The Effect of Dietary Hempseed on Atherosclerotic Plaque Formation and Contractile Function of the Aorta from Hypercholesterolemic Rabbits

By

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A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfilment of the Requirements
For the Degree of

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Abstract

Atherosclerosis is a chronic disease which has important health implications. A number of risk factors for atherosclerosis have been identified including a poor diet that is high in saturated fats and cholesterol. Omega-3 polyunsaturated fatty acids from fish oil and flaxseed have been studied for their beneficial cardiovascular effects. These include effects on atherogenesis, platelet aggregation and adhesion, and improving vascular tone following atherogenesis. Research has shown that the ratio of omega-6 to omega-3 fatty acids is important in modulating subsequent eicosanoid metabolism and the physiological effects mediated by the metabolites. Hempseed has the optimal ratio of 4:1 omega-6 to omega-3 fatty acids, which makes this plant an ideal nutraceutical with cardioprotective effects. Our hypothesis is that dietary supplementation with hempseed will decrease platelet aggregation, decrease plaque formation, and improve vascular tone in rabbits even in the presence of elevated dietary cholesterol.

This study examined the effects of various dietary interventions on vascular function of male New Zealand white rabbits. The rabbits were separated into 6 groups of 10 rabbits in each, and were fed regular rabbit chow (control), or diets supplemented with 10% hempseed, 10% hempseed cake, 0.5% cholesterol, 5% coconut oil, and 10% hempseed with 0.5% cholesterol. The diets were fed to the animals for an 8 week period after which plasma was analysed for lipid content and platelet aggregation, the aorta was removed for enface and histological analysis of atherosclerotic plaque formation, and aortic rings were removed and perfused *in vitro* to measure vascular function. The

response of the aortic rings to KCl, increasing concentrations of norepinephrine, acetylcholine, and sodium nitroprusside was measured as an indicator of vascular tone.

Both cholesterol levels and triglycerides levels increased in the cholesterol and cholesterol plus hempseed groups. Furthermore, supplementation with hempseed did not significantly alter these values at the end of the study. Dietary hempseed supplementation did not significantly increase plasma fatty acid levels at any time during the study. However, there were significant increases in the levels of both saturated and unsaturated fatty acids in the cholesterol and cholesterol plus hempseed dietary groups at the end of the study in comparison to the control diet. The presence of cholesterol appeared to facilitate the absorption of these lipids from the diet.

Platelet aggregation (both extent and rate of aggregation) was significantly increased in the cholesterol and seedcake groups in comparison to the control group when collagen and ADP were used to stimulate aggregation. Hempseed inhibited the stimulatory effect of cholesterol on platelet aggregation.

Atherosclerotic plaque deposition was significantly increased in the two dietary groups that were supplemented with cholesterol. Hempseed supplementation did not affect the amount of plaque or the thickness of the plaque that was formed.

The cholesterol, hempseed, and cholesterol plus hempseed dietary groups all had a decreased contractile response to KCl. When exposed to norepinephrine, aortic tissue samples from the cholesterol and cholesterol/hempseed diet groups exhibited a significantly attenuated tension generation when compared to the control group. To assess endothelium dependant relaxation, the tissues were treated with acetylcholine. No significant differences amongst groups were observed. However, endothelium

independent relaxation via sodium nitroprusside was significantly depressed in the cholesterol plus hempseed group at the two highest concentrations of SNP in comparison to the relaxation response of the control tissues.

As a result of data obtained from this study, we can conclude that hempseed may be a valuable dietary supplement to inhibit platelet aggregation under conditions of hypercholesterolemia. Direct evaluation of vascular function and atherogenesis did not reveal significant beneficial effects of dietary hempseed supplementation. However, in most experiments, it is just as important to note that there was also not a deleterious effect which contrasts previous reports in the literature. Additional studies should be performed, in the future, to further examine the mechanisms responsible for the potential cardioprotective effects of hempseed supplementation.

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I. Introduction

In 1999, cardiovascular diseases were the leading cause of death in Canada (1). With mortality levels at 36% that year, the resulting costs to the health care industry were significant. The direct and indirect expenditures in that year approached $18.5 billion dollars for Canada (1). There are many risk factors for cardiovascular disease that are preventable and avoidable, including smoking, inactivity, obesity, an unhealthy diet high in saturated and trans fats with low levels of fruits and vegetables, high blood pressure, and Type II diabetes (1, 2).

A great deal of research has focused on the impact of dietary changes on heart disease and atherosclerosis. An updated Food Pyramid was recently released (3) that emphasizes exercise and weight control, whole grains, fruits and vegetables, and healthy fats (monounsaturated, polyunsaturated). This represents a shift in thinking from the traditional "Western Diet", high in overall fat, saturated fat, and trans fat, high in omega-6 polyunsaturated fatty acids, low in antioxidants and low in omega-3 polyunsaturated fatty acids (4). The Mediterranean Diet is high in monounsaturated fatty acids, low in saturated fats and trans fats, with a balance between omega-6 and omega-3 fatty acids, and including many fruits and vegetables, has been associated with a decrease in cancer and heart disease, and longer life (4).

The essential fatty acids, omega-3 (alpha-linolenic acid) and omega-6 (linoleic acid) have been studied extensively, and benefits of their intake have emerged. Omega-3 essential fats have been shown to reduce heart disease, diabetes, and hypertension (4). While omega-3 fatty acids are considered healthy and beneficial for a variety of diseases, omega-6 fatty acids, in excess, are thought to be harmful. A

4: 1 (omega-6:omega-3) ratio is ideal for normal metabolism and for eicosanoid production (4). Hempseed is a rich source of macronutrients and micronutrients, phytosterols, antioxidants, and has the ideal fatty acid ratio of 4:1 (LA to ALA). Therefore, dietary consumption may have an impact on vascular tone, platelet aggregation, inflammation, and chemotaxis.

II. Review of Literature

A. The Cardiovascular System

The heart and blood vessels make up the cardiovascular system, with the heart pumping blood throughout the body, and the blood circulating nutrients and removing waste. The heart is one of the first organs to develop, and this early development is necessary so that the developing fetus can obtain nutrients from the mother's circulation via the placenta to nourish the developing organs and tissues (5). While this circulation of nutrients is especially important in the earliest stages of life, it is a crucial part of the healthy maintenance of cells throughout life.

There are 3 basic components of the cardiovascular system: the heart, the blood vessels, and the blood. The heart acts as a pump, creating a favourable pressure gradient so that the blood can flow down the pressure gradient. The blood vessels are the tubes through which the blood flows and allow the blood to reach all cells, tissues, and organs in the body. The blood itself is responsible for delivering essential nutrients to all areas of the body and for removing waste products, maintaining cellular homeostasis.

Atherosclerosis is a vascular disease which causes the arteries to develop plaque and to eventually harden. Plaque develops on the arterial walls in a progressive fashion, often resulting in a significant clinical event such as a heart attack or stroke. A number of physiological elements are significant in the development and progression of the disease, including the circulating blood, the endothelium, smooth muscle cells of the blood vessels, lipoproteins and cholesterol.

1. Blood

The composition of the blood is about 55% plasma, 45% red blood cells (erythrocytes), and less than 1% of the blood is white blood cells (leukocytes) and platelets (5). Plasma is about 90% water, which allows for the transport of water soluble organic and inorganic electrolytes (HCO_3^-, Na^+, Cl^-, K^+, and Ca^{2+}, etc.). The plasma is also an important medium for the transport of nutrients, waste products, and hormones to the appropriate location within the body.

