THE EFFECTS OF DIETARY HEMPSEED ON PLATELET AGGREGATION AND CARDIAC ISCHEMIA/ REPERFUSION INJURY

BY

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A thesis submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the Degree of

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Department of Physiology
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And the National Centre for Agri-Food Research in Medicine
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The Effects of Dietary Hempseed on Platelet Aggregation and Cardiac Ischemia/Reperfusion Injury

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Dedication

I dedicate this thesis to my parents and to Ryan.

With their love and support, I can achieve anything.

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ABSTRACT

Cardiovascular disease (CVD) is a leading cause of mortality and is greatly influenced by lifestyle choices (3). Ischemic heart disease (IHD), a prominent form of CVD (3), can therefore be reduced by healthier dietary choices. One such choice is the consumption of functional foods. Functional foods are those which impart an additional health benefit over their traditional nutritional value (47). Hempseed, a functional food, is predominantly composed of protein and several health-promoting unsaturated fatty acids, including linoleic and alpha-linolenic acids. We hypothesized that dietary supplementation with hempseed would reduce platelet aggregation and protect the heart from arrhythmias derived from ischemia/ reperfusion (I/R) challenge. In addition, we hypothesized that hempseed would reduce cholesterol-induced platelet aggregation and protect the heart against cholesterol-induced arrhythmias derived from I/R challenge.

In this study, male New Zealand white (NZW) rabbits were fed for eight weeks on one of six dietary interventions: a control diet (RG), a control diet + 10% hempseed (HP), a control diet + 10% delipidated hempseed (DHP), a control diet + 0.5% cholesterol (OL), a control diet + 0.5% cholesterol + 10% hempseed (OLHP) and a control diet + 5% coconut oil (CO). After eight weeks, blood was collected by venipuncture and used for platelet aggregation studies as well as fatty acid, cholesterol ester and triglyceride analysis. Following blood collection, the heart was removed and perfused (within 60 seconds) with Tyrode's solution bubbled with 95%O_2, 5%CO_2. A 50 minute equilibration period ensured stable readings after which the heart was subjected to

global ischemia for 30 minutes and subsequent reperfusion for 45 minutes. ECGs were continuously recorded during both ischemia and reperfusion.

We did not observe any significant differences in animal weight amongst the groups prior to study commencement or upon termination. We found that hempseed supplementation significantly and selectively increased plasma levels of gamma-linolenic acid (GLA) and 20:3n-6 from control levels while delipidated hempseed supplementation did not significantly alter levels of plasma fatty acids when compared to control levels. Cholesterol supplementation significantly increased plasma levels of all fatty acids with the exception of GLA, while cholesterol plus hempseed supplementation resulted in a significant increase in plasma levels of all fatty acids. This indicates that cholesterol stimulates a non-selective absorption of fatty acids present in the diet. Accordingly, plasma levels of cholesterol esters and triglycerides were significantly increased in the OL and OLHP groups. Coconut oil supplementation significantly increased plasma levels of myristic acid (14:0) but had no effect on plasma concentrations of either cholesterol esters or triglycerides. Many of these plasma changes were also reflected in tissue fatty acid content. This included cardiac, hepatic and renal tissues.

Cholesterol supplementation caused a significant elevation in both ADP- and collagen-induced platelet aggregation in the OL group. This detrimental effect of cholesterol was reversed by the addition of hempseed to the cholesterol diet (OLHP) which brought platelet aggregation back to control levels. Hempseed alone, delipidated hempseed or coconut oil supplementation had no significant effect on platelet aggregation.

ECG traces were analyzed for arrhythmias during I/R challenge using the guidelines set forth in the Lambeth Conventions (109). Ischemia and reperfusion were treated as two distinct entities. We found no significant differences in either incidence or duration of ischemia-derived arrhythmias. We found a significant decrease in the incidence of fibrillation during reperfusion in the OL and OLHP groups as compared to the DHP group. We did not find any significant differences in the incidence of tachycardia or the duration of either tachycardia or fibrillation during reperfusion.

This study represents the first investigation to examine the effects of dietary hempseed on cardiovascular parameters. We can make several important conclusions. Firstly, supplementation with dietary hempseed results in minimal fatty acid absorption from the diet unless co-supplemented with dietary cholesterol. Secondly, dietary cholesterol significantly, but non-selectively, enhances fatty acid absorption of all fatty acids present in the diet. Thirdly, supplementation with dietary cholesterol increases the level and rate of platelet aggregation which can be returned to control levels by co-supplementation with dietary hempseed. Finally, after delipidation, hempseed may become pro-arrhythmic which may be due to the presence of an unknown pro-arrhythmic entity present in the seed. In conclusion, the ability of hempseed to reduce platelet aggregation in hypercholesterolemic individuals indicates that hempseed may have potential in reducing the incidence of cardiovascular disease but our data do not support a strong role for hempseed in decreasing the incidence or duration of arrhythmias in this model.

ACKNOWLEDGEMENTS

I would firstly like to thank Dr. Grant Pierce for being such a fantastic supervisor. The time I spent in his laboratory has been a wonderful experience, one which wouldn't have been possible without his guidance, encouragement and dedication.

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Last but definitely not least, I would like to thank my family and Ryan. They have given me unlimited encouragement while I pursue my education and my dreams. What they mean to me cannot be put into words and I am truly blessed to have them in my life.

Thank-you.

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I. INTRODUCTION

Polyunsaturated fatty acids (PUFAs) have been shown to possess cardiovascular health benefits through careful epidemiological, clinical and experimental studies. One of the foremost studies, conducted by Bang and Dyerberg, postulated that marine-derived foodstuff were able to reduce ischemic heart disease (16). It was proposed that PUFAs, namely eicosapentaenoic and docosahexaenoic acids, were responsible for this observed effect (17,18,40). Since this study, many others have demonstrated the positive role PUFAs and PUFA-containing foods have on cardiovascular health (6,14,36,38,53,75,89). Due to these unique health benefits, the term 'functional food' has emerged and has been applied to foods that impart an additional health benefit over their traditional nutritional value (47). Plant-derived functional foods, of which an excellent example is flaxseed, may present an advantage over fish or fish-derived oils as a source of PUFAs. This is due to gastrointestinal upset after consumption of large quantities of fish oil (26).

Hempseed comes from the flowering top of the hemp plant, a variety of *Cannabis sativa*. *Cannabis sativa* L. has been cultivated in many areas of the world (73) and the seeds have been used in both Arabic (73) and traditional folk medicine (44) to treat various disorders. Recent clinical trials have demonstrated the ability of hempseed oil to treat ear, nose and throat disorders (44) and atopic dermatitis (27). The beneficial properties of hempseed may be due to its complement of PUFAs. Hempseed possesses a ratio of omega-6: omega-3 PUFAs of ~4:1. This ratio has been recommended by Health Canada to promote well-being (50). In addition, hempseed provides an good source of protein (105) and vitamin E (2). Due to the deficit of current research on dietary hempseed, it is difficult to hypothesize how it may benefit cardiovascular health. The

goal of this study was to investigate the potential of dietary hempseed to provide a positive cardiovascular benefit. Specifically, we were interested in testing whether dietary hempseed could reduce platelet aggregation and/or cardiac ischemia/reperfusion injury, either of which would have a direct effect on cardiovascular health.

II. REVIEW OF LITERATURE

(A) Excitation-contraction (EC) coupling in the heart

Cardiac excitation-contraction (E-C) coupling is the mechanism by which cell surface depolarization leads to Ca^{2+} entry through the sarcolemma causing subsequent myocyte contraction. Ca^{2+} is integral in E-C coupling as it has been shown that blockage of Ca^{2+} entry into the cell leads to electrical and mechanical uncoupling (19). Specifically, during E-C coupling, an action potential (AP) travels along the sarcolemma and down the transverse-tubules (T-tubules). T-tubules are extensions of the sarcolemma that allow the AP to be relayed far into the myocyte (59). The AP causes L-type Ca^{2+} channels (L, long-lasting) to open which triggers Ca^{2+} entry into the myoplasm due to a large electrochemical driving force (61). This initial Ca^{2+} entry is not sufficient to initiate contraction but acts on the ryanodine receptor to trigger Ca^{2+} release from the sarcoplasmic reticulum (SR) (1). Increased myoplasmic Ca^{2+} allows an interaction between the myosin cross-bridges and the actin thin filaments which leads to cross-bridge cycling and myocyte contraction (1).

Relaxation is induced by the removal of Ca^{2+} from the myoplasm. In rabbit ventricular myocytes this is accomplished mostly by the sarcoplasmic/ endoplasmic reticulum Ca^{2+} ATPase (SERCA) (70%) and the Na^{+}/ Ca^{2+} exchanger (NCX) (28%) while the remainder is removed by the sarcolemmal Ca^{2+}-ATPase and mitochondrial Ca^{2+} uniporter (1% each) (22). Figure 1 details the mechanism of E-C coupling.

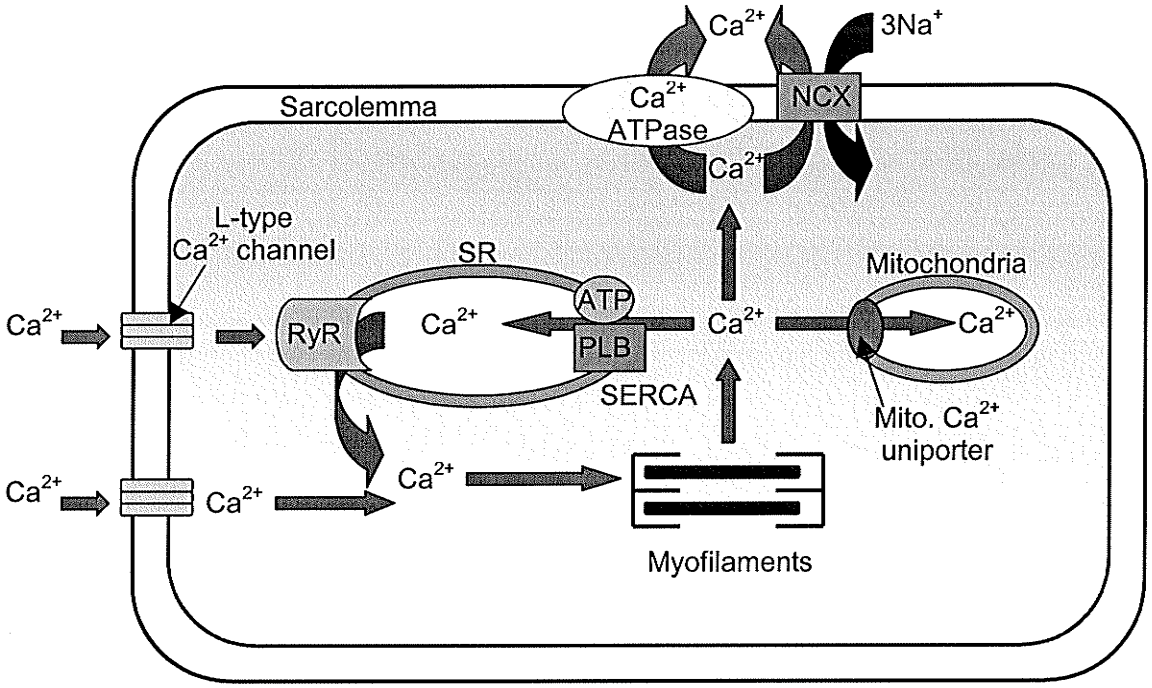
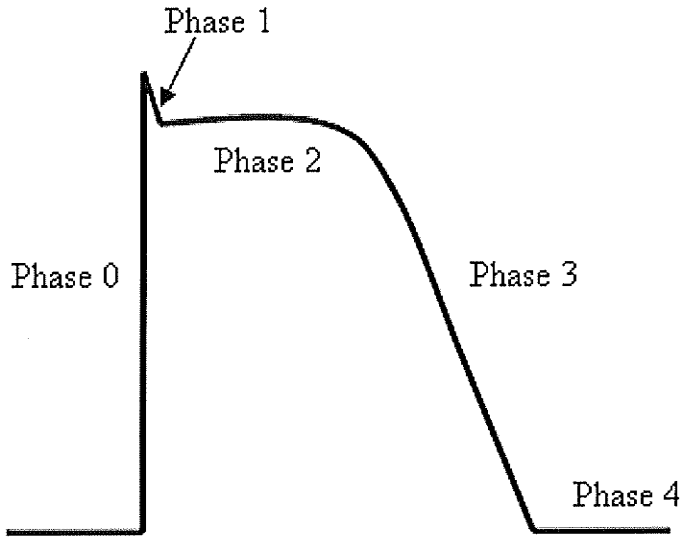


Figure 1: Excitation-contraction coupling in ventricular myocytes. Myoplasmic Ca^{2+} entry (red arrows) and exit (green arrows) during excitation and relaxation is detailed. NCX: Na^{+}/Ca^{2+} exchanger, SERCA: sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase, RyR: ryanodine receptor, PLB: phospholamban, SR: sarcoplasmic reticulum. Modified from (22).

(B) Cardiac action potential

There are two distinct types of cardiac AP, the slow response and the fast response. The slow response AP is characteristic of the sinoatrial and atrioventricular nodes. The fast response AP is characteristic of the atrial and ventricular myocytes and of the Purkinje fibres. The terms 'slow response' and fast response' refer to the rate of the action potential upstroke. The fast response AP has five distinct phases resulting from differing ion permeabilities and is represented in Figure 2.

- **Phase 0** is caused by the rapid entry of Na^+ through voltage-gated Na^+ channels ($i_{Na)}$.
- **Phase 1** is an early repolarization caused mainly by a transient outward K^+ current (i_{to1}) and, to a smaller degree, a transient inward Cl^- current (probably Ca^{2+}-activated, i_{to2}) (59).
- **Phase 2** is a plateau caused by a balance between depolarizing and repolarizing currents. *Depolarizing:* Ca^{2+} enters through the L-type Ca^{2+} channels ($i_{Ca,L}$) which open as the membrane voltage becomes progressively less negative (1). *Repolarizing:* K^+ exits through K^+ channels conducting the i_{to}, i_k and i_{k1} currents (1) and to a lesser extent through delayed rectifier channels (i_{Kr}, i_{Ks}) which open during this phase (59).
- **Phase 3** is a repolarization phase and is due to K^+ efflux through delayed rectifier channels (i_{Kr}, i_{Ks}) (59).
- **Phase 4** is the resting membrane potential and is maintained by the inward rectifying K^+ channels (i_{K1}). These channels close upon depolarization (59).



Phase 0: i_{Na}
Phase 1: i_{to1}, i_{to2}
Phase 2: Depolarizing: $i_{Ca,L}$
Repolarizing: i_{to}, i_{k}, i_{k1}, i_{Kr}, i_{Ks}
Phase 3: i_{Kr}, i_{Ks}
Phase 4: i_{K1}

Figure 2: The fast response cardiac action potential. This type of action potential is characteristic of the atria, Purkinje fibres and ventricles. Currents responsible for each phase are depicted below the schematic.

(C) Myocardial ischemia

Myocardial ischemia occurs when coronary artery flow is inadequate to meet myocardial O_2 demand (90). This phenomenon may occur via two mechanisms: 1) an increase in O_2 demand or 2) a deficit in O_2 supply (13). Rapidly after the onset of myocardial ischemia, mechanical performance (contractility) declines (8). The initial decline in contractility appears to be ATP–independent as illustrated by Figure 3. ATP levels begin to decline as substrates for oxidative phosphorylation, the principal ATP-producing pathway in the heart, become insufficient (59). Transiently, anaerobic glycolysis is increased (59) producing lactate and protons which accumulate (30). Eventually, due to an insufficient regeneration of NAD^+, anaerobic glycolysis will also cease (59). The accumulation of H^+ (acidosis) competes with Ca^{2+} for protein binding sites, which inhibits actin-myosin interactions (59). Acidosis also activates the Na^+/H^+ exchanger (NHE) (15) thereby increasing intracellular Na^+ levels (71,90). Increased intracellular Na^+ levels may raise myocyte osmotic pressure which could lead to cell membrane rupture (90). Normally, Na^+ would be removed by the Na^+/K^+ pump, but during ischemia, this pump is inhibited (15). Therefore, in an attempt to reduce intracellular Na^+ levels, Na^+ is extruded from the cell in exchange for Ca^+ which raises intracellular Ca^{2+} levels (15,106). This increase in intracellular Ca^{2+} may be countered, in part, by the ischemia-induced opening of ATP sensitive K^+ channels which decrease Ca^{2+} influx (56). Increased intracellular Ca^{2+} may cause contractile protein damage, phospholipase activation, contracture, myocyte depolarization and mitochondrial damage (90). Prolonged ischemia will lead to irreversible cardiomyocyte death (myocardial

infarction). The size of the infarcted area will continually increase with extended ischemia leading to a time dependent loss of myocardial function (24).

(D) Myocardial Reperfusion

Reperfusion restores a supply of oxygen and substrates to the myocardium while washing away metabolic wastes. Early reperfusion is essential as the rate of myocardial recovery depends on the length and/or severity of the prior ischemia (35). Paradoxically, reperfusion does not correct all ischemic pathology but may actually cause further injury such as myocardial stunning, reperfusion arrhythmias, accelerated death of damaged cells, microvascular damage (90) and apoptosis (120). For reperfusion injury to occur, two requirements must generally be satisfied: 1) reestablishment of flow and 2) oxygen delivery to the ischemic tissue (69). Reperfusion injury may be explained by several hypotheses: generation of oxygen radicals, Ca^{2+} overload, uncoupling of oxidative phosphorylation and/or sarcolemma damage (65). Reperfusion-induced production of oxygen free radicals (62,69) are hypothesized to cause membrane defects which may increase intracellular Ca^{2+} levels (41,63) thereby unifying several hypotheses. Increased intracellular Ca^{2+} causes multiple detrimental effects, as described in the previous section.

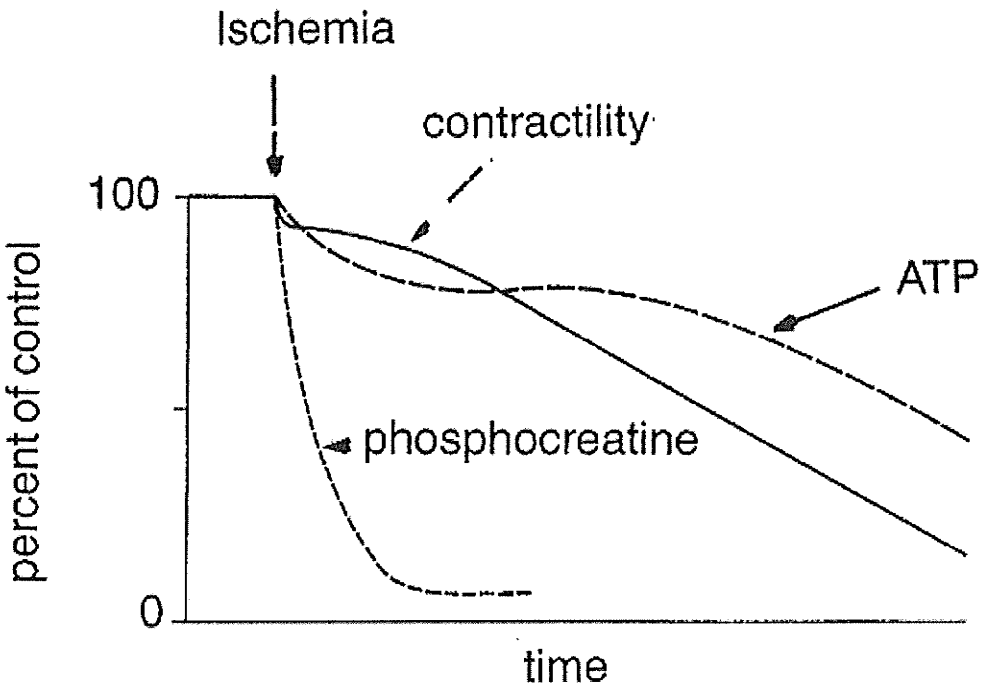


Figure 3: Ischemia onset and levels of contractility, ATP and phosphocreatine. Onset of ischemia leads to a rapid decline in contractility and phosphocreatine levels. ATP levels are more gradually depleted. (59)

(E) Cardiac conduction pathway and arrhythmias

The heart possesses automaticity and rhythmicity, i.e. the abilities to initiate and regulate its own beat, respectively (1). The main pacemaker of the heart is the sinoatrial (SA) node which is present at the junction between the superior vena cava and the right atrium. From the SA node, impulses travel throughout the atria to the atrioventricular (AV) node in the right atrium above the origin of the tricuspid valve. The impulse travels from the AV node, the source of impulses for ventricular excitation, though the Bundle of His and down the bundle branches to the Purkinje fibres to excite the ventricles.

Arrhythmias are variations from the normal heartbeat rhythm (60). Three mechanisms have been proposed to explain the development of ventricular arrhythmias: 1) abnormal automaticity, 2) re-entry and 3) afterdepolarizations (90). These mechanisms fall under two categories, disorders of impulse formation and disorders of impulse conduction. Abnormal automaticity and afterdepolarizations are disorders of impulse formation. Automaticity may develop in tissue other than nodal tissue, such as in the ventricles, which may lead to ectopic beat generation (90). Four or more consecutive ectopic beats constitute ventricular tachycardia (109) one type of ventricular arrhythmia. Afterdepolarizations can be early (EAD) or delayed (DAD). EADs occur prior to the end of the AP at a membrane voltage between -10 and -30mV while DADs occur after full repolarization (59). Both EADs and DADs can lead to ventricular arrhythmia (90). Re-entry is a disorder of impulse conduction and is when an impulse re-excites a myocardial region through which it has previously passed and which is no longer refractory (1) as seen in Figure 4. Rapid, regular re-entry circuits are one cause of ventricular tachycardia while micro-re-entry circuits are thought to trigger ventricular fibrillation (90).

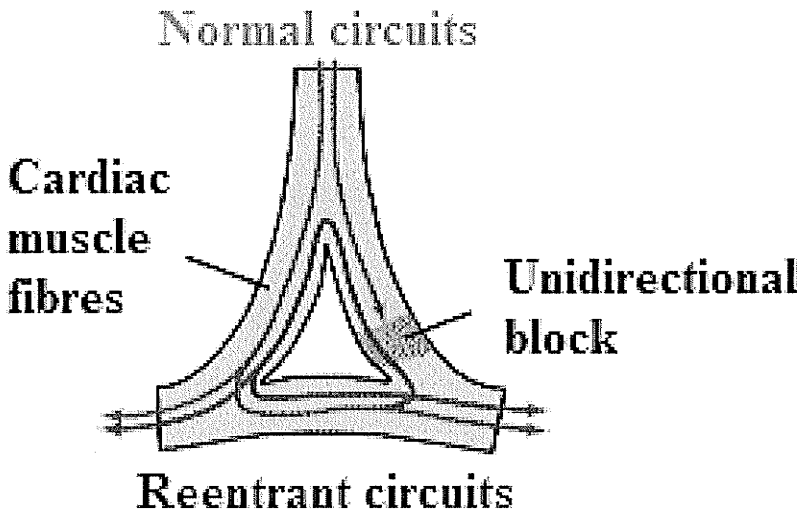


Figure 4: A schematic of a reentry circuit. In order for reentry to occur, a unidirectional block must exist which prevents conduction in one direction but not the other. As well, the area that the impulse would reenter must not be refractory (60).

(F) Fatty acids

Fatty acids are essential for health (118) and serve multiple functions such as energy storage and transport, insulation and mechanical protection (39). Fatty acids have a variable length carbon chain with a methyl terminus and a carboxylic acid head group (45). They can be categorized based on the degree of saturation of their carbon chains. Saturated fatty acids (SFAs) possess the maximum number of hydrogen atoms while monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) have one, or at least two carbon-carbon double bonds, respectively. PUFAs can be subdivided based on the position of the first double bond relative to the methyl terminus of the chain. The n-3 and n-6 families, two of the most biologically significant PUFA families, have their first double bond on either the third or sixth carbon from the methyl terminus, respectively. The methyl terminus is also known as the omega terminus (45), hence the common reference to these fatty acids as omega-3 or omega-6 PUFA. Several important PUFAs can be seen in Figure 5.

