that each rat ingests approximately 20 g of chow each day.

Due to some animal ethics, we could not take blood samples before the surgery for baseline data because of the possibility of much blood loss during the surgery. Nevertheless, using week 6 and 12 blood samples data gave us some preliminary indication as to the influences of these oils on inflammatory or lipid profiles.

Furthermore, there are important contrasts between human and rodents lipoprotein metabolism; therefore, application of our data to human is difficult. Since the number of animals was limited to 7 to 8 per group, it is possible that the sample size may have been too small to provide sufficient statistical power and detect differences among dietary treatments. Due to the low n value in the control group in phospholipid, the statistics could not be done.

7.4 FUTURE DIRECTIONS

Now that we have shown several different effects of flaxseed oil and fish oil on post-transplant complications in a rat model of heterotopic heart transplantation, investigating the underlying mechanisms of action of these oils is warranted. Further investigation would give further insights if flaxseed oil and fish oil could be included among therapeutic strategies to protect from post-transplant complications. To thoroughly perceive the mechanisms behind chronic rejection, it would be beneficial in future if we could consider the analysis of the following parameters:

1. Investigate some pro-inflammatory genes or their expressed protein such as VCAM-1, ICAM-1, leukocyte-function antigen-1, and transforming growth factor (TGF)- β 1.
2. Identify the roles of plasma and tissue PG levels in chronic rejection for instance kidney PG production (PGE2, prostacyclin, TXA2).
3. Further studies of chronic rejection and post-transplant incorporation of fatty acids in other animal models such as apo E-KO mice, will also provide additional supportive data.
4. The use of Immunohistochemistry to investigate the role oxidized LDL, VCAM-1, ICAM-1, proliferating cell nuclear antigen (PCNA), and inflammatory markers, such as (Mac-3, CD4, CD8, MHC Class II) in the development of TCA lesions and chronic rejection.
5. Investigating the potential synergetic effect of these oils with other common immunosuppressive agents e.g. Tacrolimus.

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