One of the most important parts of blood plasma is the plasma proteins which are divided into three groups: albumin, globulins, and fibrinogen, each one having a specific role (5). Albumin plasma proteins are important in binding to other molecules to transport them from one part of the body to another part of the body (5). Globulins are further subdivided into three classes, alpha, beta, and gamma globulins. The alpha and beta globulins are important for the transport of substances, such as cholesterol, and they have a role in the formation of blood clots (5). The alpha globulins are important inactive protein precursors that are activated as needed, and the gamma globulins are known as immunoglobulins, which are an important part of the immune system (5). Fibrinogen is a crucial part of the blood coagulation mechanism (5).

2. Hemostasis

Hemostasis is the process that is initiated as a result of damage to a blood vessel that stops bleeding (5). When a blood vessel is damaged, three processes occur to control and stop the bleeding. The first step is the initiation of a vascular spasm,

which constricts the vessel to slow blood flow (5). Platelets play an important role in the next step of hemostasis, which is the formation of a platelet plug. Platelets are cellular fragments derived from megakaryocytes and are stored in the spleen until they are needed (5). Given that they are not complete cellular structures (for example, they do not contain nuclei), platelets are limited to specific functions which include the ability to generate energy, make secretory products, and with a high concentration of actin and myosin, they are able to contract when necessary (5). When a blood vessel is injured, collagen is exposed, providing a surface to which platelets can adhere. As the platelets begin to stick to the collagen, they release adenosine diphosphate (ADP), which creates a "sticky" surface to which platelets may also adhere. This positive feedback loop continues until a platelet plug is formed at the injury site (**Figure 1**). A second chemical, thromboxane A_2 is released from the cell membrane of platelets as they come into contact with collagen, which also signals for the release of ADP, further supporting the formation of a platelet plug. The accumulation of platelets and the development of the platelet plug are controlled by the surrounding, healthy, normal endothelial cells which release prostacyclin, a platelet aggregation inhibitor.

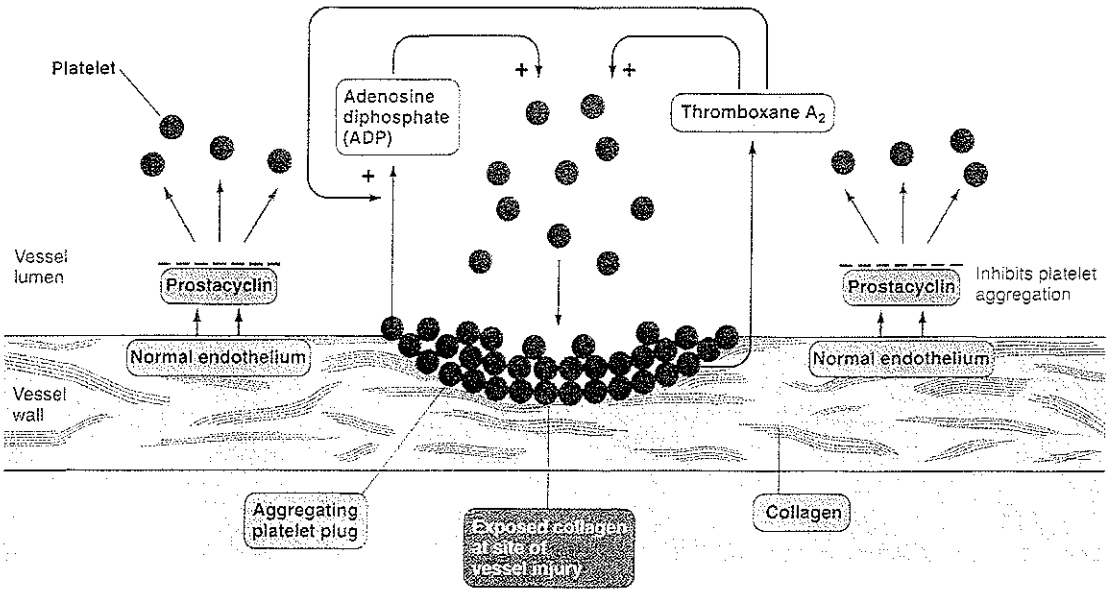


Figure 1: Platelet Plug Formation (5)

The platelet plug has three important tasks during the development of hemostasis. Once the plug is formed, actin and myosin interact within the platelets to draw the platelets closer together to form a stronger, more densely packed plug. The platelet plug maintains the vascular spasm with the release of chemicals that cause vasoconstriction, including serotonin, epinephrine, and thromboxane A_2. The third important task of the plug is to release chemicals that contribute to the formation of blood clots.

Clot formation provides added support for the platelet plug, and provides a means to slow bleeding from large injuries. The conversion of blood from a liquid to a gel is accomplished via a series of reactions, and can occur through the intrinsic or extrinsic pathways (**Figure 2**). The most significant step in clot formation is the conversion of fibrinogen to fibrin, a reaction catalyzed by the protease, thrombin. Fibrin is a stringy, insoluble protein that attaches to the damaged vessel and forms a net that traps blood cells to form a clot. The net or mesh crosslinking is strengthened via thrombin activated factor XIII, fibrin stabilizing factor. In order to reach this final, critical step, a number of reactions must occur, through the activation of 12 clotting factors.

The extrinsic pathway of the coagulation cascade is initiated when a vessel is damaged, which causes the activation of prothrombin to thrombin via factor X (activated plasma clotting factor), which is itself activated by tissue thromboplastin in response to the injury. Thrombin acts directly to activate fibrin, and the clot is