Linoleic acid (18:2n-6) and alpha-linolenic acid (18:3n-3) are essential fatty acids (EFAs) which must be acquired in the diet (45). Deficiency of EFAs can cause impaired growth and immune function, hair/skin abnormalities, kidney/liver disorders and premature death (99).

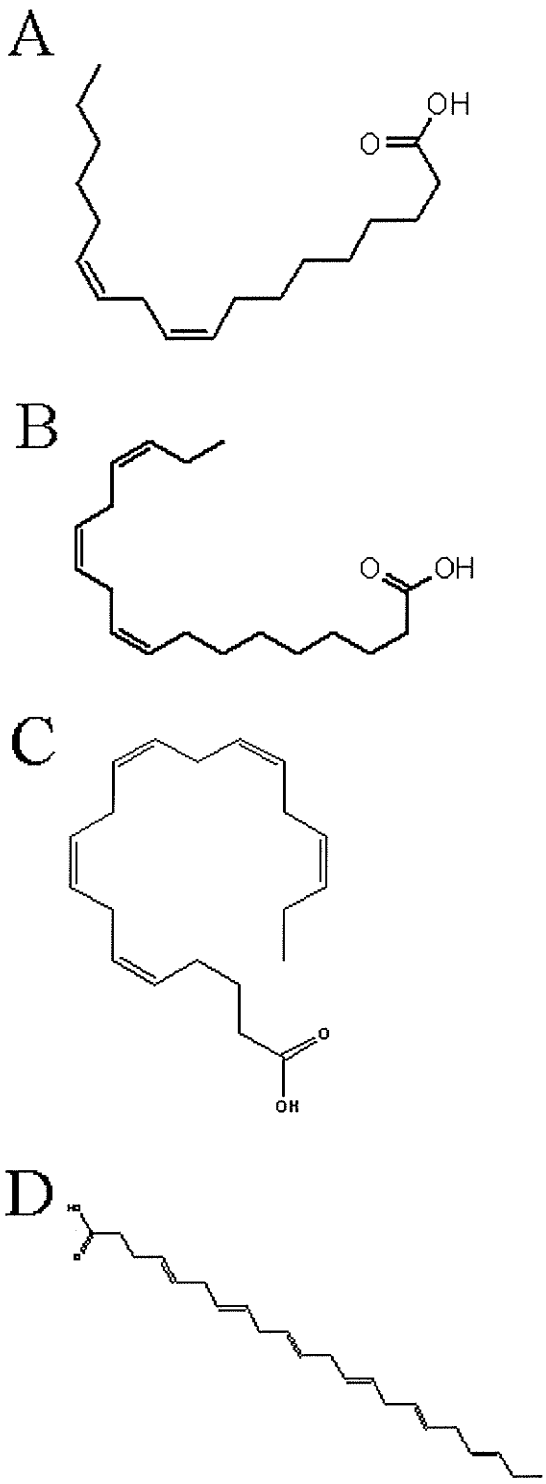


Figure 5: Structures of (A) linoleic acid, (B) alpha-linolenic acid, (C) eicosapentaenoic acid and (D) docosahexaenoic acid.

(G) Polyunsaturated fatty acid metabolism

Linoleic acid (LA) and alpha-linolenic acid (ALA) are metabolized to long chain n-6 and n-3 PUFA, respectively. In animals, downstream products are formed by sequential desaturation and elongation at the carboxyl terminus (99). PUFA metabolism is detailed in Figure 6. The desaturase and elongase enzymes are identical for both the n-3 and n-6 PUFA families (99) and this enzyme competition makes the n-6: n-3 PUFA ratio important for health. The Δ6 desaturase has been shown to decrease in activity with increasing age (29) indicating that, in older individuals, conversion of LA and ALA to their respective downstream products may be compromised.

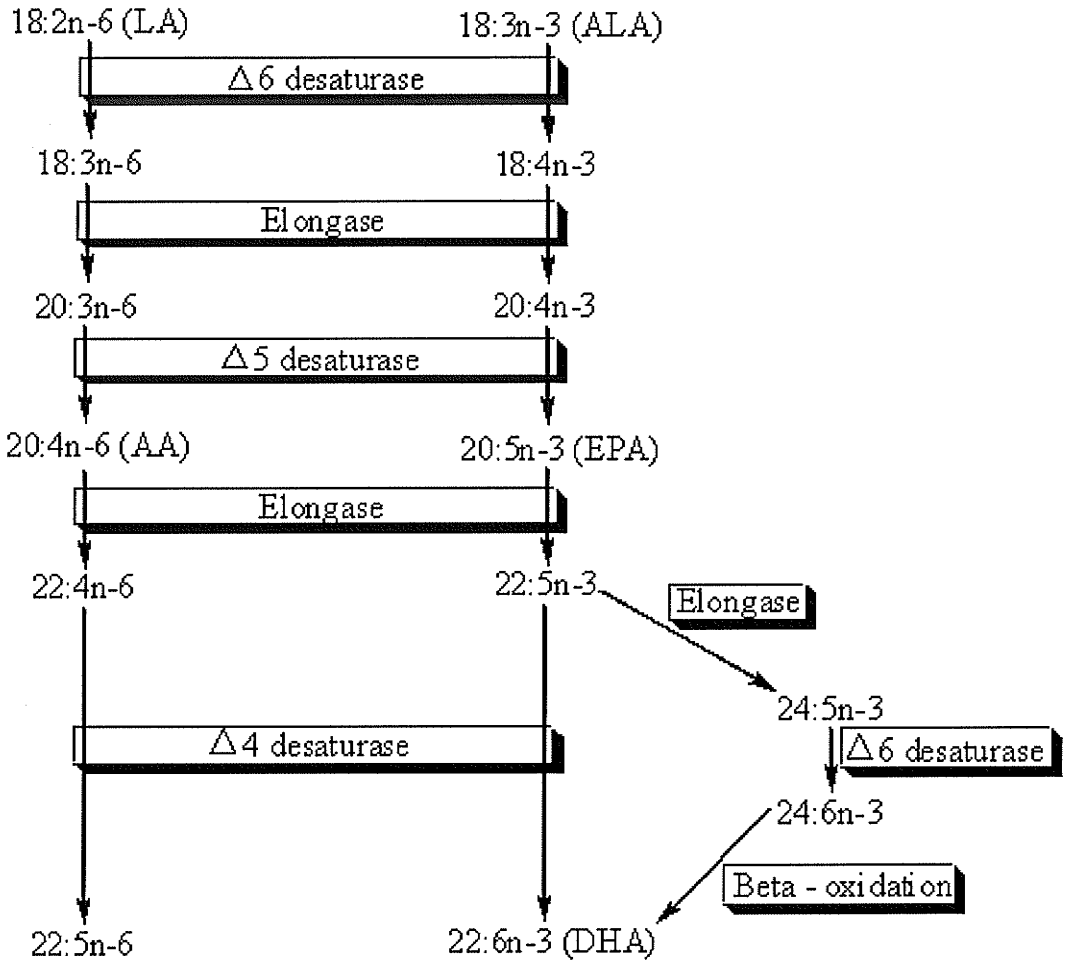


Figure 6: Metabolism of the n-6 and n-3 polyunsaturated fatty acid families. Both families use the same desaturase and elongase enzymes. This necessitates that a certain ratio of the n-6: n-3 families be present for optimal metabolism of both families.

(H) Importance of the n-6: n-3 PUFA ratio

Typically, the North American diet supplies an n-6: n-3 ratio of 10:1 – 25:1 (99). This is dramatically elevated over that recommended by Health Canada for an n-6: n-3 ratio of approximately 4:1 (50). This deviation from the recommended ratio is believed to promote disease pathogenesis (101) as this ratio appears to be an important factor determining cardiovascular health (110). Evidence from epidemiological studies supports the importance of a lowered n-6: n-3 ratio. A positive correlation has been observed between the n-6: n-3 ratio in platelet phospholipids and mortality from cardiovascular disease (100). In addition, the prevalence of NIDDM in India was decreased when the diet supplied an LA: ALA ratio of 6: 1 (101).

(I) Eicosanoids

Eicosanoids are fatty acids 20 carbon atoms in length and include the prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs). Eicosanoids are synthesized from arachidonic acid and eicosapentaenoic/ docosahexaenoic acids via two pathways, the cyclo-oxygenase (COX) pathway and the lipoxygenase (LOX) pathway. The former pathway produces prostaglandins and thromboxanes while the latter pathway produces leukotrienes. These pathways are named for their key enzymes, cyclo-oxygenase and lipoxygenase, respectively. Arachidonic and eicosapentaenoic/ docosahexaenoic acids produce different metabolites of the same families while utilizing the same enzyme systems (5). The metabolism of the n-6 and n-3 PUFA families to form eicosanoids is shown in Figure 7.

The eicosanoids have been shown to play a role in arrhythmogenesis. It was observed that during myocardial ischemia, arachidonic acid metabolites of the COX pathway were formed (91). These metabolites were found to cause arrhythmias in cultured neonatal rat cardiomyocytes (57). Conversely, prostaglandin products of the COX pathway have been shown to decrease ischemia/ reperfusion-induced arrhythmias (91). In addition to arrhythmogenesis, arachidonic acid-derived thromboxane A_2 (TXA_2) is a potent vasoconstrictor and platelet aggregator.

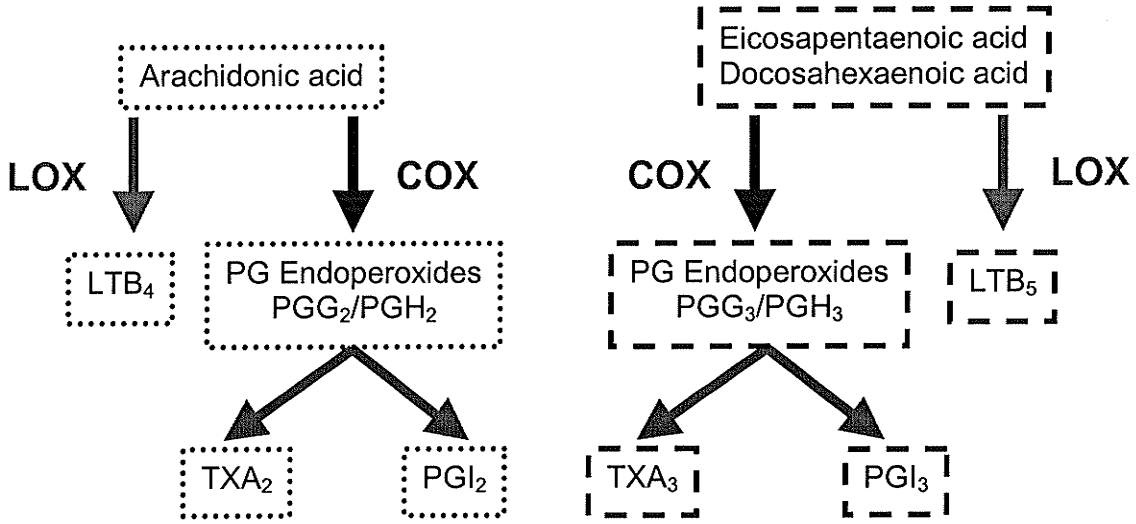


Figure 7: Eicosanoid production from arachidonic, eicosapentaenoic and docosahexaenoic acids. LOX: lipoxygenase, COX: cyclo-oxygenase, LT: leukotriene, PG: prostaglandin, TX: thromboxane. Modified from (5).

(J) Cardiovascular benefits of PUFA

PUFAs have been brought to the forefront in the prevention of cardiovascular disease as they possess hypocholesterolemic, hypotriglyceridemic, anti-thrombotic and anti-arrhythmic properties. Below is a summary of the actions of several important PUFAs from both the n-3 and n-6 families.

i) Linoleic acid (LA)

Linoleic acid is the parent fatty acid of the n-6 PUFA family and approximately 12-15g/ day is ingested in a typical North American diet (50). Human studies suggest that LA plays a role in the reduction of cardiovascular disease (CVD). The Health Professionals Follow-up Study observed an inverse relationship between LA intake and fatal coronary heart disease (CHD) (14) while in a separate study, higher adipose levels of LA have been related with lower CHD mortality (110). Myocardial infarction survivors were found to have a significantly lower quantity of LA in red-cell membrane phosphatidylcholine (which indicates long-term intake of dietary fat) as compared to individuals with no evidence of heart disease (102). The ability of LA to reduce CHD mortality may be due to its total- and LDL-cholesterol lowering ability (28,119) as cholesterol levels are an independent risk factor for CVD.

Non-epidemiological evidence also demonstrates the cardiovascular benefits of LA. In a rat model, an LA-rich diet significantly reduced the incidence and/or duration of arrhythmias during and/or after coronary artery occlusion (67,78,79,80). In addition, rats fed an LA-rich diet demonstrated a significantly reduced number of ventricular extra beats and a significant reduction in infarct size (78). Furthermore, in a non-human

primate model, an LA-rich diet significantly increased the fibrillation threshold during ischemia (82).

The role of LA as an anti-arrhythmic may be due to its action on ion channels. LA has been documented to increase $I_{K(Ca)}$ in smooth muscle cells (121) which would accelerate repolarization in situations of Ca^{2+} overload (59). Conversely, LA has also been found to significantly inhibit I_K and I_{to} (115) which would lengthen the duration of the action potential. I_{K1}, which maintains the resting membrane potential, was not inhibited by LA (115). Therefore, the ability of the cell to maintain its resting membrane potential is most likely unchanged.

In addition to anti-arrhythmic effects, LA has been shown to affect other cardiovascular parameters. In a rabbit model, supplementation with corn oil (as a source of LA) has been shown to significantly decrease both total- and LDL-cholesterol concentrations (20). In a rat model, dietary sunflower seed oil (source of LA) has been shown to significantly reduce plasma triglyceride levels in comparison to a control diet (77). Evening primrose oil (source of LA and gamma-linolenic acid) has been shown to significantly reduce total cholesterol and triglycerides and significantly raise HDL-cholesterol in rabbits fed an atherogenic diet (33). Evening primrose oil has also been shown to significantly reduce the response of platelets to pro-aggregatory factors in rabbits fed an atherogenic diet (33). However, LA cannot be wholly recommended as an agent to reduce cardiovascular disease as it is metabolized to arachidonic acid, the precursor for the pro-thrombotic and pro-aggregatory eicosanoids.

ii) Alpha-linolenic acid (ALA)

ALA is the parent fatty acid of the n-3 PUFA family and approximately 1-3g/ day is ingested in a typical North American diet (50). Several human studies have observed cardiovascular benefits associated with dietary ALA. The Lyon Diet Heart Study focused on a Mediterranean diet rich in ALA for secondary prevention. A protective effect of this diet was observed with a significant reduction in the rates of cardiovascular events (36). The Indian Experiment of Infarct Survival supplemented patients with suspected acute MI with mustard oil (as a source of ALA) and found a significant reduction in total cardiac events, non-fatal infarctions and total cardiac arrhythmias (103). In a study nested in the Cardiovascular Health Study, fatty acid levels were measured in plasma collected previous to cardiac events. It was found that in older adults, higher levels of ALA tended to lower the risk of fatal ischemic heart disease (64). The Nurses Health Study recruited registered female nurses to complete lifestyle and medical history questionnaires. It was observed that nurses who consumed higher levels of ALA had a lower relative risk of fatal ischemic heart disease (53). A study conducted in Costa Rica examined levels of ALA in adipose tissue in relation to nonfatal acute MI. It was found that ALA was associated with a significant reduction in the risk of this event (21). In a study conducted with participants of the National Heart, Lung, and Blood Institute Family Heart Study, a food frequency questionnaire was used to collect data on total linolenic acid (alpha- and gamma-) intake. It was found that total linolenic acid intake was inversely correlated with serum triacylglycerol concentrations (37). In a small clinical study, an ALA-rich diet was shown to significantly reduce levels of total- and LDL-cholesterol (119). In addition, supplementation with flaxseed oil (source of ALA) was shown to significantly reduce

platelet aggregation (10) while ALA itself was also shown to reduce platelet aggregation (104).

Non-clinical evidence provides support for the cardiovascular benefits described above. In a dog model, ALA was found to protect against ischemia-induced fatal ventricular arrhythmia (23) while in a rabbit model, dietary flaxseed (source of ALA) was found to significantly reduce the incidence of ischemia-derived ventricular fibrillation (12). In addition, canola oil supplementation (source of ALA and oleic acid) was found to significantly lower the incidence of ventricular fibrillation and mortality during reperfusion in comparison to olive oil supplementation (source of oleic acid) (83). Finally, in a rabbit model, flaxseed oil supplementation (a source of ALA) was found to significantly lower platelet aggregation (108).

The anti-arrhythmic actions of ALA may be due to its effects on ion channel conductance. ALA significantly inhibited I_{Na} in neonatal rat cardiomyocytes (112) which would hinder the generation of an action potential. In addition, ALA was found to inhibit T-type I_{Ca} (31) which would prevent Ca^{2+} entry. ALA was observed to have both stimulatory (54) and inhibitory (111) effects on I_{Ca}. This inconclusive evidence may result from a difference in species or cell age. ALA also significantly inhibited I_K and I_{to} (115) which would increase the duration of the action potential. ALA had no effect on I_{K1} (115), therefore, the ability of the cell to maintain its resting membrane potential is most likely unchanged. ALA has also been observed to stimulate Na^+-Ca^{2+} exchange (95). During ischemia, the Na^+/Ca^{2+} exchanger extrudes Na^+ in exchange for Ca^{2+} and increasing its function would reduce the Na^+ load in the cell. However, this would also lead to increased Ca^{2+} entry which is detrimental.

iii) Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)

EPA and DHA are downstream metabolites of the n-3 family. The typical North American diet provides approximately 0.10 – 0.15g/day of these fatty acids (50). The observation that Greenland Eskimos were resistant to ischemic heart disease prompted the hypothesis that marine derived foodstuff (as a source of unsaturated fatty acids) may be cardioprotective (16). Since then, several human studies have demonstrated a definite cardiovascular benefit of fish consumption and of EPA and DHA supplementation. The US Physicians Health study was a prospective cohort study which questioned male physicians about their fish consumption. An inverse relationship was observed between fish consumption and the risk of sudden cardiac death (6). The Nurses Health Study recruited registered female nurses to complete lifestyle and medical history questionnaires. A significant inverse relationship was observed for the consumption of fish and omega-3 PUFAs and the incidence of CHD events (52). The Finnish, Italian and Dutch cohorts of the Seven Countries Study demonstrated an inverse relationship between fatty fish consumption and 20-year CHD mortality (89). The Multiple Risk Factor Intervention Trial (MRFIT) was a clinical trial designed to study the primary prevention of CHD. A significant inverse relationship was observed between the predominant fatty acids found in fish and mortality from CHD, CVD and all-causes (38). GISSI-Prevenzione was a clinical trial investigating the efficacy of long-chain n-3 PUFAs (EPA and DHA) and/or vitamin E in patients with recent myocardial infarction (MI). An early, highly significant reduction in sudden cardiac death was observed with n-3 PUFA supplementation (75). The Indian Experiment of Infarct Survival Study investigated the efficacy of fish oil in patients with suspected acute MI. After one year of

supplementation, a significant reduction in total cardiac events, non-fatal infarctions and cardiac deaths were observed (103). A prospective study in men from Shanghi, China showed that those who consumed over 200g/week of fish and shellfish had a 59% reduction in the risk of fatal MI compared to those consuming less than 50 g/week (117). Finally, in individuals from a Japanese fishing village, an increased threshold to 50% maximal platelet aggregation was observed as compared to a Japanese farming village (49). This finding was observed in association with a significant elevation in plasma levels of EPA, DHA and AA as well as an increase in the EPA: AA ratio (49).

Animal studies also suggest that EPA and DHA provide protection from cardiovascular disease. In a rat model, fish oil supplementation led to a significant reduction in the incidence and/or duration of arrhythmias during both ischemia and reperfusion (79,80,93). It was found that both rat (93) and non-human primate (82) hearts from animals supplemented with fish oil had a higher fibrillation threshold. In addition, non-human primate hearts were found to have a significantly higher left ventricular ejection fraction and end diastolic volume (81). In a rat model, fish oil supplementation significantly decreased the myocardial oxygen consumption (MVO_2), significantly increased the efficiency of cardiac oxygen use and significantly increased the cardiac output of reperfused hearts compared to respective controls (94).

The anti-arrhythmic effects of fish oil may be due to the actions of EPA and DHA on ion channels. EPA and DHA have been observed to inhibit voltage-gated sodium channels (74,112,114) which would hinder the generation of an action potential. EPA and DHA were found to inhibit I_K (115) and I_{to} (74) which would lengthen the duration of the action potential. EPA and DHA were found to have no effect (115) on I_{K1} which is

responsible for maintaining the resting membrane potential. EPA and DHA had an inhibitory effect on L-type $I_{Ca,}$ (74). Inhibition of L-type $I_{Ca,}$ may shorten action potential duration thereby maintaining electrical stability and diminishing arrhythmogenesis (11). In addition, EPA and DHA were found to inhibit T-type I_{Ca} (31) which would prevent Ca^{2+} entry. EPA has been shown to inhibit the release of Ca^{2+} from the SR (86), and more specifically, both EPA and DHA have been shown to reduce the ryanodine receptor channel open probability (51). Inhibiting the release of Ca^{2+} from the SR, which may become overloaded post-ischemia, may help prevent Ca^{2+} triggered arrhythmias (86). Actions of EPA and DHA are not limited to channels as they have been shown to decrease the activity of the sodium-hydrogen exchanger (NHE) (43). The NHE is activated during ischemia and functions to exchange H^+ for Na^+. In addition, EPA and DHA have been shown to significantly inhibit the cardiac Na^+/Ca^{2+} exchanger (NCX) current (113). During ischemia, the NCX removes Na^+ in exchange for Ca^{2+} thereby raising intracellular Ca^{2+} levels.

Effects of EPA and DHA, other than those on ion channels, may also play a role in improving cardiovascular health. Platelet aggregation stimulated by thrombin, collagen and ADP was significantly lower in a fish oil supplemented diet when compared to a corn oil supplemented diet (108). It has also been observed that supplementation with fish oil elevates the concentration of inducer (ADP/ collagen/ arachidonic acid) required to reach 50% maximal platelet aggregation (70).

(K) Functional foods

Functional foods have been identified to play a key role in protection against cardiovascular disease. While no definitive classification has been applied, a functional food is considered to be any potentially healthful food that may provide a health benefit beyond its traditional nutritional value (47). This is in contrast to nutraceuticals, which are food extracts and are presented in a form not usually associated with food (i.e. pills, oils, etc.) (55). Several well-known functional foods include soybeans, oats, garlic, tea, flaxseed and fish (48). The two latter foods, flaxseed and fish, contain polyunsaturated fatty acids (PUFAs), and have been of particular interest in reducing cardiovascular disease.

(L) Hempseed

Hempseed comes from the flowering top of the hemp plant, *Cannabis sativa* L, an annual herbaceous plant which grows 4-15 feet in height (4). Canadian hempseed typically contains ~2ppm THC although Canadian regulation limits THC content to 10ppm for hempseed products (68). Hempseed can be hulled, without loss of nutritional value, to make a more malleable seed. Hulled hempseed is composed primarily of fatty acids (44g fatty acids/100g hulled hempseed) and protein (33g protein/ 100g hulled hempseed) as detailed in Figure 8. The fatty acids in hulled hempseed are principally unsaturated (39g unsaturated fatty acids/100g hulled hempseed) with only a small proportion in a saturated form (5g saturated fatty acids/100g hulled hempseed). After hempseed oil extraction, the composition of the fatty acids can be ascertained (Table 1). It can be seen that the prominent fatty acids in hempseed oil are linoleic acid and alpha-

linolenic acid which form 55.2% and 20.0% of the total fatty acids, respectively. These two fatty acids are termed essential fatty acids as they cannot be manufactured by the body. The protein content of hulled hempseed (33g protein/100g hulled hempseed) is composed mainly of edestin (65%), a legumin class reserve protein (92) while the remainder is albumin (35%) (2). The proteins present in hempseed provide all essential amino acids (105). The carbohydrates present are mostly dietary fibre (7%) although low levels of sugars (3%) are also present (2). Hempseed is a source of vitamin E (mostly gamma-tocopherol) providing 100-150 mg/100g (2) but its levels are dependant upon seed maturity (96).