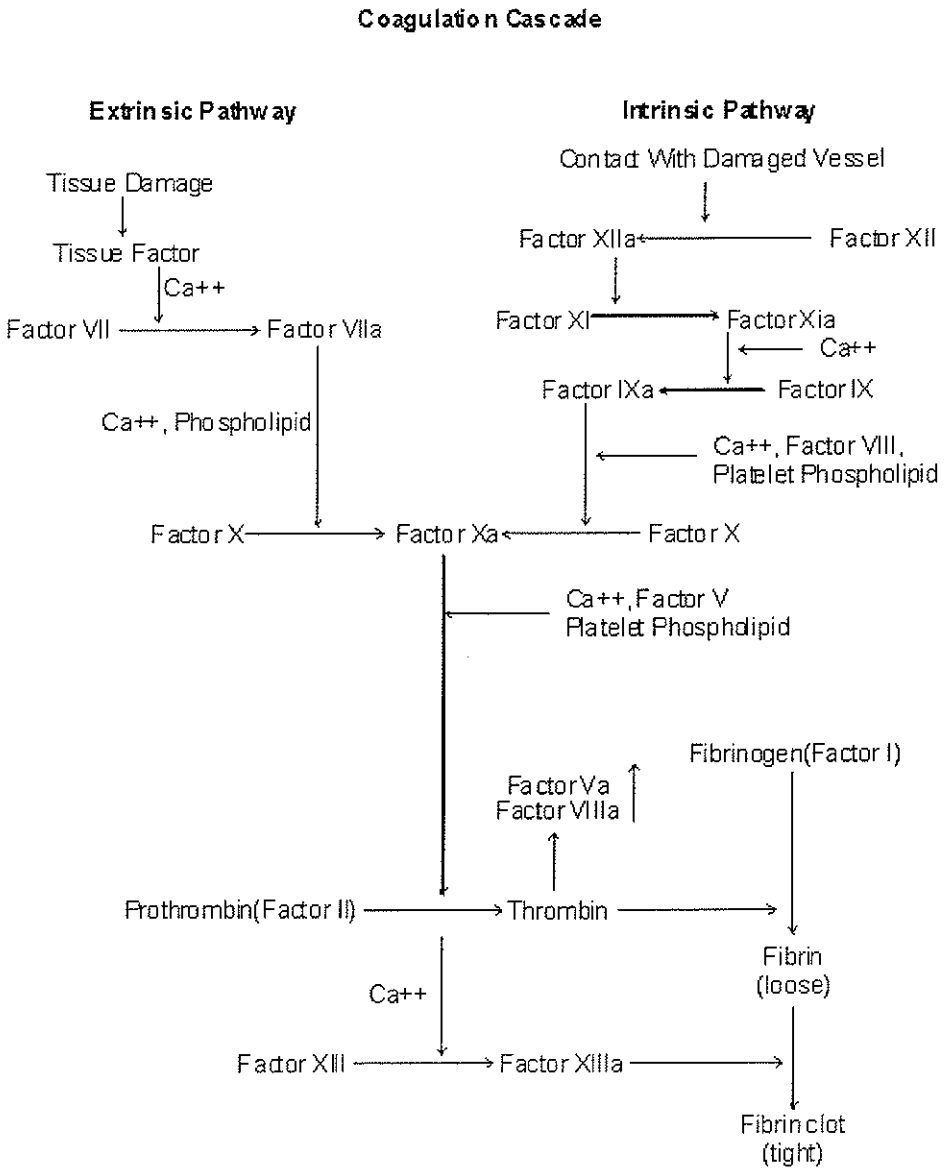


Figure 2: Steps in the clotting cascade
http://www.arn.org/docs/glicksman/eyw_040501.htm

formed. The intrinsic pathway begins with exposure to collagen, which activates the Hageman factor (factor XII), and the six subsequent reactions follow to the formation of a clot. These two pathways operate cooperatively, with the intrinsic pathway controlling the blood in the blood vessel, and the extrinsic pathway arresting the bleeding into tissues. At the termination of the clotting cascade, and when the clot is no longer necessary, plasmin, a fibrinolytic enzyme, dissolves the clot.

3. Blood Vessel Histology

Blood vessels consist of 3 regions known as *tunica adventitia, tunica media,* and *tunica intima,* with the *tunica intima* as the innermost section (**Figure 3**). Each region has varying amounts of smooth muscle tissue and elastin, depending on the requirements of the tissue. The tremendous diversity of cells, organs, and tissues perfused by the blood vessels requires them to develop some distinguishing features.

The *tunica adventitia* is the outermost layer of the vessel, and consists mostly of fibro-elastic connective tissue (6). The middle layer is the *tunica media* and it is made up, mostly, of smooth muscle tissue and elastin fibers (6). Larger arteries, with a higher amount of blood flowing through them, can also have a layer for structural support, known as the external elastic lamina (6). The *tunica intima* is the innermost layer of the blood vessel. It is made up of one layer of endothelial cells that is supported by the basement membrane, followed by a sub-endothelial layer of fibro-elastic connective tissue and a layer of internal elastic lamina for stability and flexibility (6). Surrounding the endothelial cells are pericytes, stem cells which can develop into different cell types, depending on the requirements of the region (6).

Pericytes help to maintain a balanced cellular environment for the endothelial layer of cells, and may also play a role in the contractility of the blood vessels (6).

4. The Endothelium

Endothelial cells play a critical role in the maintenance of vascular homeostasis (7) (8, 9). They have the ability to sense changes in the immediate environment, via chemical or physical stimuli, and to respond immediately by releasing vasoactive chemicals, thromboregulatory molecules, or growth factors (7) (8). These stimuli are recognized by mechanoreceptors or ligand receptors that are located on the cell membrane of endothelial cells. In response to these stimuli, the endothelial cells release thromboregulatory molecules, vasoactive chemicals, or growth factors into the lumen of the vessel, or into the space surrounding the vascular smooth muscle cells (VSMC) (7). Endothelial cells can also affect the regulation of immunological expression through various immune modulators, and the regulation of inflammatory processes through cell surface adhesion molecules (5,6,9).

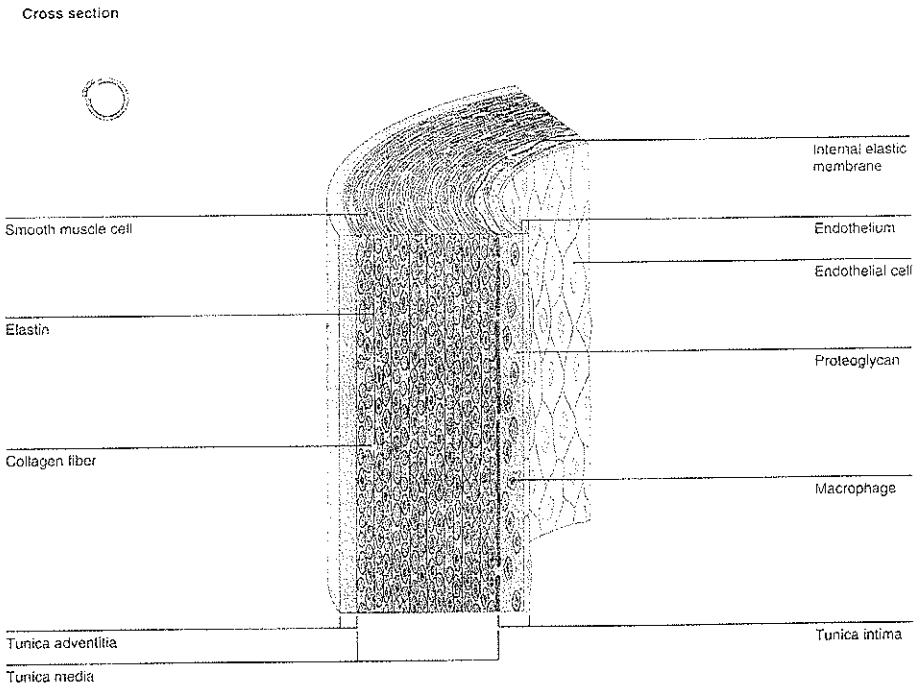


Figure 3: Cross sectional view of a blood vessel (10)

The balance between coagulation and blood fluidity is achieved through thromboregulatory molecules released by endothelial cells. Some molecules which are known to induce platelet formation include thrombin, adenosine diphosphate (ADP), and adenosine triphosphate (ATP) (7). While these molecules promote the formation of platelets and thrombosis, they also cause prostacyclin to be released from the endothelium. This is significant because prostacyclin inhibits the ability of these platelets to adhere to the endothelial cell surface, and ultimately function as platelet plugs. In regulating the release of these molecules, the endothelium is able to control blood fluidity.

Vascular tone is regulated by the endothelium through the release of vasoconstrictors and vasodilators that act on the VSMC. One of the most significant vasodilators is the endothelial derived relaxant factor (EDRF) or nitric oxide (NO) which has been shown to relax VSMCs by decreasing the intracellular levels of calcium (7). Nitric oxide is released in response to increased or turbulent blood flow, and chemical stimuli such as acetylcholine, prostacyclin, bradykinin, and thrombin (7).

Just as the endothelium can release chemicals to relax the smooth muscle cells, it can also release chemicals to contract these cells. One example of an endothelium derived contracting factor is endothelin (ET-1), which is elevated in some disease states, including hypertension, coronary artery disease, and heart failure (7).

Acetylcholine is another vasoactive factor that can act as a vasodilator or as a vasoconstrictor (7). When the endothelium has been damaged, acetylcholine acts as a vasoconstrictor, however, under normal conditions, where the endothelium is intact, acetylcholine acts as a vasodilator through a nitric oxide mediated mechanism. The reaction of L-arginine with the enzyme nitric oxide synthase (NOS) results in the formation of nitric oxide and L-citrulline (7, 11). Nitric oxide activates guanylate cyclase to increase cyclic guanosine monophosphate (cGMP), which decreases intracellular Ca^{2+} levels in smooth muscle cells (7, 12). When NO is derived from an external source, such as it is with sodium nitroprusside (SNP), this mechanism of relaxation is the same (7). Nitric oxide generation is initiated by membrane receptors interacting with acetylcholine, bradykinin, or thrombin, or by the activation of endothelial mechanoreceptors in response to changes in shear force (7, 13, 14).

5. Nitric Oxide

Nitric oxide is an important regulatory chemical due to its ability to affect homeostasis. This is achieved through dilation of vessels (as discussed above) and the inhibition of vasoconstriction, inhibition of platelet aggregation and adherence, immunological effects, and through neurotransmission (11). Given that nitric oxide is an uncharged radical, it can act as an electron donor, and as an antioxidant (11). The protective effect of NO as an antioxidant is significant given that oxidative stress has been linked to a number of diseases, including atherosclerosis.

B. Muscle Physiology

Muscles in the human body are divided into three types; skeletal, cardiac, and smooth. Skeletal muscles are striated and are connected to the bones in the body, allowing for voluntary movement. Cardiac muscles are striated muscles that are found in the heart. Smooth muscles are unstriated muscles that are found in all hollow organs and tubes within the body. The cardiac and smooth muscles respond to the autonomic nervous system and contract or relax involuntarily.

All the muscle groups have actin (thin filaments) and myosin (thick filaments) which respond to increased Ca^{2+} in the cell by contracting. A further similarity between the muscle groups is that they all acquire the energy used for cross bridge cycling from ATP. The way that the contraction occurs, the excitation-contraction coupling mechanism, and the contraction itself, differ for each muscle type. Smooth muscle cells are found in blood vessels and are the most relevant muscle group for atherosclerosis.

1. Mechanism of Contraction

Smooth muscle cells have an elongated, spindle shaped structure, with one single nucleus. These cells can be contracted after exposure to an agonist (neurotransmitter, hormones, etc) (5). This exposure activates membrane receptors and Ca^{2+} channels to induce an increase in intracellular Ca^{2+}. Alternatively, excitation can arise due to changes in the membrane potential. Vascular smooth muscle cells

are able to maintain tension with little energetic expenditure – a characteristic that is not found in cardiac and skeletal muscle cells (15).

Muscle contraction is mediated by a rise in the concentration of Ca^{2+} within the cell (**Figure 4**). Increased Ca^{2+} levels can be achieved by an influx of calcium into the cell from the extracellular fluid (ECF), or by activating Ca^{2+} channels on the sarcoplasmic reticulum (SR) within the cell. When the concentration of Ca^{2+} in the cell increases, Ca^{2+} complexes with calmodulin causing a conformational change and allowing for the activation of myosin light chain kinase (MLCK) (16). As a result of the activation of MLCK, the serine residue on the myosin light chain is phosphorylated. This phosphorylation reaction is a critical step in the contraction of smooth muscle cells. Once the myosin has been phosphorylated, it can interact with the actin filament and initiate cross-bridge cycling and muscular contraction (17).

External chemical agonists like norepinephrine, endothelin, and adenosine triphosphate (ATP) can bind to receptors in the membrane, and, when coupled with a G-protein, stimulate phospholipase C (PLC) activity. Phospholipase C reacts specifically with phosphatidylinositol 4,5-bisphosphate to produce inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 triggers the release of Ca^{2+} from the SR. In addition, when DAG is in a Ca^{2+} rich environment, it activates protein kinase C (PKC). PKC phosphorylates specific proteins that control cross-bridge cycling and thus mediating muscle cell contraction. PKC can also affect contraction by the phosphorylation of voltage dependent, L-type Ca^{2+} channels in response to depolarization of the cell membrane.

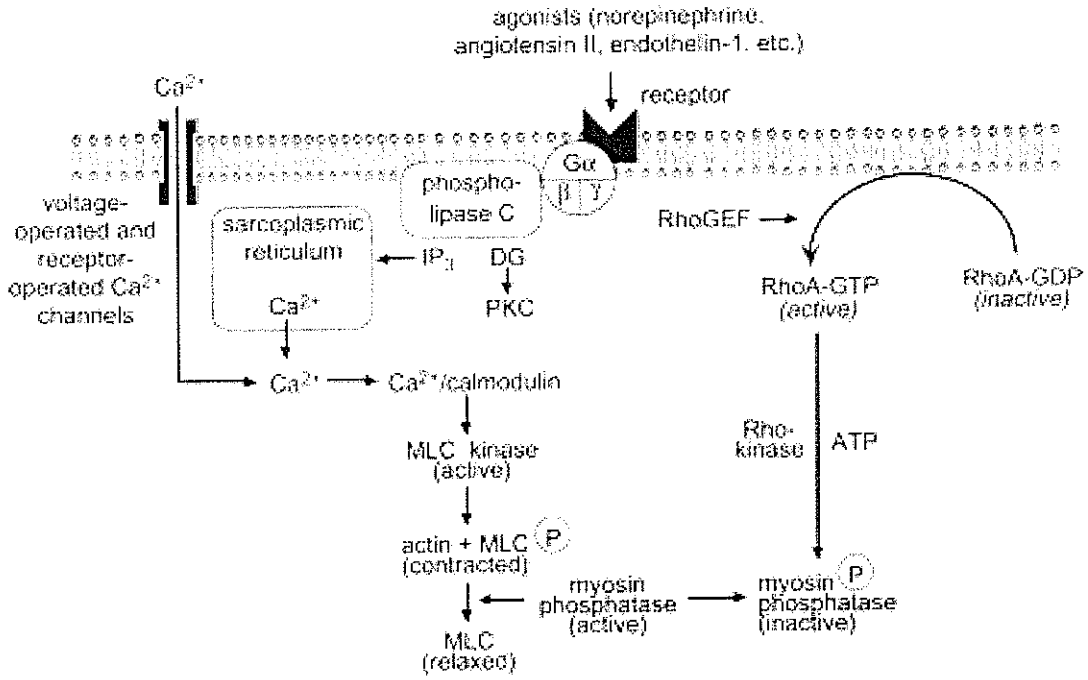


Figure 4: Mechanism of smooth muscle contraction and Ca^{2+} sensitization. (17)

2. Mechanism of Ca^{2+} Sensitization

The concept of Ca^{2+} sensitization stems from variability in the degree of smooth muscle contraction which has been shown to depend upon the pathway utilized to initiate the contraction (16). The maintenance of a contracted state is dependent upon the phosphorylated state of the myosin filament. Myosin light chain phosphatase (MLCP) is an enzyme which cleaves the phosphate group from myosin, and therefore, contributes to a relaxed state. This enzyme is inhibited by an activated G protein, RhoA and RhoA kinase. As illustrated in **Figure 4**, when RhoA is activated, RhoA kinase becomes available, and this enzyme inhibits the activity of MLCP, thereby allowing the cell to remain contracted (17).

3. Mechanism of Relaxation

Given that an increased concentration of Ca^{2+} and the phosphorylation of myosin creates an environment that supports muscle contraction, it follows that the removal of Ca^{2+} from the intracellular environment and the cleavage of phosphorus from myosin would cause relaxation (**Figure 5**) (15).