There is a limited amount of research on the health-related benefits of hempseed. Historically, hempseed has been used in both Arabic (73) and folk medicine (44). In Arabic medicine, it has been used as a diuretic, to evacuate bile and phlegm and in the treatment of tapeworms, vitiligo (a skin disorder) and leprosy (73). In folk medicine, a decoction from crushed hempseed has been used to treat urinary tract inflammation and inflammation of the prostate (44). Hempseed oil has been used in Arabic medicine to treat ear and skin diseases as well as tumours and as a painkiller (73). However, this information must be viewed cautiously as hempseed used at that time was probably not of the industrial type and may have contained higher levels of THC. In an open clinical trial, hempseed oil was used to lessen treatment time of ear, nose and throat disorders (44). In addition, clinical symptoms of atopic dermatitis were beneficially affected by dietary hempseed oil (27). This is thought to be due to the linoleic and gamma-linolenic acids present in hempseed (32).

Hempseed appears to be a promising functional food; however, due to the lack of research, it is difficult to know if hempseed may benefit cardiovascular health. This study is a necessary first step in elucidating the possible cardiovascular benefits of hempseed.

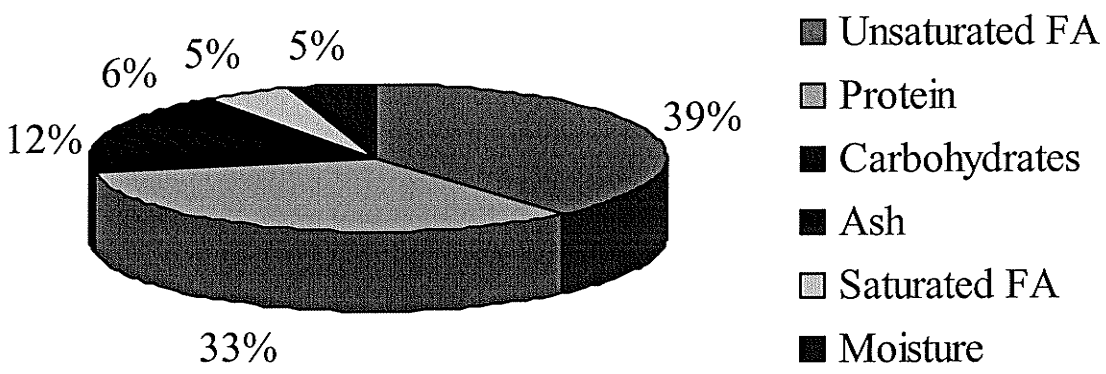


Figure 8: Composition of hulled hempseed. Every 100g of hulled hempseed provides 39g of unsaturated fatty acids, 33g protein, 12g carbohydrates, 6g ash, 5g saturated fatty acids and 5g moisture.

Modified from the Hemp Oil Canada Inc. website (2)

Table 1: Fatty acid composition of hempseed oil.

	% of total fatty acids
Saturated fatty acids	
Palmitic acid (16:0)	6.50%
Margaric acid (17:0)	0.03%
Stearic acid (18:0)	2.50%
Arachidic acid (20:0)	0.40%
Behenic acid (22:0)	0.20%
Lignoceric acid (24:0)	0.06%
Monounsaturated fatty acids	
Palmitoleic acid (16:1)	0.12%
Oleic acid (18:1n-9)	10.50%
Polyunsaturated fatty acids	
Linoleic acid (18:2n-6)	55.20%
Gamma-linolenic acid (18:3n-6)	3.10%
Alpha-linolenic acid (18:3n-3)	20.00%
Stearidonic acid (18:4n-3)	1.20%
Eicosaenoic acid (20:1)	0.50%
Total fatty acids	**≈100%**

Modified from the Hemp Oil Canada Inc. website (2)

III. HYPOTHESES

1. Supplementation with dietary cholesterol will increase platelet aggregation induced by ADP/ collagen and increase the incidence and/ or duration of arrhythmias during ischemia/reperfusion (I/R) challenge in comparison to a control diet.
2. Supplementation with dietary hempseed will induce a decrease in platelet aggregation induced by ADP/ collagen and decrease the incidence and/ or duration of arrhythmias during I/R challenge in comparison to other dietary interventions.
3. Co-supplementation of dietary hempseed and dietary cholesterol will decrease cholesterol-induced platelet aggregation and decrease the cholesterol-induced increase in incidence and/or duration of arrhythmias during I/R challenge.

IV. OBJECTIVES

1. To determine if the rate and extent of platelet aggregation is influenced by dietary cholesterol, coconut oil or hempseed using two distinct pro-aggregatory stimuli.
2. To determine if dietary cholesterol, coconut oil or hempseed could alter plasma cholesterol and triglyceride levels as well as fatty acid content and composition.
3. To determine if dietary cholesterol, coconut oil or hempseed could alter tissue (cardiac, hepatic, renal) fatty acid content and composition.
4. To determine if dietary cholesterol, coconut oil or hempseed could alter the length of the QT interval during the equilibration period.
5. To determine if dietary cholesterol, coconut oil or hempseed could alter left ventricular end diastolic pressure and left ventricular developed pressure during ischemia and reperfusion.
6. To determine if dietary cholesterol, coconut oil or hempseed could alter the incidence and duration of arrhythmias during ischemia and reperfusion.
7. To determine if dietary hempseed can reverse any or all of the electrophysiological and metabolic changes induced by dietary cholesterol.

V. MATERIALS AND METHODS

(A) Materials

All materials and equipment used in this study are listed in Table 2.

Table 2: List of materials.

Material	Source
Acetyl chloride (C_2H_3ClO)	Sigma Aldrich
AcqKnowledge 3.7.3 Software	Biopac Systems
Adenosine diphosphate (ADP)	Chrono-log Corporation
Aggregometer	Chrono-log Corporation
Analytical balance	Mettler Toledo
Benzene (C_6H_6)	Omnisolv
Calcium chloride ($CaCl_2$)	Sigma-Aldrich
Centrifuge	Eppendorf
Chloroform ($CHCl_3$)	Fisher Scientific
Cholesterol	Federal Co-operatives Limited
Cholesterol standard	Sigma Diagnostics
Coconut oil	Kissan International
Coffee Grinder	Braun
Collagen	Chrono-log Corporation
CO-OP Complete Rabbit Ration	Federal Co-operatives Limited
Custom perfusion apparatus	Dr. Anton Lukas
Delipidated hempseed	Hemp Oil Canada Incorporated
Dextrose	Fisher Scientific
Dry bath incubator	Fisher Scientific
Gas chromatograph: Varian GC/MS/MS with CP-3800 GC, CP-8400 autosampler and a Flame Ionization Detector (FID)	Varian
Hempseed	Hemp Oil Canada Incorporated

Heparin	Sigma-Aldrich
Homogenizer	Polytron
Incubator	Lab-Line
Infinity Cholesterol Liquid Stable Reagent	Thermo Electron Corporation
Magnesium chloride ($MgCl_2$)	Fisher Scientific
Male New Zealand White Rabbits	Southern Rose Rabbitry Farm
Methanol (CH_3OH)	Fisher Scientific
Microplate reader	Dynex Technologies
Microplate shaker	Labsystems
MP100 data acquisition system	Harvard Apparatus Canada
95% N_2, 5% CO_2 (gas)	Welders Supply (Winnipeg, MB)
N_2 (liquid)	Medigas
95% O_2, 5% CO_2 (gas)	Welders Supply (Winnipeg, MB)
Potassium carbonate (K_2CO_3)	Fisher Scientific
Potassium chloride (KCl)	Sigma-Aldrich
Rotary evaporator	Büchi
Sodium bicarbonate ($NaHCO_3$)	Sigma-Aldrich
Sodium chloride (NaCl)	Sigma-Aldrich
Sodium phosphate (NaH_2PO_4)	Sigma-Aldrich
Sodium sulfate (Na_2SO_4)	Fisher Scientific
Triglyceride standard	Sigma Diagnostics
Triglycerides Liquid Incorporating Dynamic Stabilization Technology	Thermo Electron Corporation
Vacutainer blood collection needles	Becton-Dickinson
Vacutainer holder	Becton-Dickinson
Vacutainers with EDTA	Becton-Dickinson
Vacutainers with sodium citrate	Becton-Dickinson
Vortex	Thermolyne
Whatman filter paper	Fisher Scientific

(B) Interventional diets

Six interventional diets were made: 1) control, 2) a control diet supplemented with (w/w) 10% hempseed, 3) a control diet supplemented with (w/w) 10% partially delipidated hempseed, 4) a control diet supplemented with (w/w) 0.5% cholesterol, 5) a control diet supplemented with (w/w) 10% hempseed and 0.5% cholesterol, 6) a control diet supplemented with (w/w) 5% coconut oil. Hempseed was ground in a Braun coffee grinder, water was added to the partially delipidated hempseed and the coconut oil was heated to 25°C to facilitate homogenous distribution with the control diet. During mixing, approximately equal amounts of water were added to each interventional diet. Once mixed, interventional diets were ground to resemble rabbit chow, spread in a thin layer and air dried over a period of 2-3 days. The diets were stored at 4°C until used.

(C) Animals

Male New Zealand white (NZW) rabbits were acquired from Southern Rose Rabbitry in Manitoba. A mandatory conditioning period of 7 days preceded interventional diet commencement. Rabbits were maintained at a constant temperature in a 12 hour light/dark cycle.

(D) Feeding

Each rabbit received 250g/day of one of six interventional diets and were continuously monitored for weight and health. Rabbits were fed their respective diets for 56 days (8 weeks).

(E) Blood/ Plasma

i) Blood collection

Approximately 20mL of blood was acquired from each rabbit by venipuncture of the jugular vein. Collection of blood was in vacutainers containing either sodium citrate or EDTA to prevent blood coagulation. Tubes containing EDTA were put immediately on ice after blood collection. Tubes containing sodium citrate remained at room temperature.

ii) Platelet aggregation analysis

Blood collected with sodium citrate remained at room temperature for 30 minutes. Following this period it was centrifuged at 100xg for 15 minutes to isolate the platelet rich plasma (PRP). The plasma was re-centrifuged at 2400xg for 15 minutes to isolate the platelet poor plasma (PPP) which was used as a blank for platelet aggregation. Either collagen (4 ug/mL) or adenosine diphosphate (10uM) was added to the PRP to induce platelet aggregation. Both the extent (% aggregation) and rate of platelet aggregation (% change/ min) was recorded with a Chrono-log Aggregometer.

iii) Plasma collection

Blood collected with EDTA was centrifuged at 1800xg for 10 minutes at 4°C. The red blood cell-free plasma was removed, aliquoted and snap-frozen in liquid nitrogen. Plasma was stored at -80°C.

iv) Triacylglycerol (TG) assay

Plasma TG levels were measured enzymatically. Triglycerides Liquid Incorporating Dynamic Stabilization Technology reagent was pre-warmed (37°C) in a 96 well micro-plate. One of 0.9% NaCl (w/v), standard (250 mg/dL TG) or plasma sample was added to the warmed reagent in a 1: 100 ratio. Gentle agitation facilitated mixing. After 5 minutes of incubation at 37°C, the absorbance of each well was measured at 500nm. Based on the standard reading, the concentration of TG was determined (see below):

$$[TG] = \frac{\text{absorbance of unknown}}{\text{absorbance of standard}} \times \text{concentration of standard (mg/dL)}$$

Any plasma sample with excess lipid was diluted in 0.9% NaCl (w/v) to prevent interference with spectrophotometric analysis. Data was corrected for any dilution performed.

v) Cholesterol ester (CE) assay

Plasma CE levels were measured enzymatically. Infinity™ Cholesterol Liquid Stable reagent was pre-warmed (37°C) in a 96 well micro-plate. One of 0.9% NaCl (w/v), standard (200 mg/dL CE) or plasma sample was added to the warmed reagent in a 1: 100 ratio. Gentle agitation facilitated mixing. After 5 minutes of incubation at 37°C the absorbance of each well was measured at 500nm. Based on the standard reading, the concentration of CE was determined (see below):

$$[CE] = \frac{\text{absorbance of unknown}}{\text{absorbance of standard}} \times \text{concentration of standard (mg/dL)}$$

Any plasma sample with excess lipid was diluted in 0.9% NaCl (w/v) to prevent interference with spectrophotometric analysis. Data was corrected for any dilution performed.

(F) Tissue Collection

One lobe of liver and one kidney was collected. All tissues were washed in 0.9% NaCl (w/v) and snap frozen in liquid nitrogen. Tissues were stored at -80°C.

The heart was collected after use in Langendorff method of ischemia/reperfusion. The heart was snap frozen in liquid nitrogen and stored at -80°C.

(G) Fatty acid analysis

i) Fatty acid extraction

Tissue (cardiac, hepatic, renal)

Fatty acids were extracted from the heart, liver and kidney by the Folch method (42). Briefly, organ pieces were minced and weighed to 1 g. These tissues were then homogenized in 5mL of 2:1 (v/v) $CHCl_3$:CH_3OH using a Polytron homogenizer (3x10 seconds at 7200 RPM). Next, the homogenate was transferred to a graduated cylinder, and the original homogenization tube was refilled with 5mL of 2:1 (v/v) $CHCl_3$:CH_3OH. The solution was homogenized again using the above method and transferred into the same graduated cylinder containing the first batch of homogenate. This step was repeated one more time. It is important to note that the homogenization step was performed in a cold room (4°C) with samples maintained on ice.

In a fume hood, the homogenate was poured into a separatory funnel. The empty tube was then rinsed with 2 x 2.5mL portions of 2:1 (v/v) $CHCl_3$:CH_3OH and the rinses added to the separatory funnel. Next, 4.2mL of 0.73% NaCl (w/v) was added. Extraction of the lipids into the organic phase resulted from vigorous agitation ensuring adequate mixing of the organic and aqueous layers. Separation of the two phases occurred overnight (~16 hours). The organic layer was then collected, dried over Na_2SO_4 and filtered into a pre-weighed round bottom flask. Removal of the $CHCl_3$ was facilitated using a rotary evaporator (40°C), after which the lipids were further dried under a steady stream of N_2(g). The round bottom flask was then reweighed to determine the overall lipid weight. Samples were then resuspended in 3mL of $CHCl_3$ and stored at -20°C.

Interventional diets

Fatty acids were extracted from the interventional diets by an adaptation of the Folch method (42). The interventional diet was ground (Braun coffee grinder), weighed to 1g in a glass beaker and then transferred to a separatory funnel. From a 40mL volume of 2:1 (v/v) $CHCl_3$:CH_3OH, an aliquot was used to rinse out the beaker and the washing was transferred into the separatory funnel. This was repeated two more times. Next, the remaining solvent was added to the funnel such that the total volume of solvent was 40mL. Next, 8.4mL of 0.73% NaCl (w/v) was added and the funnel was shaken vigorously. Following separation of the organic and aqueous phases (~16 hours), the bottom $CHCl_3$ layer was collected and then the aqueous layer was once again extracted with 40mL of the 2:1 (v/v) $CHCl_3$:CH_3OH mixture and 8.4mL of the NaCl solution. Once again, the organic layer was collected and combined with the first batch. The lipid

solution was dried over Na_2SO_4, filtered and the solvent removed via rotary evaporation (40°C). The exposed lipids were then dried under N_2(g). Lipid weights were determined using the same methodology as previously described. Samples were then stored in 3mL of $CHCl_3$ at -20°C.

ii) Fatty Acid Esterification

Fatty acids were esterified by the Lepage method (66). For plasma fatty acid esterification, samples of plasma were added to a 2mL solution of 4:1 (v/v) CH_3OH:C_6H_6 after which two-hundred microliters of C_2H_3ClO were then added and the mixture vortexed. For extracted tissue/diet esterification, an aliquot of the extract in $CHCl_3$ was placed under a stream of nitrogen to evaporate the solvent. Following this, 3:2 (v/v) CH_3OH:C_6H_6 and 5:100 (v/v) C_2H_3ClO:CH_3OH was added to tubes containing the nitrogen evaporated extracts and vortexed. From this point onward, both plasma and tissue/diet extracts follow an identical protocol. The tubes were weighed to obtain a weight prior to the esterification reaction. Next, they were heated for 1 hour at 95°C to promote methanolysis during which they were vortexed every 15 minutes. Once cool, they were re-weighed to evaluate the possibility of sample evaporation. Samples having a percent difference greater than 1% were rejected. Neutralization of the reaction occurred upon the addition of 6% K_2CO_3. Liberation of the organic solvent containing the fatty acid methyl esters (FAMEs) resulted after centrifugation for 5 minutes at 4500xg at 22°C.

iii) Gas chromatography analysis of FAMEs

An aliquot of the upper benzene layer was removed and analyzed by gas chromatography (GC) on a Varian GC/MS/MS instrument equipped with a CP-3800 GC, CP-8400 autosampler and a Flame Ionization Detector (FID).

(H) Ischemia/ reperfusion on isolated hearts

Rabbits were anesthetized with isoflurane. Once blood was collected, the rabbits were injected with heparin to prevent blood coagulation. The thoracic cavity was opened and the aorta severed to remove the heart. The heart was mounted on the canula of a custom perfusion apparatus where it was perfused at 20 mL/ min with Tyrode's solution within 60 seconds of excision, as previously described (25). Tyrode's solution contained (in mmol/L): NaCl 115.0; $NaHCO_3$ 28.0; NaH_2PO_4 0.5; Dextrose 20.0; KCl 4.0; $CaCl_2$ 2.0; $MgCl_2$ 0.7 and was continuously bubbled with 95% O_2, 5% CO_2. The right atrium was removed and the atrioventricular node crushed (by hemostat) to allow external pacing. A hook electrode placed high in the right ventricle provided external pacing at 2 Hertz (120 beats/ minute).

Left ventricular pressure was measured by placing a deflated latex balloon into the left ventricle where it was inflated to a baseline of 5 mmHg. The balloon was connected to a pressure transducer which monitored left ventricular end-diastolic pressure and left ventricular developed pressure.

An electrocardiogram was recorded by immersing the heart into a circulating bath of Tyrode's solution bubbled continuously with 95% O_2, 5% CO_2. Three electrodes

present in the walls of the bath formed Einthoven's configuration and provided ECG leads I, II and III.

Right ventricular and bath temperatures were monitored and maintained at 37 ± 0.5°C. An equilibration period of 50 minutes ensured the temperature of the right ventricle and bath was stabilized at 37°C as well as to produce stable ECG and pressure tracings. After equilibration, global ischemia was initiated by eliminating buffer flow to the heart and bubbling the bath continuously with 95% N_2, 5% CO_2. Global ischemia lasted 30 minutes and was followed by a 45 minute reperfusion period.

A schematic of the perfusion apparatus is shown in Figure 9.

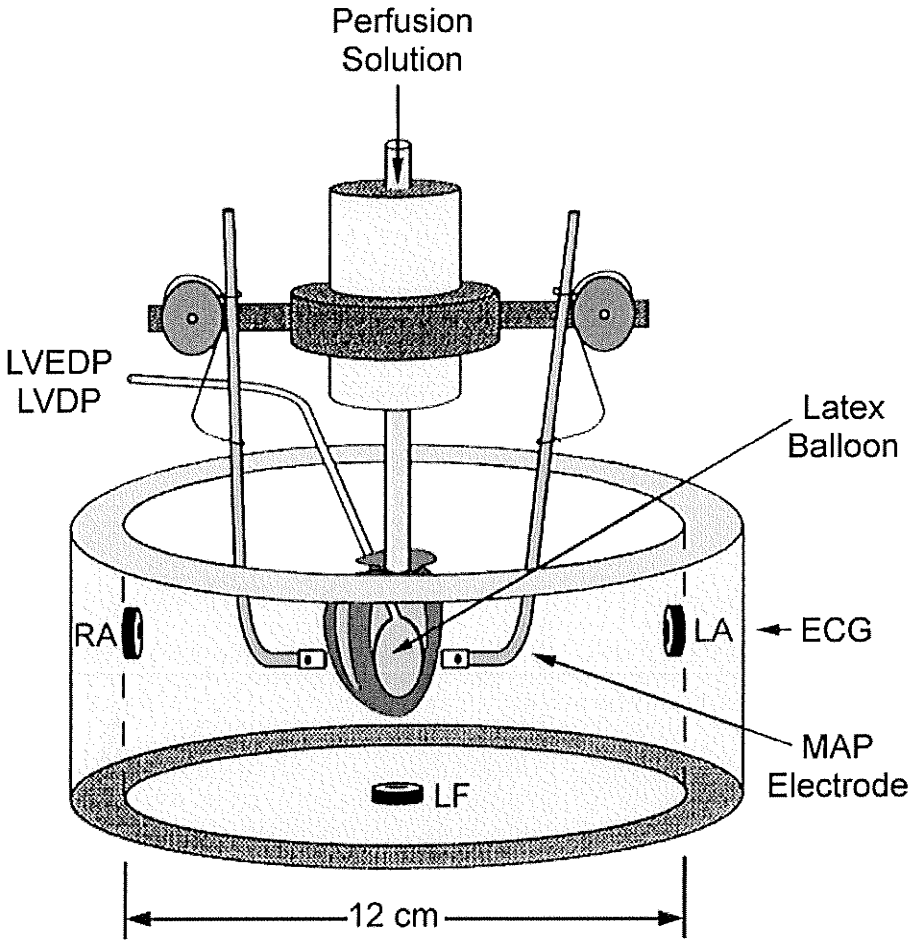


Figure 9: Modified perfusion apparatus. Diagram provided by Dr. A. Lukas. ECG: electrocardiogram; MAP: monophasic action potential; RA: right arm; LA: left arm; LF: left foot.

(I) Electrocardiogram (ECG) analysis

i) Left ventricular pressure

Left ventricular end-diastolic pressure (LVEDP) values were taken as the baseline pressure between stimulated left ventricular contractions. Left ventricular developed pressure (LVDP) values were taken as the peak pressure of a stimulated contraction minus the LVEDP for that same contraction. LVDP must achieve 30mmHg during the equilibration period in order for the ECG trace to be included for analysis.

ii) QT interval

QT intervals were measured during the last 10 seconds of the equilibration period. Three separate measurements of this interval as seen in Figure 10, from the beginning of the Q-wave to the end of the T-wave (1), were obtained for each heart. Each QRS-T complex measured was checked to ensure it was a stimulated beat (ie. fell at a 0.5 sec interval) as the duration of the QT interval depends on heart rate (59). Animals used for measurement of the QT interval must satisfy the same criteria as for ECG analysis. As well, the Q and T deflections must be readily identifiable to ensure correct quantification.

iii) Arrhythmia

ECGs were analyzed for arrhythmias based on the parameters set at the Lambeth Conventions (109). Ventricular tachycardia was defined as a "run of 4 or more consecutive ventricular premature beats" (109). Ventricular fibrillation was defined as

when "individual QRS deflections can no longer be distinguished from one another" and "a rate can no longer be measured" (109). Incidence and duration of tachycardia/ fibrillation in ischemia/ reperfusion was recorded. Incidence of tachycardia and fibrillation was classified as continuous tachycardia or fibrillation ≥ 30 seconds, respectively. The duration of tachycardia or fibrillation was the total length of tachycardia or fibrillation, respectively.

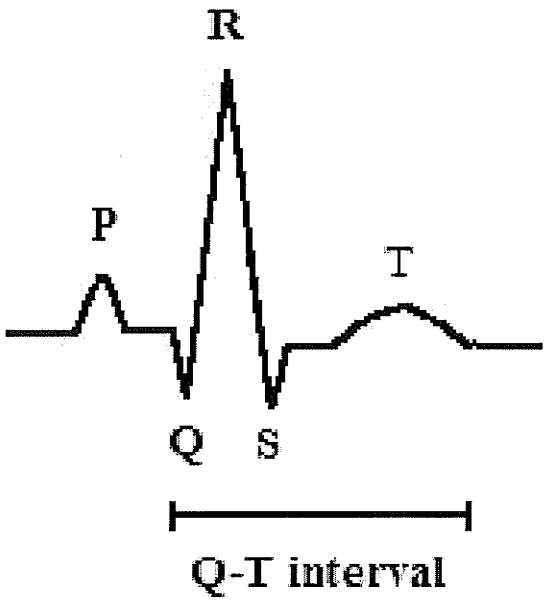


Figure 10: Schematic of the P-QRS-T complex. The Q-T interval is taken from the beginning of the Q-wave to the termination of the T-wave.