An enzyme, Ca,Mg-ATPase, on the plasma membrane and sarcoplasmic reticulum is responsible for the removal of some intracellular Ca^{2+} (17). This enzyme is activated in the presence of Mg^{2+}, and can remove two Ca^{2+} ions from the cytoplasm of the cell into the SR. The SR also has Ca^{2+} binding proteins, calreticulin and calsequestrin, which have the ability to bind Ca^{2+} and transfer it to the SR (17).

The plasma membrane, in addition to Ca,Mg-ATPase, has Na^{+}/Ca^{2+} exchangers which can play a role in the removal of Ca^{2+} from inside the cell. Calcium channels in the plasma membrane, opened due to membrane depolarization (voltage operated) and specific agonists (receptor operated), close and stop the influx of Ca^{2+} (17).

The phosphorylation of myosin is a key part of smooth muscle cell contraction. The activation of myosin light chain phosphatase (MLCP) functions to remove phosphate from myosin, and results in muscle relaxation (17). MLCP is activated by cyclic guanosine monophosphate (cGMP). Nitric oxide (NO) is a vasodilator which operates via cGMP (16). NO activates guanylate cyclase, which generates cGMP, and causes muscle relaxation (16).

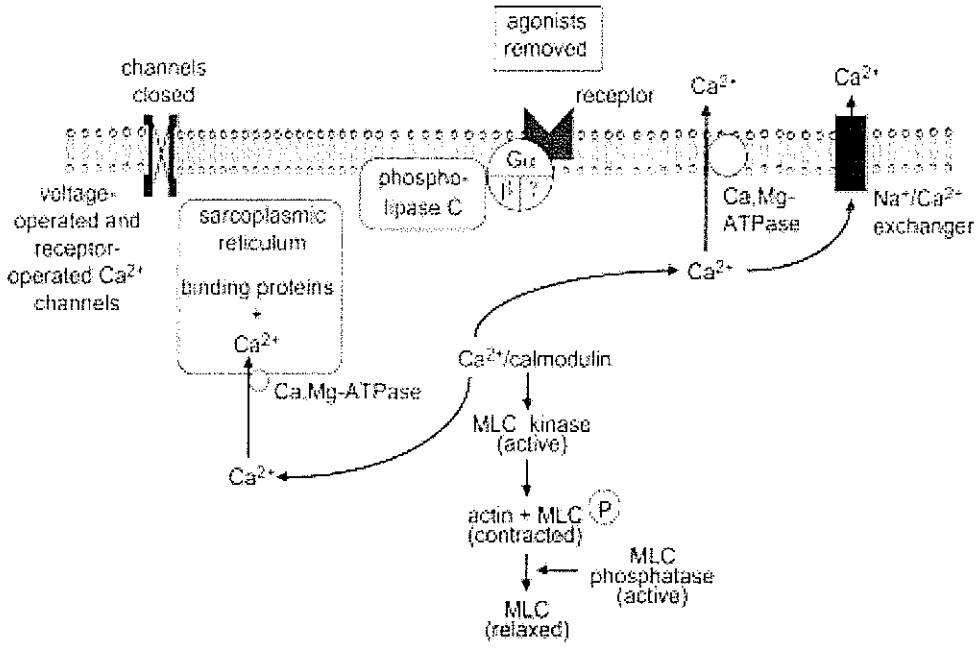


Figure 5: Mechanism of smooth muscle relaxation (17)

C. Atherosclerosis

Atherosclerosis is a disease in which cholesterol and fats are deposited on the vessel walls, resulting in intimal thickening. Functionally, this results in a decreased supply of oxygen and nutrients to tissues and organs. The cell types involved in the initiation and development of atherosclerosis include macrophages, platelets, smooth muscle cells, and T lymphocytes (18). The progression of the disease occurs over time and follows characteristic stages of development. The first stage involves the movement of cholesterol that is incorporated in low density lipoprotein (LDL) into the subendothelial space from the circulation. The LDL becomes modified into oxidized LDL, thereby allowing for its uptake by macrophages and smooth muscle cells, resulting in the formation of foam cells (19). As these foam cells accumulate, they form fatty streaks, and eventually, fibrous plaques. From the fibrous plaques, complicated atherosclerotic lesions develop, which often result in heart attacks or strokes (19). Each of these stages will be discussed in further detail.

1. Cholesterol and Lipoproteins

Cholesterol is a lipid having a characteristic steroid ring nucleus as the main component of its structure (**Figure 6**). This lipid is synthesized though a series of reactions that take place, for the most part, in the liver. The starting point of this synthetic scheme is with acetyl CoA which reacts with HMG-CoA reductase to

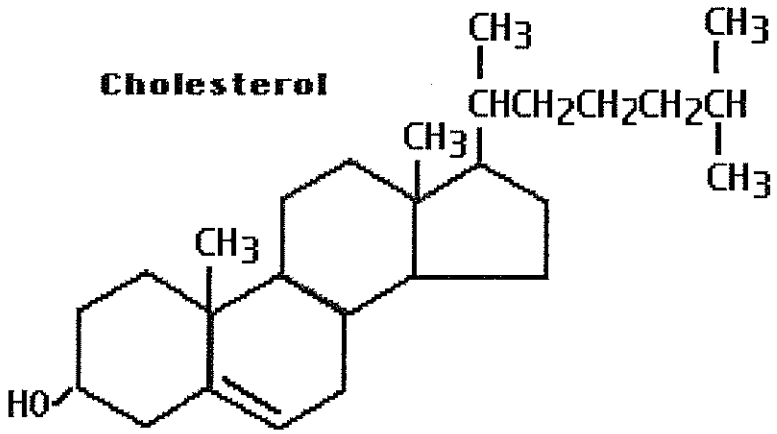


Figure 6: Chemical Structure of Cholesterol
http://faculty.uca.edu/~johnc/mole1440.htm

produce mevalonic acid (19). A number of reactions follow this rate limiting step to eventually produce cholesterol.

Cholesterol is an important lipid in the body and plays a critical role in the healthy functioning of cells. The structure and fluidity of all cell membranes are altered by the presence of cholesterol. Cholesterol is also important for the transport of a variety of substances across cell membranes (19). Cholesterol is an integral part of serum lipoproteins. This cholesterol helps in the movement of insoluble triglycerides in the aqueous serum (19). Cholesterol is an important component of bile acids, which aid in the digestion and absorption of dietary lipids and in the synthesis of adrenal steroids and sex hormones (19).

Most cholesterol is made in the liver and can be converted into bile acids, secreted into the intestine, or enter the bloodstream. Enterohepatic circulation allows cholesterol to travel between the liver and the intestine. Absorption of cholesterol into the intestine is dependent on polar lipids within the intestinal lumen, the most important of which being bile acids, which allow the cholesterol to be suspended in an aqueous medium (19). Only half of cholesterol is actually absorbed into the intestine, due to a relatively poor absorption of lipids that solubilize cholesterol. Once these lipids are absorbed, the cholesterol that remains falls out of solution, and is subsequently excreted (19). The absorbed cholesterol returns to the liver and controls, through feedback regulation, the synthesis of cholesterol (19). The rate-limiting enzyme involved in the synthesis of cholesterol, HMG-CoA reductase, is activated by a low concentration of cholesterol that returns to the liver, and suppressed when a high concentration of cholesterol returns (19).