(J) Statistical analysis

Data was analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls post hoc test. Values were expressed as mean ± standard error (SEM). Incidence of arrhythmia data was analyzed for significance by the Fisher Exact test. Within incidence of arrhythmia graphs, no error bars are present as these graphs compare proportions, not means. Statistical significance was reached at a p-value < 0.05.

Statistical significance is denoted in graphs and tables as a letter. Within a graph, if two bars have a different letter assigned to them, those bars are statistically significant. Within a table, if two numbers (within the same row) have a different letter assigned to them, those two numbers are statistically significant. This lettering system was adopted to demonstrate statistical significance between all groups instead of just between select groups.

VI. RESULTS

(A) Diet composition

Objective: To analyze diet extracts by gas chromatography to determine fatty acid content.

Each diet was sampled and extracted three individual times to increase accuracy. Table 3 shows the fatty acid nomenclature and Table 4 shows the fatty acid composition of the six diets. The hempseed (HP) diet was significantly enriched in several fatty acids including 16:0, 18:0, 20:0, 22:0, 18:1n-9, 20:1n-9, 18:2n-6 (LA), 18:3n-6 (GLA) and 18:3n-3 (ALA) in comparison to the control (RG) diet. The partially delipidated hempseed diet (DHP) diet was significantly enriched in 20:0, 20:1n-9, LA, GLA and ALA in comparison to the RG diet. The levels of these fatty acids in the DHP diet were approximately half those found in the HP diet with the exception of 20:1n-9 which was approximately at the same level. The cholesterol (OL) diet was significantly depleted in 16:0, 18:0, 16:1, 18:1n-9, 18:1n-7, 20:1n-9 in comparison to the RG diet. The cholesterol-hempseed (OLHP) diet was significantly enriched in 20:0, 22:0, 20:1n-9, LA, GLA and ALA in comparison to the RG diet. As well, the OLHP diet was significantly depleted in 16:1 in comparison to the RG diet. The CO diet was significantly enriched in 14:0, 16:0, 18:0 and 18:1n-9 in comparison to the RG diet.

Table 3: Fatty acid nomenclature.

Common name	Code
Myristic acid	14:0
Myristoleic acid	14:1
Palmitic acid	16:0
Palmitoleic acid	16:1
Stearic acid	18:0
Oleic acid	18:1n-9
Vaccenic acid	18:1n-7
Linoleic acid	18:2n-6
Gamma-linolenic acid	18:3n-6
Alpha-linolenic acid	18:3n-3
Arachidic acid	20:0
Eicosenoic acid	20:1n-9
Eicosadienoic acid	20:2n-6
Behenic acid	22:0
Methyl 8-11-14 eicosatrienoic acid	20:3n-6
Eicosatrienoic acid	20:3n-3
Arachidonic acid	20:4n-6
Eicosapentaenoic acid	20:5n-3
Erucic acid	22:1
Docosatetraenoic acid	22:4
Tetracosenoic acid	24:1n-9
Docosahexaenoic acid	22:6n-3

Table 4: Fatty acid content of the diet as a function of dietary intervention.

SFA	*RG*	*HP*	*DHP*	*OL*	*OLHP*	*CO*
14:0	364.9 ± 6.1^{a}	342.9 ± 28.8^{a}	353.3 ± 12.5^{a}	259.8 ± 6.4^{a}	264.2 ± 6.2^{a}	8259.8 ± 295.9^{b}
16:0	9423.8 ± 228.2^{a}	10938.7± 540.0^{b}	10127.0 ± 280.2^{a,b}	8095.2 ± 154.2^{c}	9741.9 ± 154.3^{a,b}	13212.6 ± 452.4^{d}
18:0	3510.7 ± 87.8^{a}	4290.5 ± 219.1^{b}	3675.0 ± 88.8^{a}	2817.7 ± 76.4^{c}	3683.5 ± 70.7^{a}	4657.1 ± 159.2^{b}
20:0	119.9 ± 4.9^{a}	440.0 ± 29.3^{b}	240.6 ± 19.0^{c}	102.3 ± 2.2^{a}	382.5 ± 9.3^{d}	138.7 ± 6.5^{a}
22:0	139.1 ± 3.8^{a}	260.2 ± 13.9^{b}	160.7 ± 33.2^{a}	132.3 ± 1.8^{a}	251.0 ± 5.9^{b}	133.9 ± 4.7^{a}
MUFA	*RG*	*HP*	*DHP*	*OL*	*OLHP*	*CO*
14:1	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
16:1	577.2 ± 15.1^{a}	542.0 ± 29.4^{a}	552.8 ± 16.4^{a}	400.9 ± 9.4^{b}	427.7 ± 7.9^{b}	541.6 ± 17.5^{a}
18:1n-9	13801.1 ± 406.3^{a}	17361.1 ± 828.8^{b}	14992.7 ± 323.0^{a,d}	11586.7 ± 116.6^{c}	15454.8 ± 263.9^{a,e}	16464.0 ± 512.0^{b,d,e}
18:1n-7	1481.0 ± 111.3^{a}	1511.2 ± 144.8^{a}	1384.5 ± 116.3^{a}	905.4 ± 10.0^{b}	1230.0 ± 29.6^{a,b}	1159.1 ± 39.4^{a,b}
20:1n-9	249.8 ± 8.9^{a}	348.5 ± 21.2^{b}	292.6 ± 7.2^{c}	170.3 ± 2.7^{d}	304.6 ± 7.6^{c}	242.6 ± 11.7^{a}
22:1	77.1 ± 8.2^{a,b}	79.5 ± 18.1^{a,b}	108.7 ± 10.4^{b}	49.6 ± 4.9^{a}	66.0 ± 5.2^{a,b}	48.8 ± 9.6^{a}
PUFA	*RG*	*HP*	*DHP*	*OL*	*OLHP*	*CO*
18:2n-6	11687.8 ± 304.7^{a}	31757.7 ± 1738.8^{b}	18282.5 ± 384.5^{c}	11453.9 ± 274.3^{a}	30676.6 ± 489.3^{b}	11381.3 ± 341.8^{a}
18:3n-6	0.0 ± 0.0^{a}	855.3 ± 60.8^{b}	347.3 ± 24.5^{c}	0.0 ± 0.0^{a}	742.7 ± 16.9^{d}	0.0 ± 0.0^{a}
18:3n-3	2631.0 ± 75.7^{a}	8243.3 ± 480.9^{b}	4334.5 ± 71.7^{c}	2152.1 ± 54.6^{a}	7860.3 ± 137.7^{b}	2381.7 ± 74.4^{a}
20:2n-6	45.6 ± 4.6^{a,b}	86.3 ± 10.4^{a}	94.3 ± 29.1^{a}	27.2 ± 0.8^{b}	68.0 ± 5.6^{a,b}	56.4 ± 2.6^{a,b}
20:3n-6	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
20:3n-3	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
20:4n-6	10.0 ± 2.7^{a,c}	10.4 ± 4.2^{a,c}	12.0 ± 3.1^{a,c}	0.8 ± 0.5^{a,b}	5.6 ± 1.5^{a}	19.6 ± 1.8^{c}

20:5n-3	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
22:6n-3	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	9.6 ± 9.6^{a}	10.4 ± 10.4^{a}	0.0 ± 0.0^{a}

Mean ± SEM are shown for each group (n = 3) and are represented as µg FAME/g diet.

Significant differences amongst groups are denoted by a different superscript.

(B) Body weight and animal health.

Objective: To analyze rabbit body weight and heath status during the eight week dietary intervention.

Animals were weighed prior to diet commencement and at termination. All animals included in the calculation of mean body weight completed the assigned diet for 56 ± 3 days. One animal in the OLHP group ceased feeding and was removed from the study at 50 days. This animal was removed from the data analysis. Figure 11 shows the pre- and post-diet animal weights. No statistically significant differences in animal weight were observed amongst the groups.

All animals were monitored daily and no animals were removed from the study due to illness.

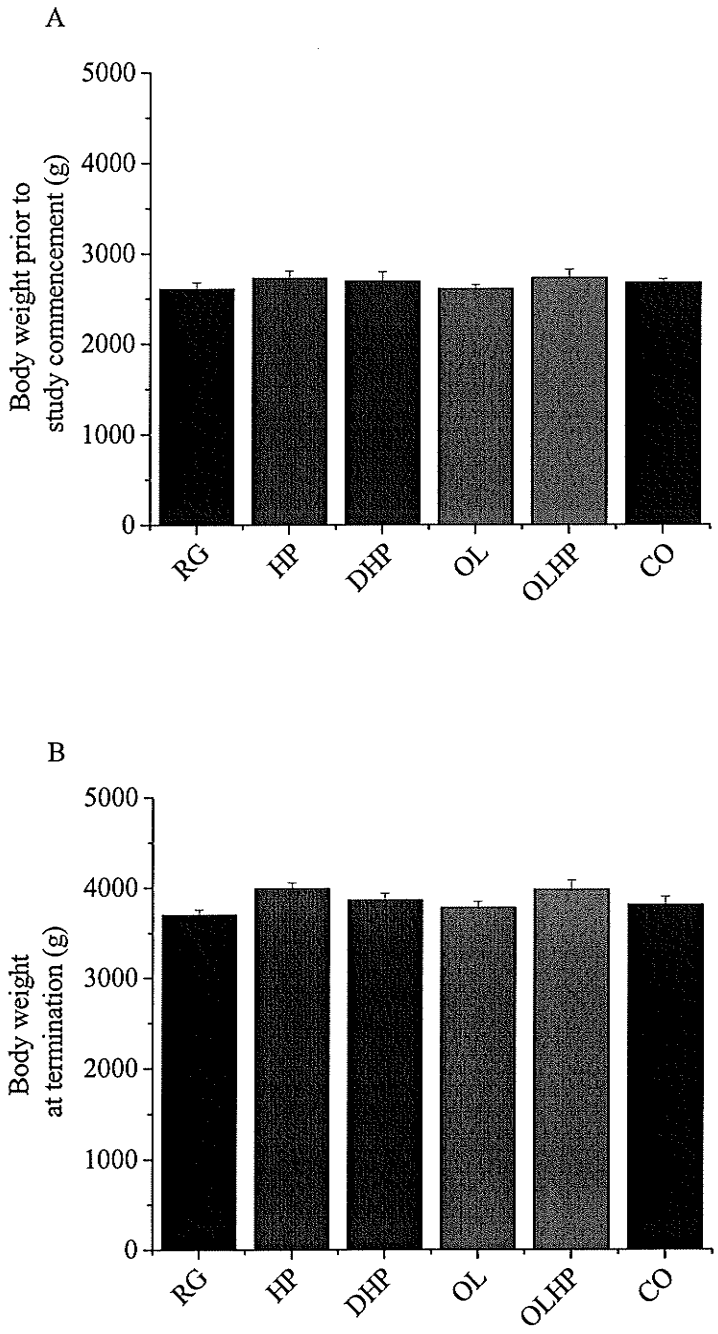


Figure 11: Rabbit body weights as a function of dietary intervention. Mean ± SEM values are shown for each group either prior to study commencement (A) or at termination (B) (n= 9-10). There were no significant differences in body weight amongst the groups at either time point ($p > 0.05$).

(C) Plasma levels of cholesterol esters, triglycerides and fatty acids.

Objective: To analyze plasma for cholesterol ester, triglyceride and fatty acid levels after an eight week dietary intervention.

Plasma was isolated from blood collected at termination. The plasma was analyzed for cholesterol ester (CE), triglyceride (TG) and fatty acid content. Figure 12 shows both the plasma CE and TG levels for each group. Significant differences exist between groups for both plasma CE and TG levels. The OL and OLHP groups had significantly elevated levels of plasma CE and TG in comparison to all other groups. As well the OLHP group had a significantly elevated plasma CE level as compared to the OL group.

Table 5 shows the plasma fatty acid content as a function of dietary intervention. Levels of 14:0 were significantly elevated in the OL, OLHP and CO groups as compared to the RG group. As well, a significant elevation of 14:0 was observed in the OL and OLHP groups as compared to the CO group. Levels of 16:0, 18:0, 20:0 and 22:0 were significantly elevated in the OL and OLHP groups when compared to all other groups. As well, the OLHP group was significantly elevated in these fatty acids over the OL group. Levels of 14:1, 16:1, 18:1n-9, 18:1n-7, 20:1n-9 and 24:1n-9 were significantly elevated in the OL and OLHP groups when compared to all other groups. In addition, the OLHP level of 24:1n-9 was significantly elevated over the OL group. Levels of LA, ALA, 20:2n-6, 20:3n-6, 20:4n-6, 20:5n-3 and 22:6n-3 were significantly elevated in the OL and OLHP groups when compared to all other groups. As well, the OLHP levels of these fatty acids were significantly elevated over the OL levels. Levels of 20:3n-6 were also

significantly elevated in the HP group as compared to the RG, DHP and CO groups but were significantly lower than the levels in the OL and OLHP groups. Levels of GLA were significantly elevated in the HP and OLHP groups as compared to the RG, DHP, OL and CO groups with the OLHP levels of this fatty acid significantly elevated over the HP levels.

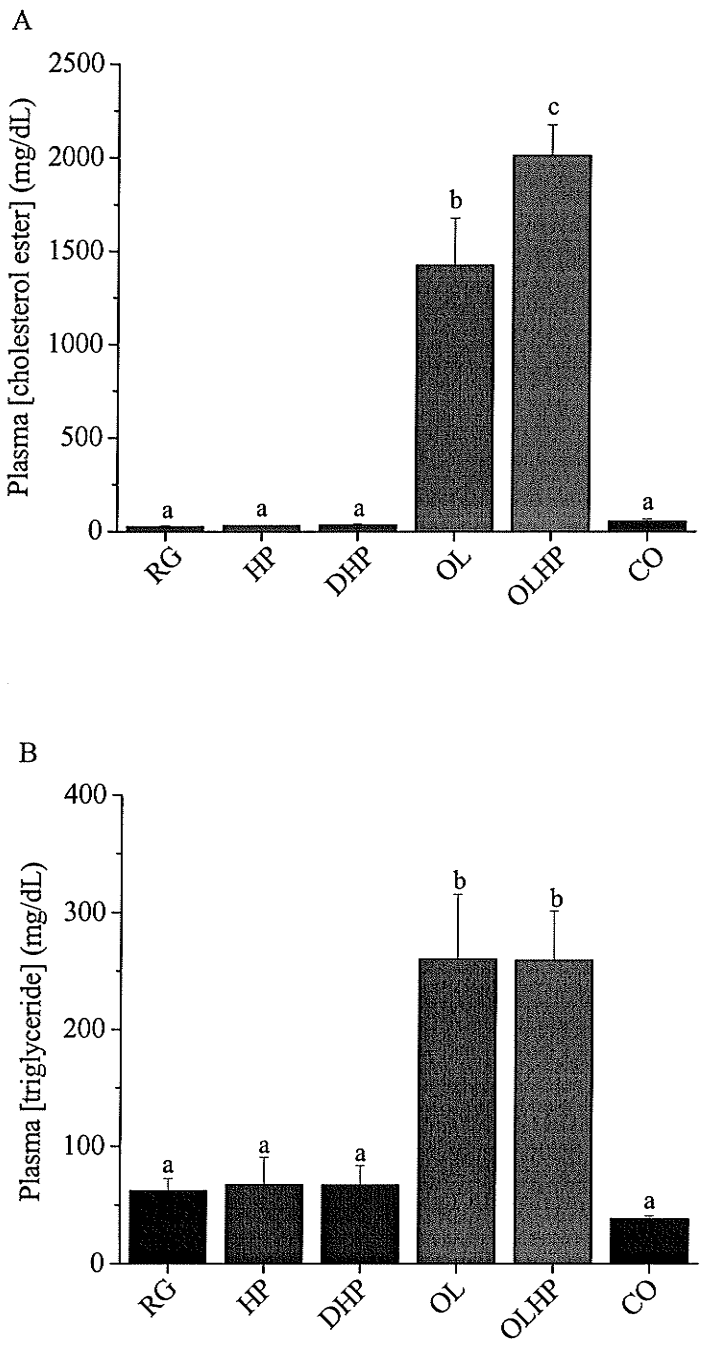


Figure 12: Plasma cholesterol ester and triglyceride levels as a function of dietary intervention. Mean ± SEM values are shown for cholesterol ester (A) and triglyceride (B) concentrations. n=4 for all groups. Significant differences amongst groups ($p < 0.05$) are denoted by a different letter.

Table 5: Fatty acid content of plasma as a function of dietary intervention.

SFA	*RG*	*HP*	*DHP*	*OL*	*OLHP*	*CO*
14:0	31.4 ± 2.0[a]	29.9 ± 4.6[a]	39.0 ± 4.5[a]	82.8 ± 5.6[b]	92.5 ± 4.1[b]	58.1 ± 2.1[c]
16:0	321.7 ± 19.8[a]	384.6 ± 40.6[a]	371.9 ± 43.2[a]	2633.9 ± 287.0[b]	3342.1 ± 82.1[c]	309.3 ± 15.0[a]
18:0	159.0 ± 6.0[a]	219.8 ± 23.0[a]	183.5 ± 15.5[a]	917.5 ± 84.1[b]	1167.5 ± 52.8[c]	184.7 ± 18.7[a]
20:0	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	56.6 ± 11.2[b]	117.5 ± 19.9[c]	0.0 ± 0.0[a]
22:0	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	60.7 ± 4.7[b]	81.1 ± 0.9[c]	0.0 ± 0.0[a]
MUFA	*RG*	*HP*	*DHP*	*OL*	*OLHP*	*CO*
14:1	0.0 ± 0.0[a]	0.0 ± 0.0[a]	5.5 ± 3.6[a]	24.7 ± 1.4[b]	16.9 ± 5.4[b]	0.0 ± 0.0[a]
16:1	46.3 ± 5.5[a]	35.3 ± 2.1[a]	44.9 ± 9.6[a]	733.3 ± 66.5[b]	707.3 ± 33.3[b]	38.8 ± 2.7[a]
18:1n-9	445.5 ± 22.0[a]	458.4 ± 47.6[a]	456.0 ± 58.9[a]	5245.2 ± 477.4[b]	5856.9 ± 78.8[b]	327.5 ± 16.9[a]
18:1n-7	33.9 ± 3.8[a]	30.9 ± 4.2[a]	38.0 ± 7.7[a]	330.2 ± 37.2[b]	330.9 ± 12.2[b]	22.3 ± 1.7[a]
20:1n-9	5.5 ± 3.5[a]	7.9 ± 3.0[a]	6.7 ± 4.4[a]	33.3 ± 3.5[b]	37.1 ± 1.1[b]	0.0 ± 0.0[a]
24:1n-9	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	108.0 ± 9.9[b]	123.1 ± 1.5[c]	0.0 ± 0.0[a]
PUFA	*RG*	*HP*	*DHP*	*OL*	*OLHP*	*CO*
18:2n-6	321.7 ± 19.7[a]	657.2 ± 109.5[a]	388.3 ± 31.1[a]	2620.7 ± 283.0[b]	6564.1 ± 129.5[c]	360.4 ± 41.7[a]
18:3n-6	0.0 ± 0.0[a]	11.6 ± 3.1[b]	0.0 ± 0.0[a]	4.5 ± 1.7[a]	52.3 ± 1.5[c]	0.0 ± 0.0[a]
18:3n-3	39.8 ± 2.4[a]	118.9 ± 27.2[a]	59.5 ± 5.8[a]	469.8 ± 46.8[b]	1656.3 ± 27.5[c]	36.0 ± 1.4[a]
20:2n-6	5.5 ± 3.5[a]	6.3 ± 3.1[a]	5.8 ± 3.8[a]	40.4 ± 4.1[b]	72.9 ± 3.0[c]	4.9 ± 3.1[a]
20:3n-6	0.0 ± 0.0[a]	19.9 ± 2.9[b]	6.4 ± 4.2[a]	45.5 ± 5.1[c]	104.6 ± 4.3[d]	0.0 ± 0.0[a]
20:4n-6	41.9 ± 2.2[a]	42.1 ± 5.3[a]	41.0 ± 5.3[a]	223.8 ± 22.8[b]	313.3 ± 13.0[c]	50.0 ± 6.8[a]
20:5n-3	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	13.7 ± 3.0[b]	19.4 ± 0.3[c]	0.0 ± 0.0[a]

22:6n-3	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	26.4 ± 5.8^{b}	36.0 ± 0.9^{c}	0.0 ± 0.0^{a}

Mean ± SEM are shown for each group (n=3 for RG, OLHP and CO while n=4 for HP, DHP and OL) and are represented as μg FAME/mL plasma. Significant differences amongst groups ($p < 0.05$) are denoted by a different superscript.

(D) Platelet aggregation.

Objective: To analyze platelet aggregation after an eight week dietary intervention.

Blood was collected from animals by venipuncture at termination (8 weeks). Platelet aggregation was stimulated by the addition of either ADP or collagen, both of which are recognized to activate platelets *in vivo* (84). ADP recruits platelets to the site of vascular injury (7) while collagen supports platelet adhesion and directly activates the cells which initiate aggregation and coagulant activity (88). Figure 13 shows total platelet aggregation (%) while Figure 14 shows the rate of platelet aggregation (% change/min). Significant differences exist amongst groups for both ADP- and collagen-induced platelet aggregation. The OL group had significantly increased ADP-induced aggregation as compared to every group except DHP. The OLHP group experienced significantly reduced collagen-induced aggregation when compared to every group except RG. In both instances the OLHP group had a significantly reduced level of aggregation compared to the OL group. Significant differences exist between groups in the rate of both ADP- and collagen-induced platelet aggregation. The OL group had a significantly increased rate of ADP-induced aggregation compared to every group except DHP. The OLHP group had a significantly decreased rate of ADP-induced aggregation compared to both DHP and OL groups. The OL group had a significantly increased rate of collagen-induced aggregation when compared to all groups. Again, in both instances the OLHP group had a significantly reduced rate of platelet aggregation compared to the OL group. All groups, with the exception of the OL group, exhibited rates of aggregation that were similar to control values.

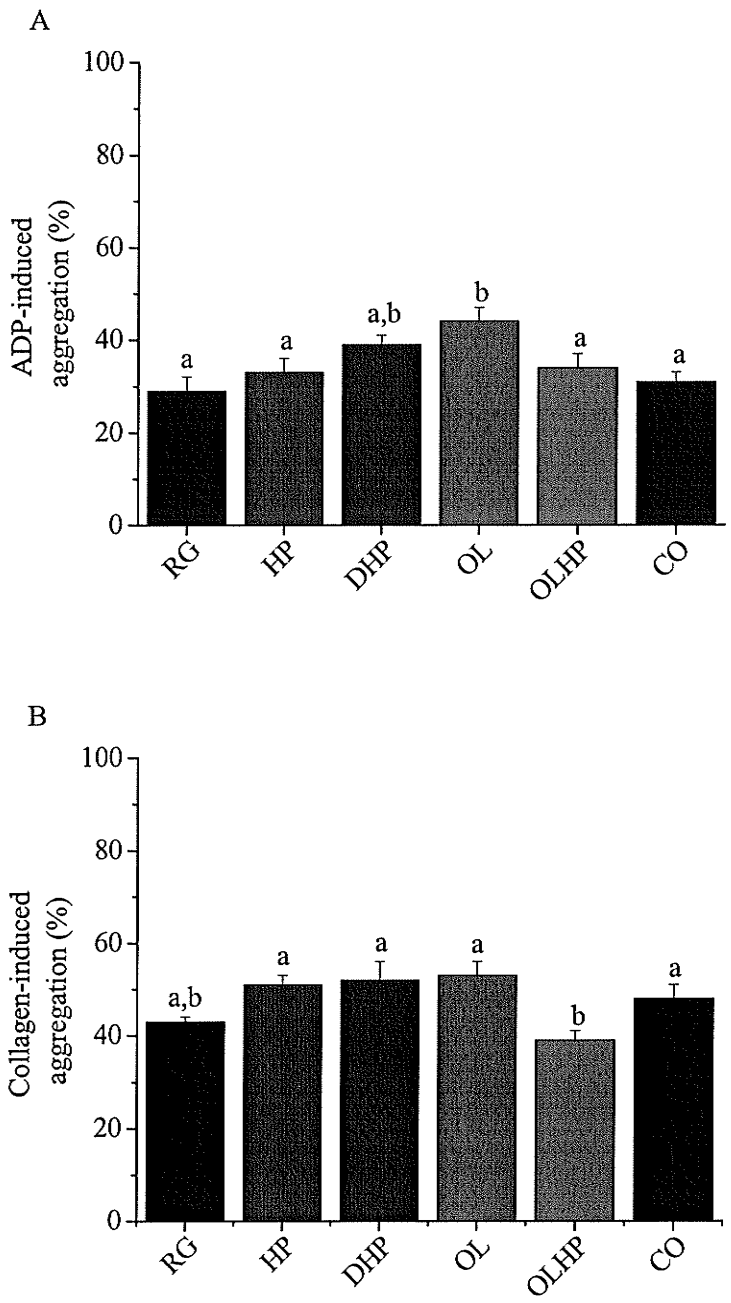


Figure 13: Total platelet aggregation as a function of dietary intervention. Mean ± SEM values are shown for each group for either ADP-induced (A) or collagen-induced (B) platelet aggregation (n = 7-10). Significant differences amongst groups ($p < 0.05$) are denoted by a different letter.

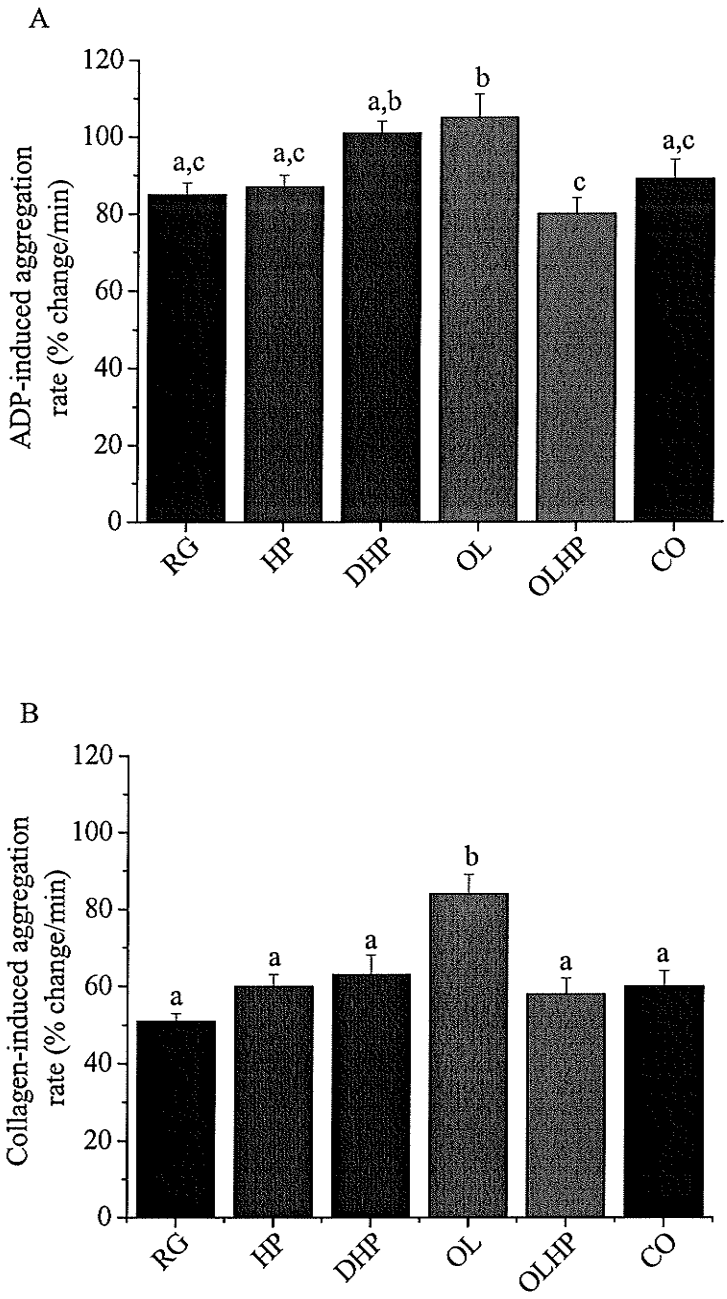


Figure 14: Rate of platelet aggregation as a function of dietary intervention. Mean ± SEM values are shown for each group for either ADP-induced (A) or collagen-induced (B) platelet aggregation. n=8 for RG, HP, OL; n=7 for DHP; n=9 for OLHP; n=10 for CO. Significant differences amongst groups ($p < 0.05$) are denoted by a different letter.

(E) Tissue levels of fatty acids

Objective: To analyze cardiac, hepatic and renal tissue for fatty acid levels after an eight week dietary intervention.

i) Cardiac:

Table 6 lists cardiac fatty acid content as a function of the different dietary interventions. Levels of 14:0 were significantly lower in the OL group as compared to the RG and CO groups. As well, the CO group had a significantly higher level of 14:0 over all other groups. Levels of 16:0 were significantly lower in the DHP and OL groups as compared to the RG group. Levels of 20:0 were significantly higher in the OL and OLHP groups as compared to the HP, DHP and CO groups. Levels of 22:0 were significantly higher in the OL and OLHP groups as compared to all other groups. Levels of 14:1 were significantly higher in the CO group as compared to all other groups. Levels of 18:1n-9 were significantly lower in the DHP and OL groups as compared to the RG group. Levels of 18:1n-7 were significantly lower in the OLHP group as compared to the RG group. Levels of 20:1n-9 were significantly higher in the OL group as compared to the DHP group. Levels of LA were significantly higher in the HP and OLHP groups as compared to the DHP and OL groups. Levels of GLA were significantly higher in the HP and OLHP groups as compared to all other groups. As well, the level of GLA in the OLHP group is significantly higher than in the HP group. Levels of ALA were significantly lower in the OL group as compared to the HP and OLHP groups. As well, levels of ALA were significantly higher in the OLHP group as compared to the DHP and CO groups. Levels of 20:2n-6 were significantly higher in the OLHP group as compared to the HP, DHP and CO groups. Levels of 20:3n-6 were significantly higher in the HP, OL and

OLHP groups as compared to the RG, DHP and CO groups. As well, the level of 20:3n-6 was significantly elevated in the OL and OLHP groups over the HP group. In addition, the level of 20:3n-6 was significantly elevated in the OLHP group over the OL group. Levels of 20:4n-6 were significantly higher in the CO group as compared to the DHP group. Levels of 22:6n-3 were significantly higher in the OL group as compared to the DHP and CO groups. Levels of 18:0, 16:1, 24:1n-9 and 20:5n-3 are unchanged.

Table 6: Fatty acid content of cardiac tissue as a function of dietary intervention.

SFA	*RG*	*HP*	*DHP*	*OL*	*OLHP*	*CO*
14:0	289.5 ± 18.5^{a}	199.0 ± 42.7^{a,b}	154.3 ± 13.6^{a,b}	90.3 ± 35.4^{b}	170.3 ± 59.4^{a,b}	1137.5 ± 64.7^{c}
16:0	4769.9 ± 287.7^{a}	3600.4 ± 500.9^{a,b}	2942.7 ± 147.6^{b}	2837.5 ± 383.5^{b}	3668.1 ± 616.1^{a,b}	3943.6 ± 190.7^{a,b}
18:0	2717.7 ± 496.5^{a}	2321.4 ± 111.4^{a}	2078.7 ± 179.2^{a}	1849.6 ± 136.8^{a}	2222.5 ± 114.8^{a}	2610.6 ± 287.5^{a}
20:0	14.8 ± 9.1^{a,b}	5.2 ± 2.8^{a}	0.0 ± 0.0^{a}	31.5 ± 9.3^{b}	32.9 ± 10.5^{b}	0.2 ± 0.2^{a}
22:0	0.7 ± 0.4^{a}	5.2 ± 2.0^{a}	0.3 ± 0.2^{a}	55.9 ± 15.8^{b}	44.7 ± 18.6^{b}	2.6 ± 1.1^{a}
MUFA	*RG*	*HP*	*DHP*	*OL*	*OLHP*	*CO*
14:1	4.8 ± 2.3^{a}	3.4 ± 2.3^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	3.3 ± 2.1^{a}	18.7 ± 3.8^{b}
16:1	506.1 ± 31.6^{a}	312.6 ± 69.8^{a}	261.4 ± 19.1^{a}	273.7 ± 71.0^{a}	295.5 ± 85.2^{a}	364.5 ± 74.8^{a}
18:1n-9	5539.8 ± 365.5^{a}	3782.4 ± 636.1^{a,b}	3217.2 ± 178.2^{b}	2876.7 ± 443.8^{b}	3883.7 ± 720.9^{a,b}	4218.2 ± 185.4^{a,b}
18:1n-7	773.7 ± 46.9^{a}	554.8 ± 78.0^{a,b}	553.9 ± 30.3^{a,b}	623.9 ± 55.3^{a,b}	507.3 ± 67.0^{b}	620.8 ± 31.5^{a,b}
20:1n-9	49.0 ± 13.0^{a,b}	25.9 ± 14.3^{a,b}	14.4 ± 4.3^{a}	60.3 ± 3.5^{b}	47.6 ± 5.5^{a,b}	37.7 ± 12.3^{a,b}
24:1n-9	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
PUFA	*RG*	*HP*	*DHP*	*OL*	*OLHP*	*CO*
18:2n-6	5908.6 ± 371.5^{a,b}	7084.6 ± 551.0^{a}	5103.3 ± 219.9^{b}	4259.0 ± 371.8^{b}	7533.2 ± 911.7^{a}	5676.1 ± 116.6^{a,b}
18:3n-6	0.0 ± 0.0^{a}	34.0 ± 7.2^{b}	0.4 ± 0.4^{a}	0.0 ± 0.0^{a}	62.2 ± 9.4^{c}	0.0 ± 0.0^{a}
18:3n-3	656.7 ± 48.3^{a,b,c}	773.0 ± 152.9^{a,c}	386.3 ± 39.0^{a,b}	283.7 ± 50.7^{b}	926.1 ± 216.6^{c}	403.4 ± 36.9^{a,b}
20:2n-6	47.2 ± 19.1^{a,b}	29.5 ± 7.9^{a}	19.8 ± 3.5^{a}	59.5 ± 6.2^{a,b}	79.8 ± 5.5^{b}	26.2 ± 6.3^{a}
20:3n-6	45.0 ± 10.2^{a}	80.8 ± 6.5^{b}	54.0 ± 4.8^{a}	111.1 ± 8.4^{c}	156.4 ± 14.8^{d}	44.9 ± 2.0^{a}
20:4n-6	2800.3 ± 173.8^{a,b}	2709.1 ± 124.4^{a,b}	2498.0 ± 153.1^{a}	2721.6 ± 137.8^{a,b}	2688.1 ± 54.7^{a,b}	3186.7 ± 84.5^{b}
20:5n-3	42.1 ± 10.1^{a}	38.8 ± 1.9^{a}	39.3 ± 1.4^{a}	43.0 ± 5.0^{a}	40.3 ± 10.6^{a}	51.4 ± 1.3^{a}

22:6n-3	81.9 ± 12.0^{a,b}	81.3 ± 10.8^{a,b}	48.9 ± 7.5^{b}	105.0 ± 10.8^{a}	70.8 ± 3.0^{a,b}	58.3 ± 9.7^{b}

Mean ± SEM are shown for each group (n=4) and are represented as μg FAME/ g tissue).

Significant differences amongst groups ($p < 0.05$) are denoted by a different superscript.

ii) Hepatic:

Table 7 lists the hepatic fatty acid content as a function of the different dietary interventions. Levels of 14:0 were significantly higher in the CO group as compared to all other groups. Levels of 16:0, 20:0, 16:1, 18:1n-9, 18:1n-7, 20:1n-9 and ALA were significantly higher in the OL and OLHP groups as compared to all other groups. As well, levels of 16:0, 20:1n-9 and 18:3n-3 were significantly higher in the OLHP group as compared to the OL group. Levels of 18:0 were significantly higher in the HP group as compared to all other groups. Levels of 22:0 were significantly higher in the OL and OLHP groups as compared to the DHP group. Levels of LA were significantly higher in the HP and OLHP groups as compared to the RG group. Levels of LA were significantly higher in the HP, OL and OLHP groups as compared to the DHP group. Levels of LA were significantly higher in the OLHP group when compared to all other groups. Levels of GLA were significantly higher in the HP and OLHP groups as compared to all other groups. As well, levels of GLA were significantly higher in the OLHP group as compared to the HP group. Levels of 20:2n-6 were significantly higher in the OLHP group as compared to all other groups. Levels of 20:3n-6 were significantly higher in the HP, DHP and OLHP group as compared to the RG and CO groups. As well, levels of 20:3n-6 were significantly higher in the HP and OLHP groups as compared to all other groups. Levels of 20:4n-6 were significantly higher in the HP group as compared to the DHP and OL groups. As well, levels of 20:4n-6 were significantly higher in the CO group as compared to the OL group. Levels of 20:5n-3 were significantly higher in the RG group as compared to the HP and DHP groups. As well, levels of 20:5n-3 were significantly higher in the OL and OLHP groups as compared to the RG, HP and DHP groups. As well, levels

of 20:5n-3 were significantly higher in the OLHP group as compared to the CO group. Levels of 22:4 were significantly higher in the HP and OLHP groups as compared to the DHP group. As well, levels of 22:4 were significantly higher in the OLHP group as compared to the OL and RG groups. Levels of 22:6n-3 were significantly higher in the HP group as compared to the OL group. Levels of 14:1 and 22:1 were unchanged.

Table 7: Fatty acid content of hepatic tissue as a function of dietary intervention.

SFA	*RG*	*HP*	*DHP*	*OL*	*OLHP*	*CO*
14:0	98.6 ± 29.9^{a}	48.0 ± 19.4^{a}	54.9 ± 16.5^{a}	231.8 ± 29.2^{a}	275.9 ± 34.5^{a}	764.5 ± 128.1^{b}
16:0	4777.2 ± 216.3^{a}	4674.7 ± 407.9^{a}	3691.1 ± 111.4^{a}	9308.9 ± 607.8^{b}	11952.6 ± 1332.8^{c}	5814.2 ± 590.0^{a}
18:0	4735.3 ± 230.8^{a}	6293.1 ± 426.7^{b}	4094.0 ± 273.7^{a}	4023.6 ± 224.8^{a}	4964.9 ± 477.5^{a}	4919.9 ± 332.7^{a}
20:0	0.0 ± 0.0^{a}	20.7 ± 10.1^{a}	0.0 ± 0.0^{a}	86.6 ± 5.7^{b}	100.5 ± 4.8^{b}	18.5 ± 11.9^{a}
22:0	73.8 ± 10.2^{a,b}	76.2 ± 17.4^{a,b}	59.7 ± 15.0^{b}	122.3 ± 9.6^{a}	118.8 ± 7.2^{a}	72.6 ± 15.7^{a,b}
MUFA	*RG*	*HP*	*DHP*	*OL*	*OLHP*	*CO*
14:1	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	26.4 ± 10.1^{a}	4.8 ± 3.1^{a}	21.6 ± 10.2^{a}
16:1	360.1 ± 65.2^{a}	195.2 ± 51.1^{a}	177.5 ± 30.8^{a}	3001.9 ± 299.4^{b}	2501.4 ± 334.7^{b}	312.8 ± 32.2^{a}
18:1n-9	5270.7 ± 352.9^{a}	3637.1 ± 322.3^{a}	3620.3 ± 204.2^{a}	20473.5 ± 1940.6^{b}	23577.8 ± 1954.9^{b}	4968.3 ± 273.8^{a}
18:1n-7	562.8 ± 57.5^{a}	448.0 ± 59.2^{a}	382.7 ± 29.5^{a}	2205.5 ± 187.8^{b}	2233.2 ± 197.6^{b}	521.5 ± 43.3^{a}
20:1n-9	13.8 ± 6.3^{a}	17.4 ± 7.2^{a}	11.4 ± 4.6^{a}	88.4 ± 10.9^{b}	140.9 ± 13.3^{c}	39.4 ± 13.1^{a}
22:1	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
PUFA	*RG*	*HP*	*DHP*	*OL*	*OLHP*	*CO*
18:2n-6	6477.5 ± 335.3^{a,c}	9183.5 ± 629.7^{b}	5729.3 ± 351.7^{c}	8358.7 ± 649.1^{a,b}	18401.9 ± 1031.9^{d}	7114.0 ± 526.6^{a,b,c}
18:3n-6	0.0 ± 0.0^{a}	46.8 ± 6.5^{b}	0.0 ± 0.0^{a}	2.7 ± 2.7^{a}	81.9 ± 6.6^{c}	0.0 ± 0.0^{a}
18:3n-3	329.6 ± 29.3^{a}	517.9 ± 70.3^{a}	288.8 ± 28.7^{a}	1095.6 ± 118.9^{b}	3229.9 ± 305.6^{c}	434.5 ± 32.5^{a}
20:2n-6	99.0 ± 7.3^{a}	153.5 ± 26.3^{a}	110.6 ± 19.5^{a}	152.9 ± 13.0^{a}	374.8 ± 28.1^{b}	126.4 ± 26.1^{a}
20:3n-6	115.8 ± 6.9^{a}	633.9 ± 53.4^{b}	275.6 ± 15.7^{c}	209.0 ± 24.7^{a,c}	628.5 ± 37.5^{b}	123.7 ± 7.7^{a}
20:4n-6	1812.1 ± 123.3^{a,c,d}	2155.5 ± 158.2^{a,d}	1455.0 ± 88.3^{c,e}	1324.9 ± 113.1^{c}	1687.4 ± 98.2^{a,c,d}	1925.4 ± 179.7^{d,e}
20:5n-3	31.0 ± 12.4^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	65.7 ± 5.5^{c,d}	74.5 ± 7.0^{c}	46.9 ± 3.8^{a,d}

22:4	183.5 ± 16.8^{a,c}	265.7 ± 33.1^{a,b}	173.0 ± 15.9^{c}	184.7 ± 22.5^{a,c}	282.5 ± 16.6^{b}	207.0 ± 15.5^{a,b,c}
22:6n-3	86.7 ± 13.1^{a,b}	141.8 ± 23.5^{a}	87.6 ± 9.0^{a,b}	64.5 ± 16.9^{b}	82.5 ± 5.5^{a,b}	101.1 ± 18.6^{a,b}

Mean ± SEM are shown for each group (n=4) and are represented as μg FAME/g liver.

Significant differences amongst groups ($p < 0.05$) are denoted by a different superscript.

iii) Renal:

Table 8 lists renal fatty acid content as a function of the different dietary interventions. Levels of 14:0 were significantly elevated in the CO group as compared to all other groups. Levels of 18:0 were significantly higher in the HP group as compared to the DHP, OL and CO groups. As well, the OLHP group had a significantly higher level of 18:0 over the OL and CO groups. Levels of 20:0 were significantly higher in the HP and OLHP groups as compared to the RG group. A significantly elevated level of 22:1 was found in the OL group as compared to all other groups. Significantly higher levels of LA and GLA were found in the HP and OLHP groups as compared to all others. There was a significantly higher level of 20:2n-6 found in the OLHP group as compared to the CO group. There were significantly higher levels of 20:3n-6 found in the HP, DHP, OL and OLHP groups as compared to the RG and CO groups. As well, the levels of 20:3n-6 in the HP and OLHP groups were significantly higher than those found in the DHP and OL groups. A significantly higher level of 20:3n-3 was found in the HP group as compared to all other groups. A significantly higher level of 20:5n-3 was found in the HP and CO groups as compared to the DHP and RG groups. A significantly higher level of 22:4 was found in the OL and OLHP groups as compared to the RG, HP and DHP groups. As well, the level of 22:4 in the OLHP group is significantly elevated over the CO group. Levels of 22:5 in the HP group are significantly elevated over the DHP group. As well, levels of 22:5 are significantly highest in the OLHP group as compared to all others. Levels of 16:0, 22:0, 14:1, 16:1, 18:1n-9, 18:1n-7, 20:1n-9, ALA, 20:4n-6 and 22:6n-3 were all unchanged.

Table 8: Fatty acid content of renal tissue as a function of dietary intervention.

SFA	*RG*	*HP*	*DHP*	*OL*	*OLHP*	*CO*
14:0	172.5 ± 43.2^{a}	182.9 ± 74.9^{a}	110.7 ± 31.9^{a}	219.7 ± 60.6^{a}	211.4 ± 58.0^{a}	450.6 ± 85.7^{b}
16:0	4650.8 ± 532.2^{a}	5114.9 ± 858.1^{a}	3854.0 ± 441.3^{a}	5242.3 ± 715.6^{a}	5292.2 ± 762.9^{a}	3880.0 ± 284.1^{a}
18:0	2995.8 ± 136.9^{a,b,c,d}	3428.1 ± 69.1^{a}	2880.6 ± 146.8^{b,c,d}	2538.9 ± 149.3^{c}	3119.4 ± 128.5^{a,b}	2500.3 ± 158.4^{c,d}
20:0	24.7 ± 2.8^{a}	48.7 ± 3.3^{b}	33.6 ± 4.7^{a,b}	36.7 ± 6.1^{a,b}	45.7 ± 4.5^{b}	40.9 ± 2.2^{a,b}
22:0	64.2 ± 4.0^{a}	69.7 ± 2.9^{a}	75.3 ± 5.6^{a}	73.5 ± 11.8^{a}	82.2 ± 6.0^{a}	70.5 ± 4.5^{a}
MUFA	*RG*	*HP*	*DHP*	*OL*	*OLHP*	*CO*
14:1	18.9 ± 6.2^{a}	31.2 ± 12.7^{a}	15.7 ± 8.8^{a}	31.2 ± 12.1^{a}	49.6 ± 17.7^{a}	33.9 ± 9.7^{a}
16:1	744.1 ± 161.6^{a}	791.3 ± 300.3^{a}	589.4 ± 182.6^{a}	1049.7 ± 246.1^{a}	1228.4 ± 304.9^{a}	381.3 ± 92.6^{a}
18:1n-9	5749.4 ± 803.6^{a}	5838.4 ± 1289.4^{a}	4506.0 ± 579.6^{a}	6611.5 ± 1142.5^{a}	6448.7 ± 1023.0^{a}	3967.0 ± 380.5^{a}
18:1n-7	743.0 ± 68.3^{a}	765.1 ± 157.8^{a}	666.4 ± 64.0^{a}	965.9 ± 110.1^{a}	769.0 ± 100.7^{a}	525.5 ± 25.5^{a}
20:1n-9	41.5 ± 10.2^{a}	41.5 ± 18.4^{a}	29.1 ± 5.5^{a}	74.5 ± 21.3^{a}	48.3 ± 12.5^{a}	23.4 ± 4.8^{a}
22:1	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	12.1 ± 4.7^{b}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
PUFA	*RG*	*HP*	*DHP*	*OL*	*OLHP*	*CO*
18:2n-6	6117.0 ± 452.3^{a}	8547.5 ± 914.6^{b}	6041.5 ± 421.9^{a}	6345.2 ± 677.0^{a}	8956.4 ± 736.7^{b}	4902.0 ± 297.0^{a}
18:3n-6	0.0 ± 0.0^{a}	42.3 ± 10.2^{b}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	40.3 ± 12.2^{b}	0.0 ± 0.0^{a}
18:3n-3	464.3 ± 111.4^{a}	906.9 ± 297.0^{a}	427.5 ± 102.5^{a}	616.1 ± 169.3^{a}	1029.0 ± 212.1^{a}	299.1 ± 67.2^{a}
20:2n-6	91.8 ± 10.5^{a,b}	111.1 ± 15.8^{a,b}	95.7 ± 5.8^{a,b}	97.0 ± 16.6^{a,b}	128.6 ± 8.0^{a}	71.5 ± 8.0^{b}
20:3n-6	120.9 ± 7.0^{a}	323.6 ± 18.2^{b}	242.4 ± 16.7^{c}	222.3 ± 16.1^{c}	367.4 ± 21.3^{b}	145.6 ± 22.0^{a}
20:3n-3	6.0 ± 2.3^{a}	29.7 ± 6.8^{b}	6.7 ± 1.8^{a}	9.4 ± 4.3^{a}	14.4 ± 2.7^{a}	4.5 ± 2.3^{a}
20:4n-6	2965.3 ± 144.7^{a}	2968.1 ± 130.1^{a}	2807.0 ± 136.1^{a}	2315.2 ± 198.8^{a}	2888.1 ± 97.5^{a}	2679.4 ± 214.1^{a}

20:5n-3	7.2 ± 2.7^{a}	40.9 ± 8.7^{b}	6.0 ± 3.1^{a}	$21.1 \pm 5.9^{a,b}$	$23.2 \pm 3.2^{a,b}$	36.1 ± 7.9^{b}
22:4	$73.0 \pm 7.0^{a,d}$	$76.6 \pm 6.2^{a,d}$	$61.1 \pm 4.5^{a,d}$	$117.7 \pm 15.7^{b,c}$	140.6 ± 10.9^{b}	$90.3 \pm 10.5^{c,d}$
22:5	$83.8 \pm 5.9^{a,b}$	115.2 ± 4.4^{a}	78.9 ± 6.8^{b}	$103.5 \pm 13.6^{a,b}$	141.8 ± 9.9^{c}	$95.4 \pm 3.1^{a,b}$
22:6n-3	67.8 ± 8.1^{a}	91.5 ± 11.0^{a}	75.8 ± 8.5^{a}	56.5 ± 11.3^{a}	73.9 ± 8.0^{a}	77.1 ± 4.8^{a}

Mean ± SEM are shown for each group (n=4) and are represented as μg FAME/g kidney. Significant differences amongst groups ($p < 0.05$) are denoted by a different superscript.