Since the low solubility of cholesterol in aqueous systems affects its ability to enter the bloodstream, lipoproteins carry cholesterol in the circulation. Lipoproteins are complexes of proteins and lipids which allow cholesterol molecules to enter and travel through the plasma. The structure of a lipoprotein is illustrated in **Figure 7**. The main structural elements include the lipid core, which is composed of cholesterol esters and triglycerides, and the polar surface coat, made up of cholesterol, phospholipids, and apoproteins, which permit movement within the aqueous plasma (19). The apoproteins are important in the synthesis, secretion, stabilization, modification of lipoproteins, and the interaction of the lipoprotein with specific membrane receptors for removal of lipoproteins from the circulation when necessary (19). Lipoproteins are classified based on their density. These include chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL).

Chylomicrons have a high concentration of triglycerides and some cholesterol esters. They are involved in the transport of these lipids to the liver after absorption from the intestine (19). Triglycerides are released into the plasma when chylomicrons come into contact with lipoprotein lipase, and the cholesterol remains within the chylomicron remnant until it is absorbed by the liver (19).

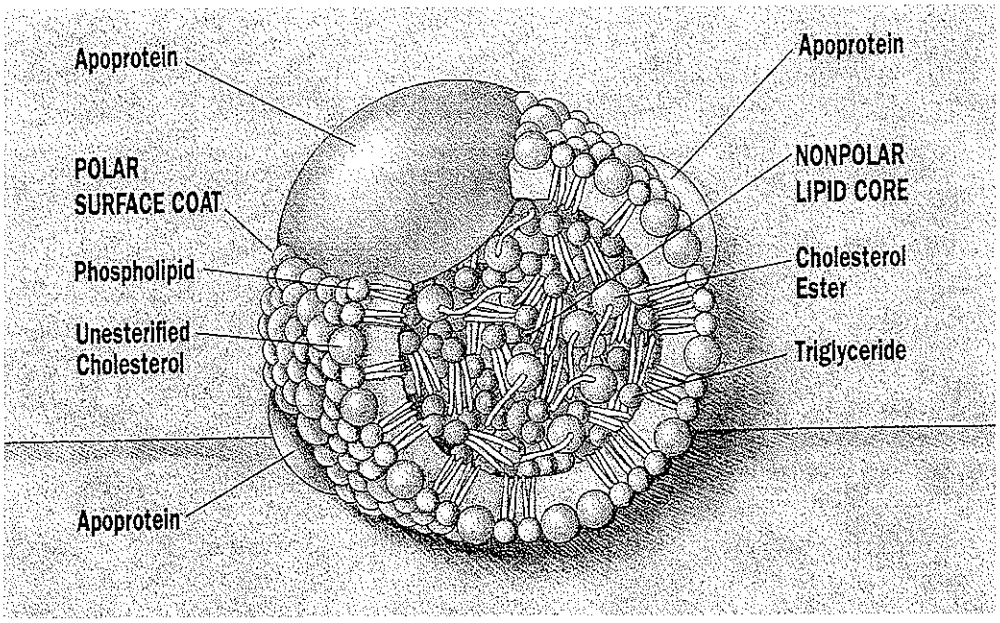


Figure 7: Lipoprotein Structure (19)

VLDL particles are produced in the liver. VLDL also have a high concentration of triglycerides and some cholesterol esters. The breakdown of VLDL, similar to that of chylomicrons, includes interaction with lipoprotein lipase to release triglycerides into the circulation, leaving VLDL remnants with a core of cholesterol esters (19). The VLDL remnants have the apoproteins, apo B-100 and apo E, which interact with LDL receptors on the liver. These receptors remove the VLDL from the circulation (19). Some VLDL remnants can be converted to cholesterol rich LDL through the action of hepatic triglyceride lipase (19).

The lipoprotein that has a lipid core composed primarily of cholesterol is LDL and it is of great significance in the study of atherogenesis. Phospholipids, cholesterol, and apo B-100 together form the outer coat of LDL to allow the movement of cholesterol in the bloodstream (19). The liver controls serum concentrations of LDL by regulation of VLDL synthesis and LDL receptor synthesis given that VLDL can interact with LDL receptors on the liver (19). Removal of LDL from circulation can occur through two mechanisms. The most common is via LDL receptors on the cell surface (19).

LDL receptors can be found on the cell surface, in coated pits, and when LDL in the bloodstream comes into contact with receptors, they form a complex which moves into the cell as lysosomes (19). This complex ultimately dissociates and the receptors return to the cell surface to interact with any additional circulating LDL molecules (19). Once the LDL has been internalized, it is degraded. The apo B-100 protein is reduced to amino acids, and the cholesterol can be incorporated into the cell

membrane, stored as cholesterol esters, or excreted (19). Receptor synthesis is controlled and limited by the amount of cholesterol in the cell (19).

Lipoprotein(a) is similar in structure to LDL, with the addition of apolipoprotein(a) which can link covalently with the apo B 100 on LDL in the liver (20). As a result of the apo(a) functional group, the LDL particle becomes more likely to cause atherosclerosis by interacting with the endothelium and macrophages (20). Levels of lipoprotein(a) depend upon genetic makeup, however hormonal changes, renal disease, and diet can affect Lp(a) levels in the circulation serum (20).

High-density lipoproteins have a lipid core which is mainly composed of cholesterol esters, with the surface containing apoproteins (apo A-I, apo A-II, apo C, and apo E) and phospholipids (19). In the synthesis of HDL, the liver and intestine produce an initial nascent HDL particle, which can absorb cholesterol, and esterify it through the action of lecithin-cholesterol acyltransferase (LCAT) (19). This series of reactions continues, creating a lipid core of cholesterol esters (19). Regulation of cholesterol levels in tissue and the blood stream can be affected by HDL particles, as they are able to internalize cholesterol from tissues and cells, and they are able to deliver the excess cholesterol to the liver (21).

All of the lipoproteins discussed above can modulate the movement of cholesterol to and from the liver. The liver is important because it is the only tissue that is capable of degrading and removing cholesterol from tissues and the bloodstream (19). Reverse cholesterol transport is the process by which cholesterol can be cycled to and from the liver (19). HDL plays a role in reverse cholesterol transport by accumulating cholesterol and converting it to cholesterol ester by the

action of LCAT. Cholesterol ester transfer protein (CETP) has the ability to transfer or exchange cholesterol esters from one lipoprotein to another, and in this case, CETP acts to transfer some cholesterol esters to VLDL from HDL (19). VLDL can be removed from circulation through LDL receptors on the liver, or it can be transformed into LDL, which can be removed in the same way (19).

2. Cholesterol and Atherosclerosis

The progression of atherosclerosis is initiated when circulating LDL molecules are able to enter into the intimal space, where they can then be entrapped and modified (**Figure 8**). Injury to the endothelial layer of cells can provide the opening through which these molecules can move from the circulation into the intima of the cell (19, 22). The apo B-100 portion of the LDL molecules can interact with glycosaminoglycans (GAG) within the intima, causing the LDL to become trapped within the subendothelial space (19).

LDL that is trapped within the intima can then be modified through oxidation or derivatization (19, 23). The apo B-100 on the LDL molecule can be oxidatively degraded by superoxides (19), lipoxygenases, and nitric oxide (22) released by macrophages, endothelial cells, or smooth muscle cells (24).