(F) Left ventricular end diastolic and developed pressures

Objective: To analyze left ventricular end diastolic pressure (LVEDP) and left ventricular developed pressure (LVDP) in each rabbit heart after an eight week dietary intervention.

Left ventricular diastolic and systolic pressure readings were taken every five minutes during the experiment. Diastolic and systolic pressures became indistinguishable between 10 – 30 minutes of ischemia. Readings were only taken from stimulated beats (at 0.5sec intervals) and data was not included if it was thought to be altered by a non-stimulated beat in close proximity. There were no significant differences detected amongst groups for either LVEDP (Figure 15) or LVDP (Figure 16) at any time point prior to ischemia, during ischemia or during reperfusion.

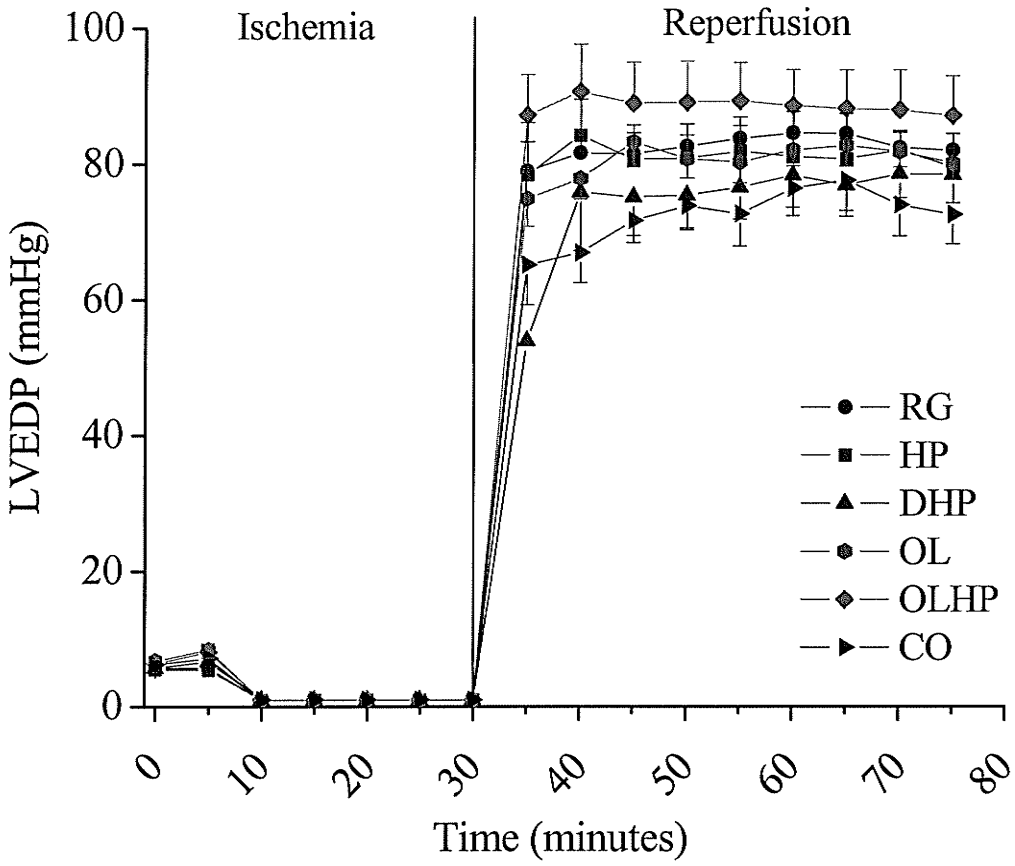


Figure 15: Left ventricular end diastolic pressure (LVEDP) as a function of dietary intervention. Mean ± SEM values are shown for each group at each time point (n = 4–10). There were no significant differences amongst the groups ($p > 0.05$).

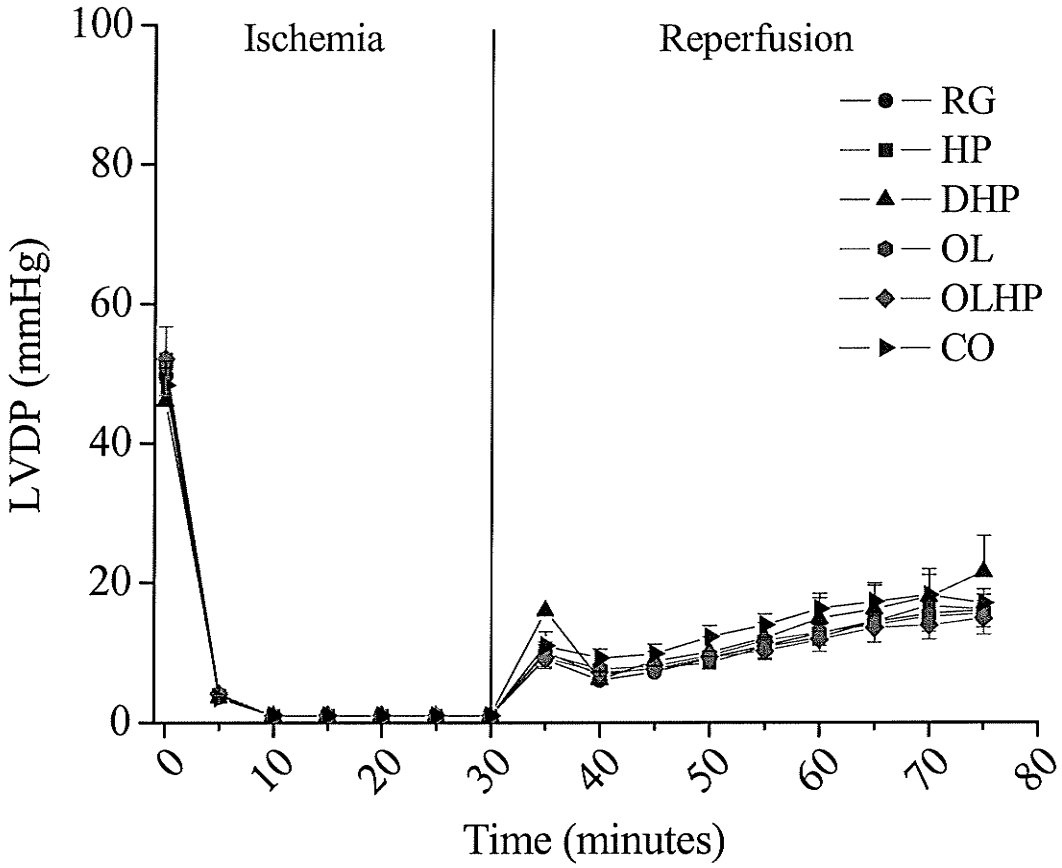


Figure 16: Left ventricular developed pressure (LVDP) as a function of dietary intervention. Mean ± SEM values are shown for each group at each time point (n = 4-10). There were no significant differences amongst the groups ($p > 0.05$).

(G) QT intervals

Objective: To analyze the duration of the QT interval in each rabbit heart after an eight week dietary intervention.

QT interval measurements were taken during the last 10 seconds of the equilibration period (ie. before the onset of ischemia). For each heart, three separate QT interval measurements were done and the mean taken. Figure 17 shows the mean QT interval duration for each group. Although the HP group demonstrated a trend to have the shortest QT interval, there were no significant differences amongst the groups.

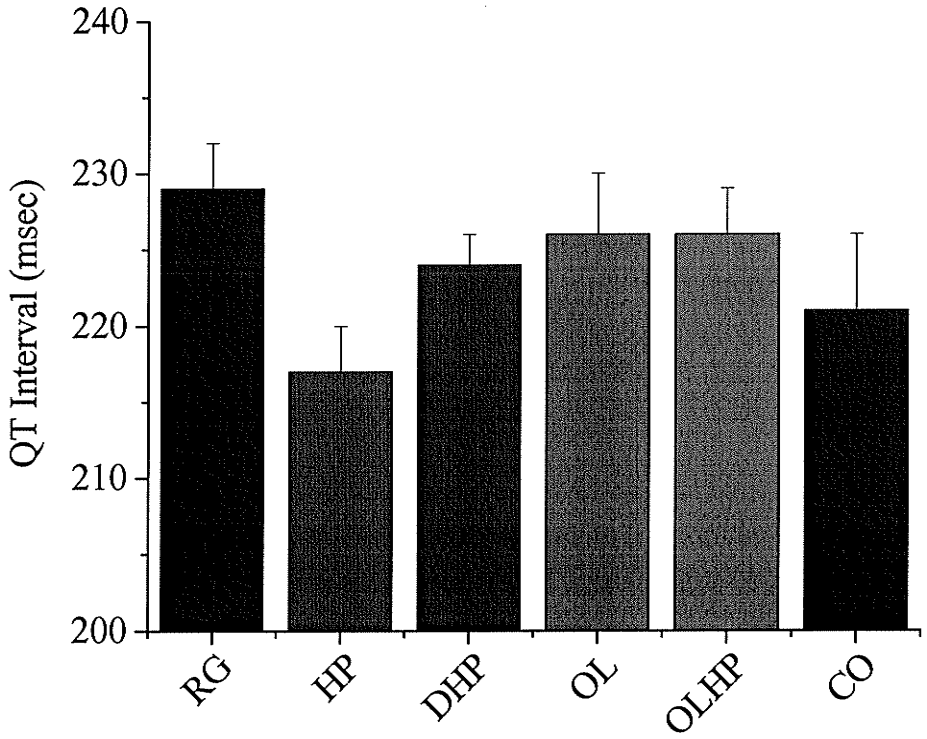


Figure 17: Duration of cardiac QT interval as a function of dietary intervention. Mean ± SEM values are shown for each group (n = 5-8). There were no significant differences amongst the groups ($p > 0.05$).

(H) Arrhythmias

Objective: To analyze incidence and duration of arrhythmias during ischemia/reperfusion challenge after an eight week dietary intervention.

ECG traces from ischemic challenge and reperfusion were analyzed for arrhythmias based on the parameters set at the Lambeth Conventions (109). Ischemia and reperfusion were treated as two distinct entities. The incidence of arrhythmia was defined as an occurrence of either tachycardia or fibrillation ≥30 sec. The duration of arrhythmia was taken as the total period of either tachycardia or fibrillation. There were no significant differences in the incidence of tachycardia or fibrillation during ischemia (Figure 18). There was no significant difference in the incidence of tachycardia for reperfusion-derived arrhythmias (Figure 20 A). The incidence of reperfusion-derived fibrillation was significantly lower in the OL and OLHP groups as compared to the DHP group (Figure 20 B). There were no significant differences in the duration of tachycardia or fibrillation for either ischemia- (Figure 19) or reperfusion- (Figure 21) derived arrhythmias.

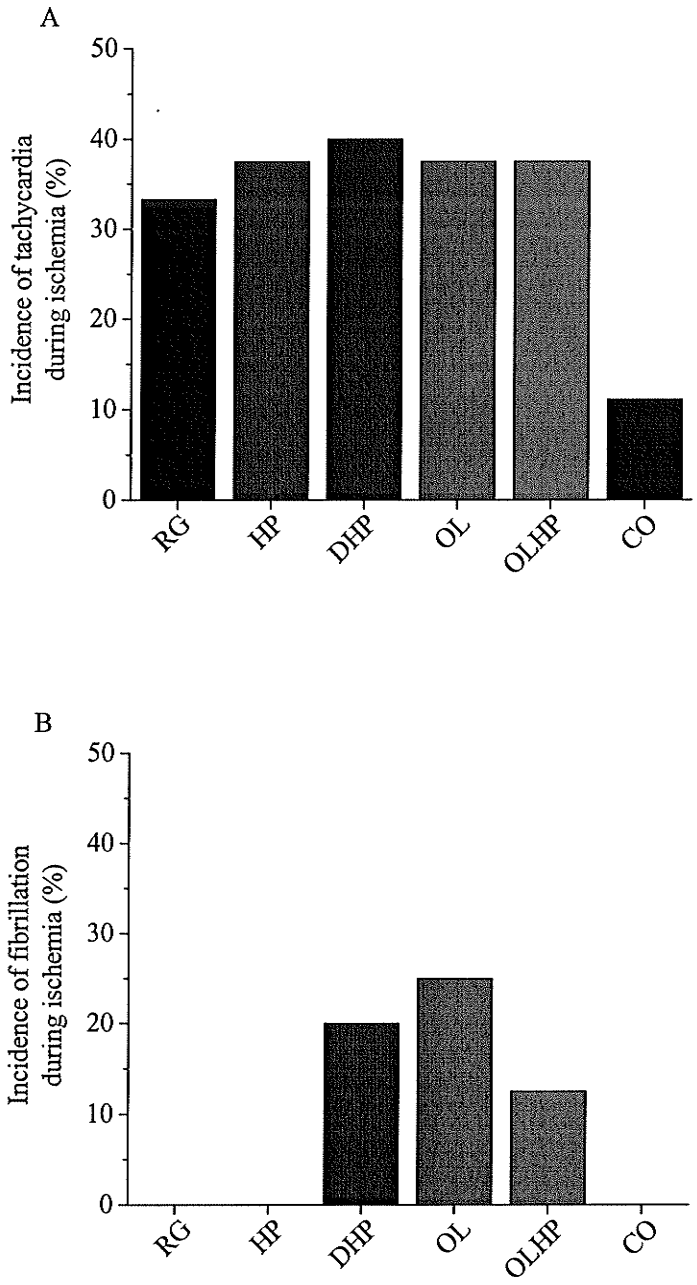


Figure 18: Incidence of ischemia-derived arrhythmias as a function of dietary intervention. Values are shown as a percentage of positive incidence (%) for either tachycardia (A) or fibrillation (B) (n = 8-10). There were no significant differences amongst the groups ($p > 0.05$).

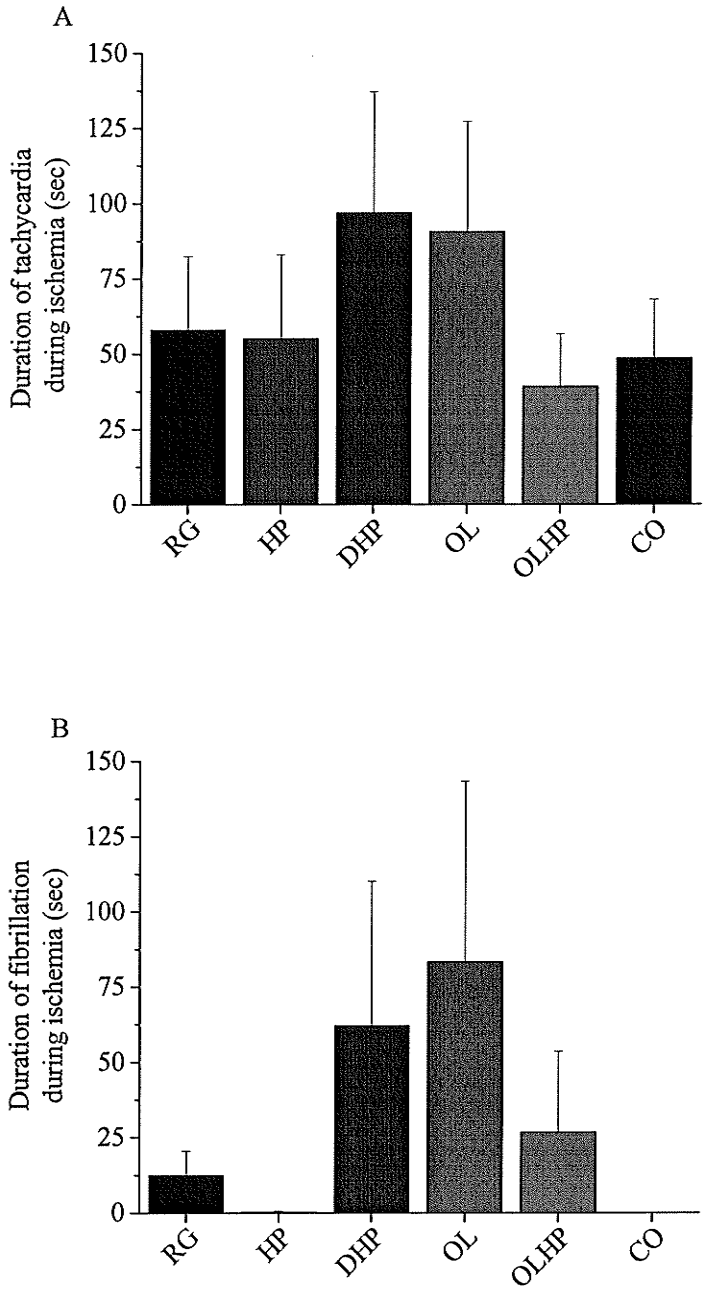


Figure 19: Duration of ischemia-derived arrhythmias as a function of dietary intervention. Mean ± SEM values are shown for each group for tachycardia (A) and fibrillation (B) (n = 8-10). There were no significant differences amongst the groups ($p > 0.05$).

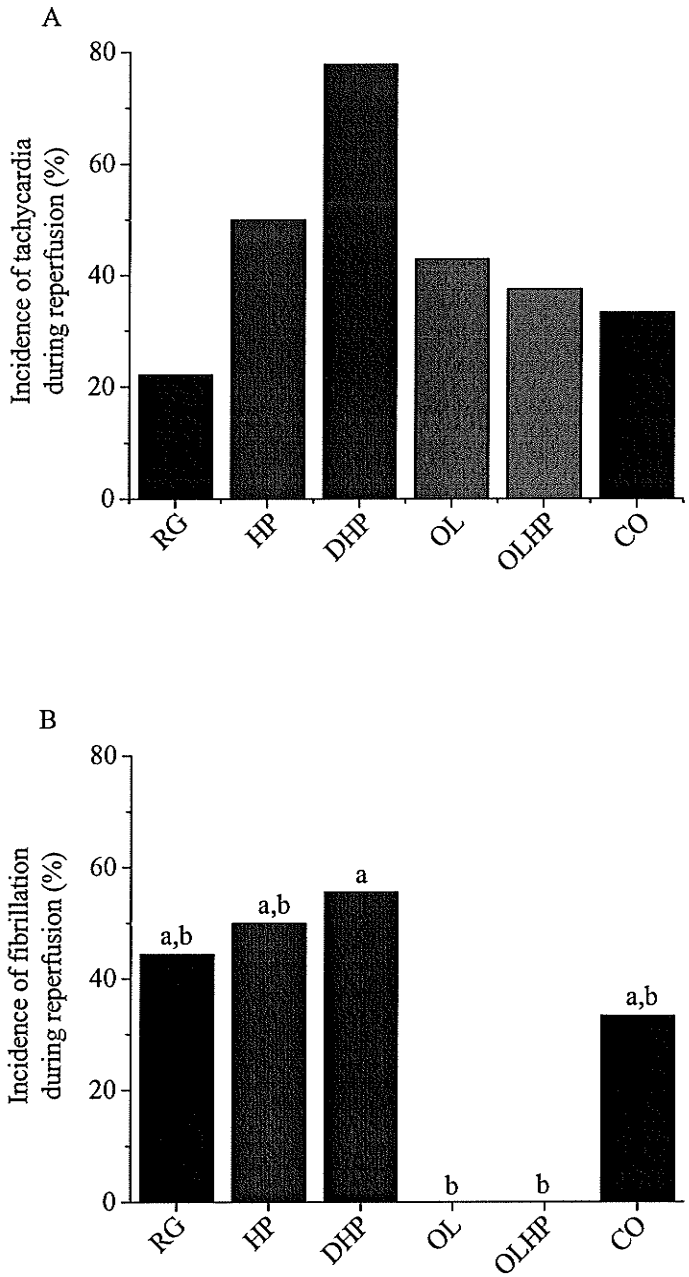


Figure 20: Incidence of reperfusion-derived arrhythmias as a function of dietary intervention. Values shown are the percentage of positive incidence (%) for tachycardia (A) or fibrillation (B) (n = 7-9). No significant differences exist in (A) ($p > 0.05$); significant differences amongst groups in (B) ($p < 0.05$) are denoted by a different letter.

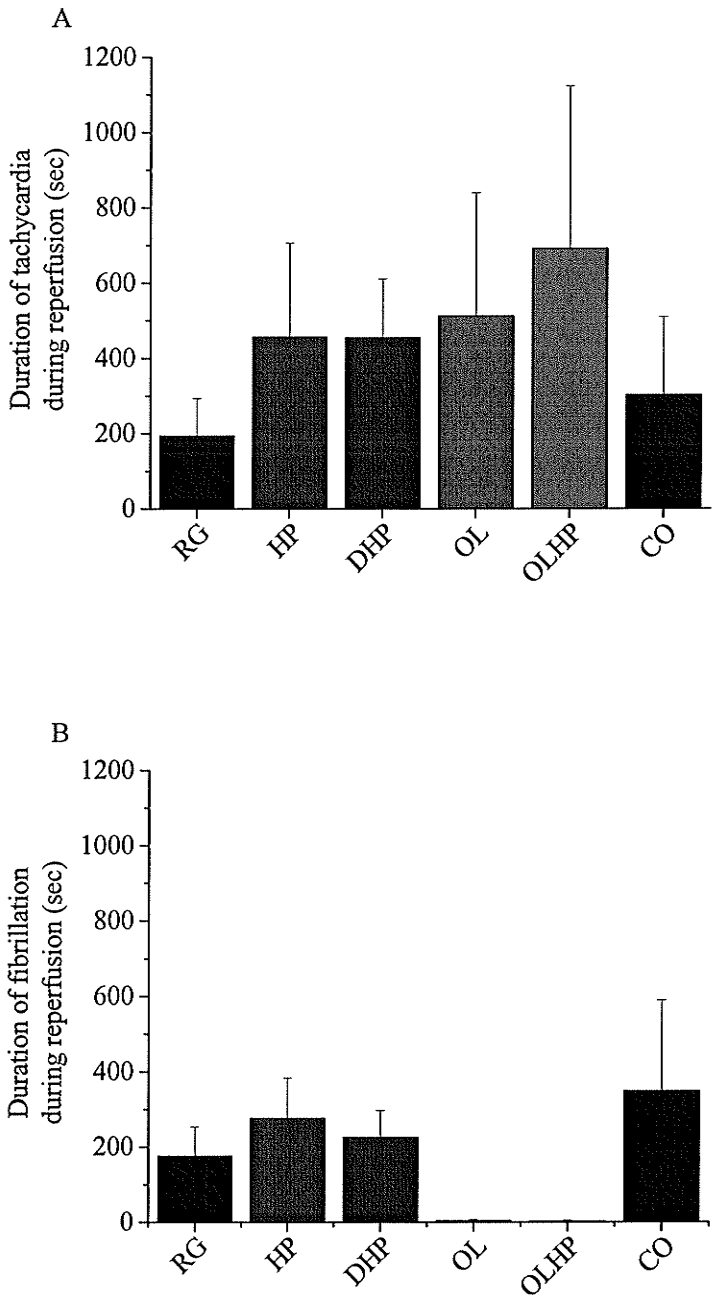


Figure 21: Duration of reperfusion-derived arrhythmias as a function of dietary intervention. Mean ± SEM values are shown for tachycardia (A) and fibrillation (B) (n = 7-9). There were no significant differences amongst the groups ($p > 0.05$).

VII. DISCUSSION

Three basic hypotheses were evaluated in this study. First, we tested the hypothesis that supplementation with dietary cholesterol would increase platelet aggregation induced by ADP/ collagen and increase the incidence and/ or duration of arrhythmias during ischemia/reperfusion (I/R) challenge in comparison to a control diet. Second, we tested the hypothesis that dietary supplementation with ground hempseed would induce a decrease in ADP/ collagen induced platelet aggregation and reduce the incidence and/ or duration of arrhythmias during I/R challenge in comparison to other dietary interventions. Third, we hypothesized that hempseed would reduce the detrimental effects of cholesterol on the two aforementioned parameters. Specifically, it was hypothesized that the unique composition of fatty acids present in hempseed would provide the mechanism responsible for these cardioprotective effects.

The hypothesis that hempseed would produce functional effects on the aforementioned parameters depends upon the efficient absorption of hempseed-derived fatty acids after eight weeks of dietary supplementation. After ingestion and subsequent translocation to the intestinal mucosal cells, fatty acids are processed based on chain length. A chain length of $\leq$12 carbon atoms allows direct fatty acid entry into the portal blood while longer chains are further processed into triacylglycerols, phosphatidylcholine and cholesteryl esters (45). Resynthesized lipids combine with protein and carbohydrates forming chylomicrons which enter the lymphatic circulation and, eventually, the blood (45). Therefore, plasma fatty acid levels should give an excellent indication of fatty acid absorption.

The hempseed supplemented (HP) diet provided significantly elevated levels of 16:0, 18:0, 20:0, 22:0, 18:1n-9, 20:1n-9, LA, GLA and ALA over control levels. Plasma derived from HP rabbits exhibited significantly elevated levels of only GLA and 20:3n-6 over control levels. This is surprising for two reasons: 1) 20:3n-6 was not present in the HP diet and 2) LA and ALA, the two most abundant fatty acids in hempseed were only marginally elevated ($p > 0.05$). The elevation in 20:3n-6 can be readily explained as 20:3n-6 is a downstream metabolite of GLA. However, the lack of a significant increase in plasma levels of LA or ALA suggests either a limited absorption of these fatty acids from the HP diet or a rapid metabolism of LA and ALA into tissue storage sites.

After entering the blood stream, chylomicrons and endogenously formed very-low density lipoprotein (VLDL) are transported throughout the body. They undergo site-specific hydrolysis via lipoprotein lipase to release free fatty acids and diacylglycerols which are readily absorbed (45). In accordance with fatty acid levels in HP plasma, we saw significant elevations in only GLA and 20:3n-6 in the HP cardiac tissue over control levels. However, the only cardiac tissue available had already undergone I/R challenge, as our main interest was in cardiac function after I/R challenge and the role that hempseed-derived fatty acids may have. Since ischemia-activated phopholipase A_2 (116) cleaves fatty acids from membrane phospholipids (1), cardiac fatty acid levels may not be an accurate representation of tissue fatty acid levels under non-I/R challenge conditions. Therefore, it was important to examine fatty acid levels in other tissues not exposed to I/R challenge. We also examined fatty acid levels in two other major organs, the liver and the kidney. In these organs, levels of fatty acids are preserved, unlike those from cardiac tissue, as they were immediately removed from the body, snap-frozen and stored at -

70°C. Furthermore, as the liver plays a major role in lipoprotein metabolism it would most accurately reflect tissue accumulation of all fatty acids. Hepatic fatty acid levels were altered in the HP group. We observed significantly elevated levels of 18:0, LA, GLA and 20:3n-6 over control levels, three of which were significantly elevated in the diet. The latter, 20:3n-6, was not present in the diet but is formed by GLA metabolism. As stated previously, only GLA and 20:3n-6 were significantly elevated in the plasma. This suggests that 18:0 and LA may be rapidly cleared from the plasma and stored in hepatic tissue. Our hepatic fatty acid levels are consistent, to some extent, with renal fatty acid levels. We observed a significant elevation in renal levels of LA, GLA and 20:3n-6 as compared to the control. However, we also saw significant increases in 20:0, 20:3n-3 and 20:5n-3 of which only 20:0 was present at detectable levels in the HP diet.

The cholesterol supplemented (OL) diet had no significant elevations in fatty acid levels as compared to the control. In contrast, plasma levels of all fatty acids were significantly elevated as compared to the control with the notable exception of GLA. Concurrently, plasma levels of triglycerides and cholesterol esters were significantly elevated over control, HP, DHP and CO levels. Dietary cholesterol had a dramatic influence on dietary fatty acid absorption as several of the significantly elevated plasma fatty acids were not present at detectable levels in the OL diet. This finding is comparable to a study by Thomson *et al*, who observed that transient feeding of a high-cholesterol diet (2%) increased jejunal permeability to fatty acids (107). However, the significant increase in most plasma fatty acid levels in the OL group was not translated to increased cardiac fatty acid levels. We observed that over 60% of the fatty acids in cardiac tissue were either present at decreased levels or not present at all compared to control levels.

Only two fatty acids, 22:0 and 20:3n-6, showed significant increases in cardiac tissue as compared to the control. These findings may be due to phospholipase A_2-mediated cleavage of fatty acids within cardiac tissue, resulting in their washout by the perfusate. Fatty acids levels within hepatic and renal tissue also changed due to cholesterol supplementation. We saw significant increases in 16:0, 20:0, 16:1, 18:1n-9, 18:1n-7, 20:1n-9, ALA and 20:5n-3 in hepatic tissue while in renal tissue, we observed a significant increase in 22:1, 20:3n-6 and 22:4 (as compared to hepatic/renal controls, respectively). The elevations observed in hepatic and renal tissue are dissimilar suggesting that these tissues may have distinctively different affinities for fatty acid uptake.

The cholesterol-hempseed supplemented (OLHP) diet provided significantly elevated quantities of 20:0, 22:0, 20:1n-9, LA, GLA and ALA as compared to the control. The dietary level of these fatty acids was similar to the HP diet. However, unlike the HP group, where plasma levels of only two fatty acids were significantly elevated, plasma levels of all fatty acids were significantly elevated in the OLHP group as compared to the control. Concurrently, plasma cholesterol ester levels were significantly elevated over all other groups while plasma triglyceride levels were significantly elevated over control, HP, DHP and CO levels. The observation of increased fatty acid absorption in OLHP plasma is similar to our findings in OL plasma and is assumed to be due to the same mechanism of cholesterol-facilitated fatty acid absorption. In the OLHP plasma we found that levels of several fatty acids (16:0, 18:0, 20:0, 22:0, 24:1n-9, LA, GLA, ALA, 20:2n-6, 20:3n-6, 20:4n-6, 20:5n-3 and 22:6n-3) were significantly elevated over those in the OL plasma. As levels of these fatty acids surpass those in either the HP or OL plasma,

there must be an additive effect of co-supplementation of hempseed and cholesterol which enhances absorption of hempseed-derived fatty acids. The increased levels of all plasma fatty acids did not directly translate to increased cardiac fatty acid levels. This is similar to our findings for the OL group. Once more, over 60% of the cardiac fatty acid levels in the OLHP group were either decreased (as compared to the control) or not present. Only three fatty acids, 22:0, GLA and 20:3n-6 were significantly increased in the cardiac tissue as compared to the control. Notably, this is comparable to the HP group where we saw a significant increase in GLA and 20:3n-6 over control levels. This suggests that these two fatty acids may be preferentially absorbed by cardiac tissue from the plasma. Fatty acid levels within the liver and kidney were also altered in the OLHP group. Unlike cardiac tissue, we observed significant elevations in hepatic levels of several fatty acids (16:0, 20:0, 16:1, 18:1n-9, 18:1n-7, 20:1n-9, LA, GLA, ALA, 20:2n-6, 20:3n-6, 20:5n-3 and 22:4) as compared to the control. This finding was not confirmed in the renal tissue where levels of only 20:0, LA, GLA, 20:3n-6, 22:4 and 22:5 were significantly elevated over control levels. However, all fatty acids significantly elevated in renal tissue were significantly elevated in hepatic tissue, with the exception of 22:5. Therefore, hepatic tissue may be better able to absorb plasma-derived fatty acids. This may be due to its more direct role in lipoprotein metabolism.

In order to differentiate the role of hempseed-derived fatty acids from hempseed itself, we provided a diet including partially delipidated hempseed (DHP). We expected our DHP diet to provide levels of fatty acids comparable to the control diet. However, in the DHP diet, we saw significant increases in several fatty acids (20:0, 20:1n-9, LA, GLA and ALA) over control levels. While significantly increased, these fatty acids were

present at approximately 50% of the levels present in the HP diet (with the exception of 20:1n-9). This depletion of fatty acids in the DHP diet transferred to the plasma where there were no significant changes in plasma fatty acid levels as compared to the control. GLA was not significantly elevated in the plasma (as it was in the HP group) as compared to the control group. This suggests that a minimum level of GLA is required in the diet in order to translate to increased plasma levels. In agreement with the lack of change in plasma fatty acid levels, there were no significant increases in fatty acid levels in either cardiac tissue, hepatic tissue (with the exception of 20:3n-6) or renal tissue (with the exception of 20:3n-6) as compared to the control. It is difficult to explain the increases in 20:3n-6 levels in these tissues. It is possible that an increased affinity for 20:3n-6, over other fatty acids, exists in these tissues. In summary, these results indicate that the DHP diet was appreciably depleted in fatty acids which lead to minimal changes in plasma and tissue fatty acids from control levels and serves as a suitable dietary control in this experimental model.

Two reasons existed for including a coconut oil supplemented (CO) diet. First, this diet provided ~5% SFA which is comparable to saturated fatty acid (SFA) levels in dietary hempseed (~5%). We were interested to see if this level of dietary SFA had its own effect on our study parameters. Secondly, we were interested to see if this level of dietary SFA would provide comparable effects to cholesterol supplementation as both dietary cholesterol and dietary SFA provide a mechanism to raise plasma cholesterol levels. We expected SFA levels in the CO diet to be significantly elevated over the control diet. In agreement, we observed a significant increase in levels of 14:0, 16:0 and 18:0. However, only 14:0 was significantly elevated in the plasma over control levels. As

previously discussed, this limited increase in plasma fatty acids may reflect either limited fatty acid absorption or rapid metabolism into tissue storage sites. Due to minimal levels of SFA in the CO plasma, an SFA-mediated increase in cholesterol levels may not be possible. In agreement, we observed no significant difference in plasma cholesterol ester or triglyceride levels as compared to the control. This finding was in contrast to the study of Nevin *et al* which provided a CO supplemented diet and found significant decreases in both serum cholesterol and triglycerides (compared to a ground nut control) (87). The discrepancy between our study and the one conducted by Nevin *et al* may be due to limited fatty acid absorption from the CO diet in the present study or the different control groups used as a reference point. Tissue fatty acid levels correlated well with our plasma observations in the CO group. In agreement with plasma results, levels of 14:0 were significantly elevated in cardiac, hepatic and renal tissue as compared to their respective controls. We also observed a significant increase of 14:1 in cardiac tissue and 20:5n-3 in renal tissue as compared to their respective controls. These results suggest that 14:0 may be preferentially absorbed, and therefore available to the tissue, over other SFA.

Our hypothesis that dietary hempseed would reduce ADP- and collagen-induced platelet aggregation was based upon previous observations of a reduction of platelet aggregation by LA, GLA, dihomo-GLA, ALA, EPA and DHA (10,33,46,49,97,104,108). As stated previously, our HP, DHP and CO groups did not demonstrate a significantly altered profile for most plasma fatty acids. In addition, these groups did not raise plasma cholesterol levels. Therefore, we did not expect, nor did we observe, a difference in platelet aggregation in these three groups as compared to the control. In contrast, in the OL group, we observed a significant increase in platelet aggregation from control levels.

This increase in platelet aggregation was most likely due to the significantly increased plasma cholesterol levels in this group. This is in agreement with a study by Renaud *et al*, which demonstrated a positive correlation between platelet cholesterol and ADP-induced platelet aggregation (98). This is also in agreement with other studies which demonstrated an increase in platelet aggregation in hyperlipidemic rabbits (33,34) and hypercholesterolemic patients (85). However in the OLHP group, where plasma cholesterol levels remained elevated, we observed no change in platelet aggregation from control values. This indicates that another mechanism, other than a reduction in plasma cholesterol, must be responsible for reducing platelet aggregation in this group. In the OLHP plasma, significant increases in levels of most SFA, one MUFA and all PUFA were seen compared to OL plasma levels. However, the most striking difference was in GLA levels where we observed a ~12-fold increase in the OLHP group, over the OL group. This fatty acid was present in the hempseed supplemented diets but not present in the RG, OL or CO dietary interventions. It is suggested, therefore, that this PUFA in the hempseed may have inhibited the cholesterol-induced stimulation of platelet aggregation. In agreement with our findings, it has been shown that evening primrose oil (EPO), as a source of LA and GLA, can inhibit platelet aggregation. De La Cruz *et al* have shown that supplementation with EPO significantly elevates the concentration of inducer (ADP/ collagen) required to reach 50% maximal platelet aggregation (EC_{50}) in hypercholesterolemic rabbits (33). In addition, EPO supplementation has been shown to significantly reduce thrombin-induced platelet aggregation in butter fed rabbits (97). However, this may have been due to the observed significant reduction in plasma total cholesterol and triglycerides (97) which we did not see in our study. Finally, in

hyperlipidemic patients, EPO supplementation has been shown to significantly reduce platelet aggregation (46). However, the observed significant reduction in serum total-cholesterol, LDL-cholesterol and triglycerides may have provided the mechanism responsible (46). Nonetheless, GLA may be responsible for the observed reduction in platelet aggregation in the OLHP group. As platelet aggregation is thought to be associated with myocardial infarction and sudden ischemic death (76) and platelet aggregation is increased in hypercholesterolemic individuals (33,34,85,98), this effect of dietary hempseed may have a significant potential for reducing the incidence of cardiovascular disease.

Our hypothesis that dietary hempseed would reduce ischemia/reperfusion injury was based on previous observations of a protective effect of LA, ALA, DHA and EPA during I/R challenge (12,23,67,78,79,80,81,82,83,93). The first parameter we assessed was left ventricular end diastolic pressure (LVDEP), the pressure within the ventricle after filling. Ventricular filling depends, in part, on ventricular compliance during diastole. During ischemia, calcium overload can lead to contracture, a state of sustained excess contraction (90) which restricts ventricular compliance. The second parameter we assessed was LV developed pressure (LVDP), a measure of the efficiency of LV contraction. During ischemia, measurement of either LVDP or LVEDP is difficult due to an early, severe depression of ventricular developed pressure (9). Therefore, during this period of depressed systolic function, accurate measurements of these parameters were not possible. During reperfusion, recovery of ventricular developed pressure allowed us to accurately measure LVEDP and LVDP. We found that LVEDP was elevated in all post-ischemic, reperfused hearts but we detected no significant differences amongst the

groups. In addition, we did not observe any significant differences amongst the groups in the post-ischemic recovery of LVDP. These results are in agreement with the results of Ander *et al* (unpublished results) where dietary supplementation with flaxseed (another dietary source of PUFA) did not induce any changes in post-ischemic end diastolic or systolic function.

The third parameter we assessed was the duration of the QT interval. Measurement of this interval gives a valuable index of ventricular action potential duration (59). Prolonged QT intervals are associated with transient, rapid, polymorphic ventricular tachycardia (torsades de pointes) which can clinically manifest as syncope (58). During the P-QRS-T interval, as depicted in Figure 10, the start of the Q-wave represents the initiation of ventricular depolarization while the termination of the T-wave indicates full ventricular repolarization. We took measurements of the QT interval during the last 10 seconds of the equilibration phase, as ischemia may cause abnormalities of action potential duration (90). There were no significant differences in QT interval duration amongst the groups. This is in contrast to Ander *et al* who found a significantly longer QT interval due to dietary cholesterol supplementation and a significantly shorter QT interval due to dietary flaxseed supplementation and dietary flaxseed + cholesterol supplementation, as compared to control values (12). We did observe a trend for the HP hearts to have the shortest QT interval duration, which is similar to the aforementioned study by Ander *et al* after dietary flaxseed supplementation (source of ALA) (12). Therefore, dietary hempseed may provide protection from arrhythmias by shortening the duration of the QT interval. A larger sample size, however, may be needed to establish statistical significance.

The fourth parameter we assessed was the incidence and duration of cardiac arrhythmias during both global ischemia and reperfusion. Cardiac arrhythmias are the result of an inability of the heart to maintain its rhythm (60). Certain arrhythmias, such as ventricular fibrillation, are lethal if left untreated. Ischemic challenge may cause cardiac arrhythmias, as ischemia modifies ion handling, and therefore electrical conduction, in the heart. In our study, we induced global ischemia in the heart to identify the effects of our dietary interventions on arrhythmia generation. We did not observe any significant differences in either incidence or duration of arrhythmias during global ischemia. This was anticipated for the HP, DHP and CO groups due to minimal fatty acid uptake, into both plasma and cardiac tissue. This is in agreement with our platelet aggregation results where we also observed no significant differences in these three groups from the control. In contrast to our hypothesis, the OL group showed no significant increase in either incidence or duration of arrhythmias during ischemia over control levels. This is surprising as it is well established that hypercholesterolemia, which our cholesterol-fed animals clearly demonstrated, increases susceptibility to ventricular arrhythmia (12,72). The reason for this discrepancy between our findings with cholesterol-fed animals and those previously reported is not clear. However, due to the inability of hypercholesterolemia to increase ventricular arrhythmia, we did not observe a significant decrease in arrhythmia generation due to hempseed and cholesterol co-supplementation as compared to cholesterol supplementation alone. During reperfusion, we did not observe any significant differences in the incidence of tachycardia. However, we did observe a significant decrease in the incidence of fibrillation in the OL and OLHP groups as compared to the DHP group. This finding was unexpected for two reasons. Firstly, in

contrast with our hypothesis, this finding demonstrates the possibility that hempseed may be pro-arrhythmic when it contains a reduced lipid level. Secondly, the OL group showed no incidence of fibrillation. As stated previously, hypercholesterolemia increases the susceptibility of the heart to ventricular arrhythmia (12,72). Therefore, we expected an increased incidence of arrhythmia in this group. Conversely, the observation that hempseed and cholesterol co-supplementation significantly decreases the incidence of fibrillation as compared to delipidated hempseed supplementation demonstrates the beneficial properties of hempseed-derived fatty acids. As mentioned previously, the OLHP group demonstrated significant elevations in plasma levels of all fatty acids compared to the control while the DHP group demonstrated no significant elevations in any plasma fatty acid levels over the control. This indicates that the OLHP group should benefit from the fatty acids present in hempseed while the DHP group should obtain very limited benefits. As stated previously, the OLHP hearts had a significantly elevated level of GLA as compared to the DHP hearts. This elevated cardiac GLA level may protect against I/R derived arrhythmias. This is comparable to Charnock, who saw a significant reduction in ischemia/reperfusion-derived arrhythmias after dietary EPO or borage oil supplementation (sources of GLA) in comparison to a saturated fat control (29). Therefore, the absence of incidence of fibrillation in the OLHP group during reperfusion may reflect a potential anti-arrhythmic effect of dietary hempseed in hypercholesterolemic individuals.

From this study, which is the first to examine the effects of dietary hempseed on cardiovascular parameters, we can make several important conclusions. Firstly, supplementation with dietary hempseed results in minimal fatty acid absorption from the

diet unless co-supplemented with dietary cholesterol. Secondly, dietary cholesterol significantly, but non-selectively, enhances fatty acid absorption of all fatty acids present in the diet. Thirdly, supplementation with dietary cholesterol increases the level and rate of platelet aggregation which can be returned to control levels by co-supplementation with dietary hempseed. Finally, after removing a portion of its lipids, hempseed may possess pro-arrhythmic properties. This may be due to the presence of an unknown pro-arrhythmic entity present in the seed. In conclusion, the ability of hempseed to reduce platelet aggregation in hypercholesterolemic individuals suggests that hempseed may have potential in reducing the incidence of cardiovascular disease. This would not appear to occur by decreasing the incidence or duration of arrhythmias.

VIII. BIBLIOGRAPHY

(1) R. M. Berne and M. N. Levy (1998). Physiology, Mosby, Inc.

(2) Hemp Oil Canada Inc. (2001) http://www.hempoilcan.com

(3) Heart and Stroke Foundation of Canada (2003) The Growing Burden of Heart Disease and Stroke in Canada

(4) Manitoba Agriculture, Food and Rural Initiatives (2006) http://www.gov.mb.ca/agriculture/crops/hemp/bko01s00.html

(5) Abeywardena, M. Y. and R. J. Head (2001). "Longchain n-3 polyunsaturated fatty acids and blood vessel function." Cardiovasc Res **52**((3)): 361-71.

(6) Albert, C. M., C. H. Hennekens, C. J. O'Donnell, U. A. Ajani, V. J. Carey, W. C. Willett, J. N. Ruskin and J. E. Manson (1998). "Fish consumption and risk of sudden cardiac death." JAMA **279**(1): 23-8.

(7) Alexy, T., A. Toth, Z. Marton, B. Horvath, K. Koltai, G. Feher, G. Kesmarky, T. Kalai, K. Hideg, B. Sumegi and K. Toth (2004). "Inhibition of ADP-evoked platelet aggregation by selected poly(ADP-ribose) polymerase inhibitors." J Cardiovasc Pharmacol **43**(3): 423-31.

(8) Allen, D. G., S. P. Cairns, S. E. Turvey and J. A. Lee (1993). Intracellular Calcium and Myocardial Function During Ischemia. Interactive Phenomena in the Cardiac System, Plenum Press.

(9) Allen, D. G. and C. H. Orchard (1987). "Myocardial contractile function during ischemia and hypoxia." Circ Res **60**(2): 153-68.

(10) Allman, M. A., M. M. Pena and D. Pang (1995). "Supplementation with flaxseed oil versus sunflowerseed oil in healthy young men consuming a low fat diet: effects on platelet composition and function." Eur J Clin Nutr **49**(3): 169-78.

(11) Ander, B. P., C. M. C. Dupasquier, M. A. Prociuk and G. N. Pierce (2003). Polyunsaturated fatty acids and their effects on cardiovascular disease. Exp Clin Cardiol. **8:** 164-72.

(12) Ander, B. P., A. R. Weber, P. P. Rampersad, J. S. Gilchrist, G. N. Pierce and A. Lukas (2004). "Dietary flaxseed protects against ventricular fibrillation induced by ischemia-reperfusion in normal and hypercholesterolemic rabbits." J Nutr **134**(12): 3250-56.

(13) Asano, G., E. Takashi, T. Ishiwata, M. Onda, M. Yokoyama, Z. Naito, M. Ashraf and Y. Sugisaki (2003). "Pathogenesis and Protection of Ischemia and Reperfusion Injury in Myocardium." J Nippon Med Sch **70**(5): 384-92.

(14) Ascherio, A., E. B. Rimm, E. L. Giovannucci, D. Spiegelman, M. Stampfer and W. C. Willett (1996). "Dietary fat and risk of coronary heart disease in men: cohort follow up study in the United States." BMJ **313**(7049): 84-90.

(15) Avkiran, M. (2003). Basic biology and pharmacology of the cardiac sarcolemmal sodium/hydrogen exchanger. J Card Surg. **18 Suppl 1:** 3-12.

(16) Bang, H. O. and J. Dyerberg (1972). "Plasma lipids and lipoproteins in Greenlandic west coast Eskimos." Acta Med Scand **192**(1-2): 85-94.