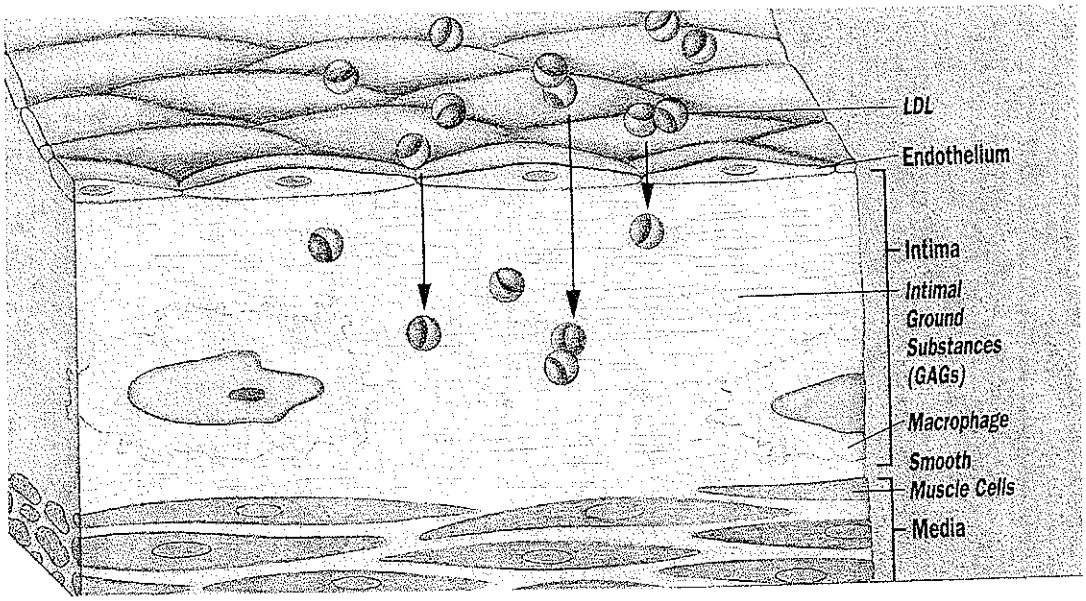


Figure 8: Infiltration and entrapment of LDL particles in the intimal space of the vessel (19)

Since one important role of apoproteins is to stabilize the structure of the lipoprotein (19), as apo B-100 is degraded, so is the LDL itself degraded. Derivatization can take place with the addition of chemical moieties to the apo B-100, including monoaldehyde, or glycosylation (19). When the endothelium is exposed to oxidized LDL, its normal function is altered, and monocytes, macrophages, and T lymphocytes can adhere to the endothelium more readily (18) (20). This may allow them access to the subendothelial space and the entire atherosclerotic process is stimulated again.

Modified LDL can be more readily absorbed by macrophages and smooth muscle cells than native LDL, causing the formation of foam cells (19). Macrophages and smooth muscle cells have receptors for modified LDL, and lack the feedback control that exists for LDL receptors (19). Cholesterol esters will continue to enter the cell and accumulate within the macrophages and smooth muscle cells causing the cells to acquire a foam-like appearance (19).

When macrophages contain oxidized LDL, this oxidized LDL can stimulate proliferation of monocytes, smooth muscle cell (25, 26) and endothelial cells(22). The scavenger receptor for oxidized LDL on macrophages is not down regulated and therefore contributes to foam cell formation (27). Oxidized LDL is also chemotactic for monocytes (18, 27), smooth muscle cells, and endothelial cells (22). High concentrations of oxidized LDL are toxic to cells which may contribute to cell necrosis and endothelial injury (27). All of these processes are important in the initiation and progression of atherosclerosis.

Foam cells, intercellular lipid, smooth muscle cells, and T lymphocytes can group together in the intima to form the fatty streak (18, 19). This lesion represents the first stage of plaque formation, and does not impede the luminal space of the blood vessel. As atherosclerosis progresses and lipid continues to accumulate in the cells, the fatty streak grows into a fibrofatty lesion, and then into the fibrous plaque (18, 19). The smooth muscle cells migrate into the intima, where they can proliferate and contribute to lesion growth (22). When in this proliferative state, smooth muscle cells affect the formation of fibrous connective tissue which creates the fibrous cap of the plaque (22). The fibrous plaque is composed of layers of smooth muscle cells, macrophages, and a lipid rich center, with a fibrous cap (18, 19). Growth factors are released which promote smooth muscle proliferation and the fibrous plaque grows further into the arterial lumen, and can begin to impede blood flow in the area (18, 19). The most advanced atherosclerotic plaque is the complicated lesion that involves the development of calcium deposits within the lesion, platelet aggregation at the site of endothelial injury, hemorrhage, ulceration, and thrombus, and further obstruction of the lumina (19). These stages of atherosclerosis are illustrated in **Figure 9**.

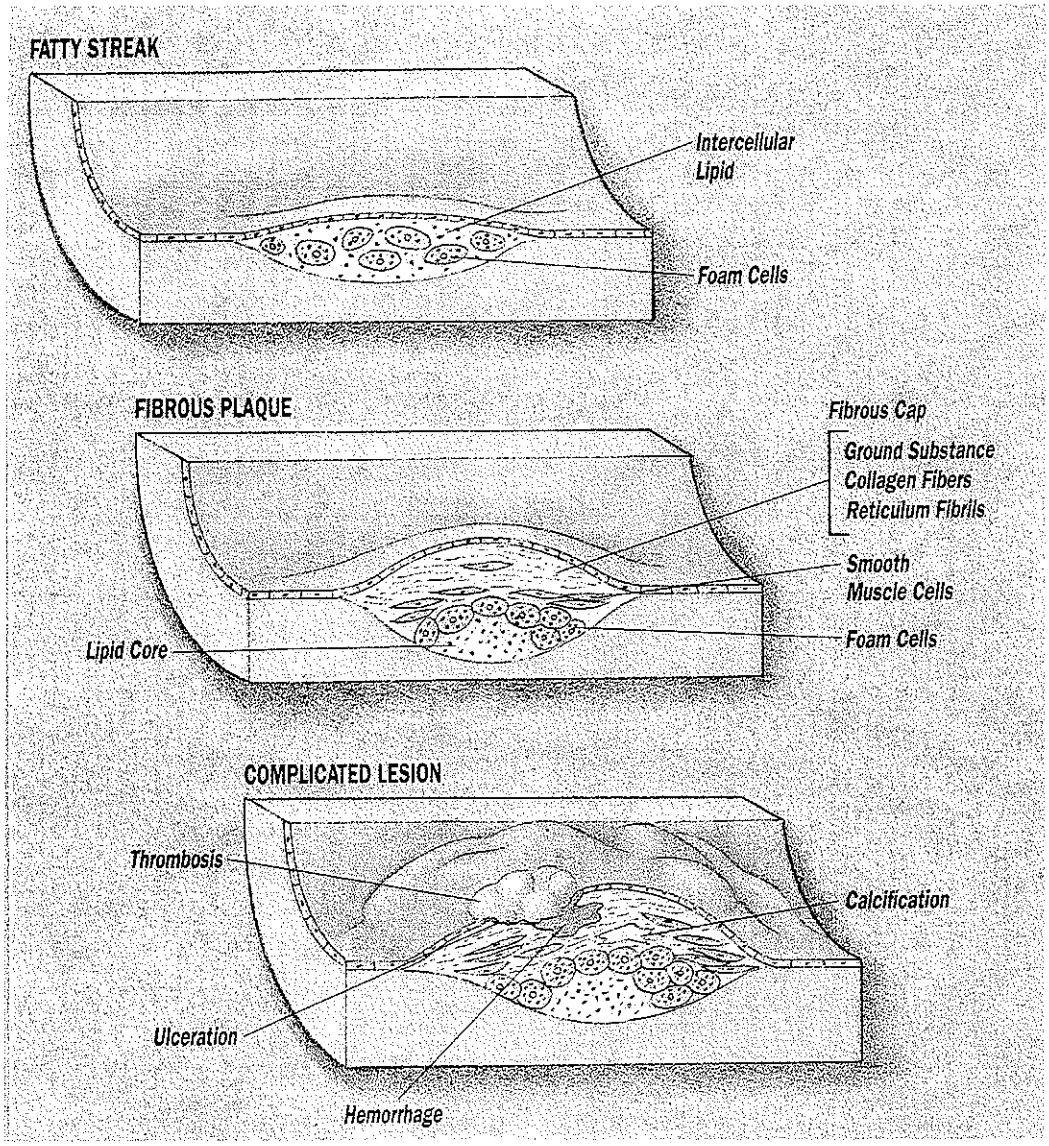


Figure 9: Stages of Atherosclerosis (19)

3. The Process of Atherogenesis

Two main theories that have been proposed to explain the development of atherosclerotic lesions. One theory, referred to as the "Lipid Hypothesis", associates high levels of cholesterol in the bloodstream with abnormal levels of lipoprotein metabolism (21). The increased concentration of cholesterol interacts with the endothelium, thereby initiating the atherosclerotic process (21).