(17) Bang, H. O., J. Dyerberg and N. Hjorne (1976). "The Composition of Food Consumed by Greenland Eskimos." Acta Med Scand **200**: 69-73.

(18) Bang, H. O., J. Dyerberg and H. M. Sinclair (1980). "The composition of the Eskimo food in north western Greenland." Am J Clin Nutr **33**: 2657-61.

(19) Bass, O. and J. A. S. McGuigan (1975). "Electrical activity and excitation-contraction coupling in mammalian ventricular muscle." Basic Res Cardiol **70**: 585-95.

(20) Bayindir, O., D. Ozmen, I. Mutaf, N. Turgan, S. Habif, C. Gulter, Z. Parildar and A. Uysal (2002). "Comparison of the Effects of Dietary Saturated, Mono-, and n-6 Polyunsaturated Fatty Acids on Blood Lipid Profile, Oxidant Stress, Prostanoid Synthesis and Aortic Histology in Rabbits." Ann Nutr Metab **46**: 222-28.

(21) Baylin, A., E. K. Kabagambe, A. Ascherio, D. Spiegelman and H. Campos (2003). "Adipose tissue alpha-linolenic acid and nonfatal acute myocardial infarction in Costa Rica." Circulation **107**(12): 1586-91.

(22) Bers, D. M. (2002). "Cardiac excitation-contraction coupling." Nature **415**(6868): 198-205.

(23) Billman, G. E., J. X. Kang and A. Leaf (1999). "Prevention of sudden cardiac death by dietary pure omega-3 polyunsaturated fatty acids in dogs." Circulation **99**(18): 2452-57.

(24) Boersma, E., N. Mercado, D. Poldermans, M. Gardien, J. Vos and M. L. Simoons (2003). "Acute myocardial infarction." Lancet **361**(9360): 847-58.

(25) Botsford, M. W. and A. Lukas (1998). "Ischemic preconditioning and arrhythmogenesis in the rabbit heart: effects on epicardium versus endocardium." J Mol Cell Cardiol **30**: 1723-33.

(26) Bruera, E., F. Strasser, J. L. Palmer, J. Willey, K. Calder, G. Amyotte and V. Baracos (2003). "Effect of Fish Oil on Appetite and Other Symptoms in Patients With Advanced Cancer and Anorexia/Cachexia: A Double-Blind, Placebo-Controlled Study." J Clin Oncol **21**: 129-34.

(27) Callaway, J., U. Schwab, I. Harvima, P. Halonen, O. Mykkanen, P. Hyvonen and T. Jarvinen (2005). "Efficacy of dietary hempseed oil in patients with atopic dermatitis." J Dermatolog Treat **16**(2): 87-94.

(28) Chan, J. K., V. M. Bruce and B. E. McDonald (1991). "Dietary alpha-linolenic acid is as effective as oleic acid and linoleic acid in lowering blood cholesterol in normolipidemic men." Am J Clin Nutr **53**: 1230-34.

(29) Charnock, J. S. (2000). "Gamma-linolenic acid provides additional protection against ventricular fibrillation in aged rats fed linoleic acid rich diets." Prostaglandins Leukot Essent Fatty Acids **62**(2): 129-34.

(30) Cross, H. R., K. Clarke, L. H. Opie and G. K. Radda (1995). "Is lactate-induced myocardial ischaemic injury mediated by decreased pH or increased intracellular lactate?" J Mol Cell Cardiol **27**(7): 1369-81.

(31) Danthi, S. J., J. A. Enyeart and J. J. Enyeart (2005). "Modulation of native T-type calcium channels by omega-3 fatty acids." Biochem Biophys Res Comm **327**: 485-93.

(32) Das, U. N. (1999). "Essential Fatty Acids in Health and Disease." J Assoc Physicians India **47**(9): 906-11.

(33) De La Cruz, J. P., M. Martin-Romero, J. A. Carmona, M. A. Villalobos and F. Sanchez de la Cuesta (1997). "Effect of Evening Primrose Oil on Platelet Aggregation in Rabbits Fed an Atherogenic Diet." Thromb Res **87**(1): 141-49.

(34) De La Cruz, J. P., M. A. Villalobos, J. A. Carmona, M. Martin-Romero, J. M. Smith-Agreda and F. Sanchez de la Cuesta (2000). "Antithrombotic Potential of Olive Oil Administration in Rabbits with Elevated Cholesterol." Thromb Res **100**: 305-15.

(35) de Leiris, J. and F. Boucher (1990). "Ischemic myocardial cell necrosis: calcium overload or oxygen free-radicals?" Rev Port Cardiol **9**(2): 153-58.

(36) de Lorgeril, M., P. Salen, J. L. Martin, I. Monjaud, J. Delaye and N. Mamelle (1999). "Mediterranean diet, traditional risk factors, and the rate of cardiovascular complications after myocardial infarction: final report of the Lyon Diet Heart Study." Circulation **99**(6): 779-85.

(37) Djousse, L., S. C. Hunt, D. K. Arnett, M. A. Province, J. H. Eckfeldt and R. C. Ellison (2003). "Dietary linolenic acid is inversely associated with plasma triacylglycerol: the National Heart, Lung, and Blood Institute Family Heart Study." Am J Clin Nutr **78**: 1098-102.

(38) Dolecek, T. A. and G. Granditis (1991). "Dietary polyunsaturated fatty acids and mortality in the Multiple Risk Factor Intervention Trial (MRFIT)." World Rev Nutr Diet **66**: 205-16.

(39) Drevon, C. A., M. S. Nenseter, I. R. Brude, H. S. Finstad, S. O. Kolset and A. C. Rustan (1995). "Omega-3 fatty acids - Nutritional aspects." Can J Cardiol **11 (Suppl)**: 47G-54G.

(40) Dyerberg, J. and H. O. Bang (1979). "Haemostatic function and platelet polyunsaturated fatty acids in eskimos." Lancet **September 1**: 433-35.

(41) Ferrari, R. (1995). "Metabolic Disturbances During Myocardial Ischemia and Reperfusion." Am J Cardiol **76**: 17B-24B.

(42) Folch, J., M. Lees and G. H. Sloane Stanley (1957). "A simple method for the isolation and purification of total lipides from animal tissues." J Biol Chem **226**: 497-509.

(43) Goel, D. P., T. G. Maddaford and G. N. Pierce (2002). "Effects of omega-3 polyunsaturated fatty acids on cardiac sarcolemmal Na+/H+ exchange." Am J Physiol Heart Circ Physiol **283**: H1688-H94.

(44) Grigoriev, O. V. (2002). "Application of Hempseed (Cannabis sativa L.) Oil in the Treatment of Ear, Nose and Throat (ENT) Disorders." J Industrial Hemp **7**(2): 5-15.

(45) Groff, J. L. and S. S. Gropper (2000). Advanced Nutrition and Human Metabolism.

(46) Guivernau, M., N. Meza, P. Barja and O. Roman (1994). "Clinical and Experimental Study on the Long-term Effect of Dietary Gamma-linolenic Acid on Plasma Lipids, Platelet Aggregation, Thromboxane Formation, and Prostacyclin Production." Prostaglandins Leukot Essent Fatty Acids **51**: 311-16.

(47) Hasler, C. M., A. S. Bloch, C. A. Thomson, E. Enrione and C. Manning (2004). "Position of the American Dietetic Association: Functional foods." J Am Diet Assoc **104**(5): 814-26.

(48) Hasler, C. M., S. Kundrat and D. Wool (2000). "Functional Foods and Cardiovascular Disease." Curr Atheroscler Rep **2**: 467-75.

(49) Hirai, A., T. Hamazaki, T. Terano, T. Nishikawa, Y. Tamura, A. Kamugai and J. Jajiki (1980). "Eicosapentaenoic acid and platelet function in Japanese." Lancet **2**(8204): 1132-33.

(50) Holub, B. J. (2002). "Clinical nutrition: 4. Omega-3 fatty acids in cardiovascular care." CMAJ **166**(5): 608-15.

(51) Honen, B. N., D. A. Saint and D. R. Laver (2003). "Suppression of Calcium Sparks in Rat Ventricular Myocytes and Direct Inhibition of Sheep Cardiac RyR Channels by EPA, DHA and Oleic Acid." J Memb Biol **196**: 95-103.

(52) Hu, F. B., L. Bronner, W. C. Willett, M. J. Stampfer, K. M. Rexrode, C. M. Albert, D. Hunter and J. E. Manson (2002). Fish and omega-3 fatty acid intake and risk of coronary heart disease in women. Jama. **287:** 1815-21.

(53) Hu, F. B., M. J. Stampfer, J. E. Manson, E. B. Rimm, A. Wolk, G. A. Colditz, C. H. Hennekens and W. C. Willett (1999). "Dietary intake of alpha-linolenic acid and risk of fatal ischemic heart disease among women." Am J Clin Nutr **69**(5): 890-97.

(54) Huang, J. M., H. Xian and M. Bacaner (1992). "Long-chain fatty acids activate calcium channels in ventricular myocytes." Proc Natl Acad Sci U S A **89**(14): 6452-56.

(55) Jones, P. J. (2002). "Clinical nutrition: 7. Functional foods - more than just nutrition." CMAJ **166**(12): 1555-63.

(56) Jovanovic, S. and A. Jovanovic (2004). "Sarcolemmal K(ATP) channels in ageing." Ageing Res Rev **3**(2): 199-214.

(57) Kang, J. X. and A. Leaf (2000). Prevention of fatal cardiac arrhythmias by polyunsaturated fatty acids. Am J Clin Nutr. **71:** 202S-7S.

(58) Kass, R. S. and A. J. Moss (2003). "Long QT syndrome: novel insights into the mechanisms of cardiac arrhythmias." J Clin Invest **112**: 810-15.

(59) Katz, A. (2001). Physiology of the Heart.

(60) Keating, M. T. and M. C. Sanguinetti (2001). "Molecular and cellular mechanisms of cardiac arrhythmias." Cell **104**(4): 569-80.

(61) Kristian, T. and B. K. Siesjo (1998). "Calcium in Ischemic Cell Death." Stroke **29**: 705-18.

(62) Lefer, D. J. and D. N. Granger (2000). "Oxidative stress and cardiac disease." Am J Med **109**(4): 315-23.

(63) Leifert, W. R., A. Jahangiri and E. J. McMurchie (1999). Antiarrhythmic fatty acids and antioxidants in animal and cell studies. J Nutr Biochem. **10:** 252-67.

(64) Lemaitre, R. N., I. B. King, D. Mozaffarian, L. H. Kuller, R. P. Tracy and D. S. Siscovick (2003). "n-3 Polyunsaturated fatty acids, fatal ischemic heart disease, and nonfatal myocardial infarction in older adults: the Cardiovascular Health Study." Am J Clin Nutr **77**(2): 319-25.

(65) Lemasters, J. J., J. M. Bond, E. Chacon, I. S. Harper, S. H. Kaplan, H. Ohata, D. R. Trollinger, B. Herman and W. E. Cascio (1996). "The pH paradox in ischemia-reperfusion injury to cardiac myocytes." EXS **76**: 99-114.

(66) Lepage, G. and C. C. Roy (1986). "Direct transesterification of all classes of lipids in a one-step reaction." J Lipid Res **27**: 114-20.

(67) Lepran, I. and L. Szekeres (1992). "Effect of Dietary Sunflower Seed Oil on the Severity of Reperfusion-Induced Arrhythmias in Anesthetized Rats." J Card Pharm **19**: 40-44.

(68) (2000) Evaluating Interference of THC in Hemp Food Products With Employee Drug Testing. Study Summary.

(69) Levine, R. L. (1993). "Ischemia: from acidosis to oxidation." FASEB J **7**(13): 1242-6.

(70) Li, X. L. and M. Steiner (1990). "Fish oil: a potent inhibitor of platelet adhesiveness." Blood **76**(5): 938-45.

(71) Linz, W. J. and A. E. Busch (2003). NHE-1 inhibition: from protection during acute ischaemia/reperfusion to prevention/reversal of myocardial remodelling. Naunyn Schmiedebergs Arch Pharmacol. **368:** 239-46.

(72) Liu, Y.-B., C.-C. Wu, L.-S. Lu, M.-J. Su, C.-W. Lin, S.-F. Lin, L. S. Chen, M. C. Fishbein, P.-S. Chen and Y.-T. Lee (2003). "Sympathetic Nerve Sprouting, Electrical Remoldeling, and Increased Vulnerability to Ventricular Fibrillation in Hypercholesterolemic Rabbits." Circ Res **92**: 1145-52.

(73) Lozano, I. (2001). "The Therapeutic Use of Cannabis sativa (L.) in Arabic Medicine." J Cannabis Therapeutics **1**(1): 63-70.

(74) Macleod, J. C., A. D. Macknight and G. C. Rodrigo (1998). "The electrical and mechanical response of adult guinea pig and rat ventricular myocytes to omega3 polyunsaturated fatty acids." Eur J Pharmacol **356**(2-3): 261-70.

(75) Marchioli, R., F. Barzi, E. Bomba, C. Chieffo, D. Di Gregorio, R. Di Mascio, M. G. Franzosi, E. Geraci, G. Levantesi, A. P. Maggioni, L. Mantini, R. M. Marfisi, G. Mastrogiuseppe, N. Mininni, G. L. Nicolosi, M. Santini, C. Schweiger, L. Tavazzi, G. Tognoni, C. Tucci and F. Valagussa (2002). "Early protection against sudden death by n-3 polyunsaturated fatty acids after myocardial infarction: time-course analysis of the results of the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI)-Prevenzione." Circulation **105**(16): 1897-903.

(76) Massberg, S., C. Schulz and M. Gawaz (2003). "Role of Platelets in the Pathophysiology of Acute Coronary Syndrome." Semin Vasc Med **3**(2): 147-61.

(77) McIntosh, G. H., P. L. McLennan, C. A. Lawson, F. H. Bulman and J. S. Charnock (1985). "The Influence of Dietary Fats on Plasma Lipids, Blood Pressure and Coagulation Indices in the Rat." Atherosclerosis **55**: 125-34.

(78) McLennan, P. L., M. Y. Abeywardena and J. S. Charnock (1985). "Influence of dietary lipids on arrhythmias and infarction after coronary artery ligation in rats." Can J Physiol Pharmacol **63**(11): 1411-7.

(79) McLennan, P. L., M. Y. Abeywardena and J. S. Charnock (1988). "Dietary fish oil prevents ventricular fibrillation following coronary artery occlusion and reperfusion." Am Heart J **116**(3): 709-17.

(80) McLennan, P. L., M. Y. Abeywardena and J. S. Charnock (1989). "The influence of age and dietary fat in an animal model of sudden cardiac death." Aust N Z J Med **19**(1): 1-5.

(81) McLennan, P. L., L. R. Barnden, T. M. Bridle, M. Y. Abeywardena and J. S. Charnock (1992). "Dietary fat modulation of left ventricular ejection fraction in the marmoset due to enhanced filling." Cardiovasc Res **26**(9): 871-7.

(82) McLennan, P. L., T. M. Bridle, M. Y. Abeywardena and J. S. Charnock (1992). "Dietary lipid modulation of ventricular fibrillation threshold in the marmoset monkey." Am Heart J **123**(6): 1555-61.

(83) McLennan, P. L. and J. A. Dallimore (1995). "Dietary Canola Oil Modifies Myocardial Fatty Acids and Inhibits Cardiac Arrhythmias in Rats." J Nutr **125**: 1003-09.

(84) Mutanen, M. and R. Freese (1996). "Polyunsaturated fatty acids and platelet aggregation." Curr Opin Lipidol **7**: 14-19.

(85) Naqvi, T. Z., P. K. Shah, P. A. Ivey, M. D. Molloy, A. M. Thomas, S. Panicker, A. Ahmed, B. Cercek and S. Kaul (1999). "Evidence That High-Density Lipoprotein Cholesterol Is an Independent Predictor of Acute Platelet-Dependent Thrombus Formation." Am J Cardiol **84**: 1011-17.

(86) Negretti, N., M. R. Perez, D. Walker and S. C. O'Neill (2000). "Inhibition of sarcoplasmic reticulum function by polyunsaturated fatty acids in intact, isolated myocytes from rat ventricular muscle." J Physiol **523**(2): 367-75.

(87) Nevin, K. G. and T. Rajamohan (2004). "Beneficial effects of virgin coconut oil on lipid parameters and in vitro LDL oxidation." Clin Biochem **37**(9): 830-5.

(88) Nieswandt, B. and S. P. Watson (2003). "Platelet-collagen interaction: is GPVI the central receptor?" Blood **102**(2): 449-61.

(89) Oomen, C. M., E. J. Feskens, L. Rasanen, F. Fidanza, A. M. Nissinen, A. Menotti, F. J. Kok and D. Kromhout (2000). "Fish consumption and coronary heart disease mortality in Finland, Italy, and The Netherlands." Am J Epidemiol **151**(10): 999-1006.

(90) Opie, L. (1998). The Heart. Physiology, from Cell to Circulation.

(91) Parratt, J. R., C. L. Wainwright, S. J. Coker and I. J. Zeitlin (1988). "Eicosanoids and cardiac arrhythmias." Biomed Biochim Acta **47**(10-11): S13-8.

(92) Patel, S., R. Cudney and A. McPherson (1994). "Crystallographic Characterization and Molecular Symmetry of Edestin, a Legumin from Hemp." J Mol Biol **235**: 361-63.

(93) Pepe, S. and P. L. McLennan (1996). "Dietary fish oil confers direct antiarrhythmic properties on the myocardium of rats." J Nutr **126**(1): 34-42.

(94) Pepe, S. and P. L. McLennan (2002). "Cardiac membrane fatty acid composition modulates myocardial oxygen consumption and postischemic recovery of contractile function." Circulation **105**(19): 2303-8.

(95) Philipson, K. D. and R. Ward (1985). "Effects of fatty acids on Na+-Ca2+ exchange and Ca2+ permeability of cardiac sarcolemmal vesicles." J Biol Chem **260**(17): 9666-71.

(96) Przybylski, R. and K. Fitzpatrick (2002). "Does hempseed need to be the forbidden crop?" inform **13**: 489-91.

(97) Renaud, S., L. McGregor, R. Morazain, C. Thevenon, C. Benoit, E. Dumont and F. Mendy (1982). "Comparative Beneficial Effects on Platelet Functions and

Atherosclerosis of Dietary Linoleic and Gamma-Linolenic Acids in the Rabbit." Atherosclerosis **45**: 43-51.

(98) Renaud, S., R. Morazain, F. Godsey, E. Dumont, C. Thevenon, J. L. Martin and F. Mendy (1986). "Nutrients, platelet function and composition in nine groups of French and British farmers." Atherosclerosis **60**(1): 37-48.

(99) Salem Jr, N. (1999). "Introduction to Polyunsaturated Fatty Acids." Backgrounder **3**(1): 1-8.

(100) Simopoulos, A. P. (1999). Essential fatty acids in health and chronic disease. Am J Clin Nutr. **70:** 560S-69S.

(101) Simopoulos, A. P. (2002). The importance of the ratio of omega-6/omega-3 essential fatty acids. Biomed Pharmacother. **56:** 365-79.

(102) Simpson, H. C. R., K. Barker, R. D. Carter, E. Cassels and J. I. Mann (1982). "Low dietary intake of linoleic acid predisposes to myocardial infarction." Br Med J **285**(11 September): 683-84.

(103) Singh, R. B., M. A. Niaz, J. P. Sharma, R. Kumar, V. Rastogi and M. Moshiri (1997). Randomized, double-blind, placebo-controlled trial of fish oil and mustard oil in patients with suspected acute myocardial infarction: the Indian experiment of infarct survival--4. Cardiovasc Drugs Ther. **11:** 485-91.

(104) Smith, D. L., A. L. Willis, N. Nguyen, D. Conner, S. Zahedi and J. Fulks (1989). "Eskimo Plasma Constituents, Dihomo-gamma-linolenic Acid, Eicosapentaenoic Acid and Docosahexaenoic Acid Inhibit the Release of Atherogenic Mitogens." Lipids **24**(1): 70-75.

(105) Tanelian, D. (2001). Hemp: Nature's Forgotten Nutraceutical. Natural Pharmacy Magazine.

(106) Theroux, P. (1999). "Protection of the Myocardial Cell During Ischemia." Am J Cardiol **83**: 3G-9G.

(107) Thomson, A. B., M. Keelan and M. Tavernini (1987). "Early feeding of a high-cholesterol diet enhances intestinal permeability to lipids in rabbits." Pediatr Res **21**(4): 347-51.

(108) Vas Dias, F. W., M. J. Gibney and T. G. Taylor (1982). "The effect of polyunsaturated fatty acids of the n-3 and n-6 series on platelet aggregation and platelet and aortic fatty acid composition in rabbits." Atherosclerosis **43**(2-3): 245-57.

(109) Walker, M. J., M. J. Curtis, D. J. Hearse, R. W. Campbell, M. J. Janse, D. M. Yellon, S. M. Cobbe, S. J. Coker, J. B. Harness, D. W. Harron and et al. (1988). "The Lambeth Conventions: guidelines for the study of arrhythmias in ischaemia, infarction and reperfusion." Cardiovasc Res **22**(7): 447-55.

(110) Wijendran, V. and K. C. Hayes (2004). Dietary n-6 and n-3 fatty acid balance and cardiovascular health. Annu Rev Nutr. **24:** 597-615.

(111) Xiao, Y. F., A. M. Gomez, J. P. Morgan, W. J. Lederer and A. Leaf (1997). "Suppression of voltage-gated L-type Ca2+ currents by polyunsaturated fatty acids in adult and neonatal rat ventricular myocytes." Proc Natl Acad Sci U S A **94**(8): 4182-7.

(112) Xiao, Y. F., J. X. Kang, J. P. Morgan and A. Leaf (1995). "Blocking effects of polyunsaturated fatty acids on Na+ channels of neonatal rat ventricular myocytes." Proc Natl Acad Sci U S A **92**(24): 11000-4.

(113) Xiao, Y.-F., Q. Ke, Y. Chen, J. P. Morgan and A. Leaf (2004). "Inhibitory effect of n-3 fish oil fatty acids on cardiac Na+/Ca2+ exchange currents in HEK293t cells." Biochem Biophys Res Comm **321**: 116-23.

(114) Xiao, Y.-F., Q. Ke, S.-Y. Wang, Y. Yang, Y. Chen, G. K. Wang, J. P. Morgan, B. Cox and A. Leaf (2004). "Electrophysiologic properties of lidocaine, cocaine, and n-3 fatty-acids block of cardiac Na+ channels." Eur J Pharmacol **485**: 31-41.

(115) Xiao, Y. F., J. P. Morgan and A. Leaf (2002). "Effects of polyunsaturated fatty acids on cardiac voltage-activated K(+) currents in adult ferret cardiomyocytes." Acta Physiologica Sinica **54**(4): 271-81.

(116) Xiao, Y. F., A. J. Zeind, V. Kaushik, C. L. Perreault-Micale and J. P. Morgan (2000). Mechanism of suppression of cardiac L-type Ca(2+) currents by the phospholipase A(2) inhibitor mepacrine. Eur J Pharmacol. **399:** 107-16.

(117) Yuan, J.-M., R. K. Ross, Y.-T. Gao and M. C. Yu (2001). "Fish and Shellfish Consumption in Relation to Death from Myocardial Infarction among Men in Shanghai, China." Am J Epidemiol **154**: 809-16.

(118) Zamaria, N. (2004). "Alteration of polyunsaturated fatty acid status and metabolism in health and disease." Reprod Nutr Dev **44**(3): 273-82.

(119) Zhao, G., T. D. Etherton, K. R. Martin, S. G. West, P. J. Gillies and P. M. Kris-Etherton (2004). "Dietary Alpha-Linolenic Acid Reduces Inflammatory and Lipid

Cardiovascular Risk Factors in Hypercholesterolemic Men and Women." J Nutr **134**: 2991-97.

(120) Zhao, Z. Q. (2004). "Oxidative stress-elicited myocardial apoptosis during reperfusion." Curr Opin Pharmacol **4**(2): 159-65.

(121) Zheng, H. F., X. L. Li, Z. Y. Jin, J. B. Sun, Z. L. Li and W. X. Xu (2005). Effects of unsaturated fatty acids on calcium-activated potassium current in gastric myocytes of guinea pigs. World J Gastroenterol. **11:** 672-5.