A second theory focuses on the immune response that appears to be an important factor in atherogenesis. This theory was first presented in 1973 by Ross and Glomset (22), and was modified to the "Response-to-Injury Hypothesis" (18, 22, 28). The focus of this theory is on a number of inflammatory responses that are involved in the development of atherosclerotic lesions. Endothelial injury, and the resulting endothelial dysfunction, can be caused by a number of events including increased permeability, increased adhesion, and the release of regulatory factors (22). Macrophages and T lymphocytes are molecules that are released in response to inflammation, and they are also significantly involved in the development of the fatty streak (22). The induced proliferation of smooth muscle cells, aggregation of platelets, activation of macrophages, release of T lymphocytes and the release of a variety of regulatory chemicals from the endothelium are all normal protective responses to injury (22). The theory postulates that this normal response becomes destructive when the injured site is not mended, and this reaction becomes excessive, resulting in the development of atherosclerotic lesions (22)

D. Risk Factors for Atherosclerosis

There are a number of risks factors associated with the development of heart disease and atherosclerosis, some which can be controlled and some which cannot. Controllable risk factors include cigarette smoking, high cholesterol or blood lipid levels, high blood pressure, obesity, unhealthy diet including decreased consumption of fruits and vegetables and increased consumption of saturated fats, physical inactivity, and Type II diabetes, and diabetes mellitus (1, 2, 29, 30). This list consists of the most significant and accepted risk factors for heart disease, however, additional risks factors have been identified, including depression, stress, excessive consumption of alcohol, poverty, the use of certain medications (hormone replacement therapy and oral contraceptive use), and left ventricular hypertrophy (2). There are a number of risk factors which can contribute to an increased risk for developing heart disease, which cannot be controlled or altered, and those include increasing age, heredity, gender, (2) (1) and race (2, 29). Additional risk factors that are becoming increasingly important in the study of cardiovascular disease include C-reactive protein levels, infections, inflammation (31), and oxidative stress. This discussion will focus on the impact of modifiable risk factors on atherogenesis.

1. Risk Factors for Atherosclerosis - Cigarette Smoking

Tobacco use and smoking is considered to be "the major cause of preventable death in Canada" (1) and is a significant contributor to the development of heart disease and atherosclerosis. The mechanisms through which smoking acts include endothelial damage which contributes to plaque formation, thrombosis, higher levels of LDL cholesterol, lower levels of HDL, accelerated heart rate, hypertension, and vasoconstriction due to increased levels of norepinephrine in the body (2, 10).

2. Risk Factors for Atherosclerosis - Physical Inactivity

A low level of physical activity and fitness is closely linked with obesity, endothelial dysfunction, oxidative stress, and increased inflammation (9). Increased levels of activity burns excess calories, and stored fats, and contribute to a healthy cardiovascular system. Some studies have shown that the endothelial dysfunction which plays an important role in hypertension and heart disease is improved following exercise, due to increased NO availability (9) (32).

3. Risk Factors for Atherosclerosis - Diabetes Mellitus

This disease contributes to atherogenesis because of several deleterious factors associated with the disease. An increased concentration of VLDL triglycerides (19), high cholesterol levels, insulin resistance, high blood pressure, and obesity are

all frequently associated with diabetic patients and these are also thought to contribute separately to atherosclerotic process. Higher than normal concentrations of VLDL triglycerides can occur as a result of increased production, or decreased metabolism. Production of VLDL triglycerides can be stimulated by free fatty acids and glucose (19). The enzyme responsible for the metabolism of triglycerides is lipoprotein lipase, the activity of which is dependent on insulin levels (19).

4. Risk Factors for Atherosclerosis – Hypertension

Hypertension is associated with an abnormal endothelial response, and one study has shown that high concentrations of endothelin-1 (ET-1) may cause high blood pressure (7). Hypertension as a risk factor for atherosclerosis is affected by its association with other risk factors including obesity, reduced HDL concentrations, increased LDL, high triglycerides, hyperinsulinemia, and glucose intolerance (33). As the number of risk factors increases, in addition to the presence of hypertension, the risk of a cardiovascular event increases (33).

5. Risk Factors for Atherosclerosis - Dietary Risk Factors

Three important dietary risk factors for atherosclerosis include the consumption of high cholesterol foods, a diet rich in saturated fatty acids, and obesity as a result of a high caloric diet (19, 30).

Cholesterol in the diet is obtained solely from animal sources, including eggs, shellfish, organ meats, and to a lesser degree, animal meats (19). Fruit, vegetables and grains are not a dietary source of cholesterol (2, 19). A high cholesterol diet impacts upon the circulating LDL concentration and LDL receptor synthesis (19). As cholesterol is ingested, it is absorbed into the liver, which has an inhibitory effect on LDL receptor synthesis and receptor activity through a negative feedback regulation (19). Because of the decrease in LDL receptor density, LDL and VLDL remnants are not removed from circulation as readily, and, therefore, an increased concentration of LDL cholesterol builds up in the bloodstream (19). The LDL concentration in the circulation correlates positively with plaque formation and atherogenesis.

A diet high in animal fats can be a source for both cholesterol and saturated fatty acids (19). Saturated fatty acids (SFA) in the diet can contribute to increased serum cholesterol levels by the same mechanism as discussed with dietary cholesterol – by suppressing the LDL receptor (19). A second effect of SFA consumption is a decreased level of HDL cholesterol in the blood (34). The SFAs that are thought to contribute the most to an elevation in blood cholesterol include the long chain fatty acids palmitic and myristic (19).

The consumption of a high calorie diet, without sufficient physical activity to utilize the calories, can result in weight gain and, if severe enough, obesity (19). One major effect of obesity is on the metabolism of all lipoproteins (VLDL, LDL, and HDL) in the blood (19). The liver responds to obesity by releasing additional apolipoprotein B molecules into the bloodstream (19). Hepatic release of VLDL is increased which can result in hypertriglyceridemia if the activity of lipoprotein lipase is not increased (19). For some obese individuals, lipoprotein lipase (important for the breakdown of lipids, including triglycerides) levels are increased, thereby maintaining the triglycerides levels within a normal, healthy range (19). Without an increase in the activity of this enzyme, hypertriglyceridemia will result (19).

LDL cholesterol levels can increase with obesity due to increased consumption of dietary cholesterol, which represses LDL receptor activity, and due to conversion of VLDL to LDL, thereby increasing the concentration of LDL in the blood stream (19). As with obesity induced lipolysis of VLDL triglycerides, obesity can result in an increase in LDL receptor activity as a response to high cholesterol levels (19). If the receptor activity does not increase, then hypercholesterolemia may result (19).

Obese individuals often have decreased concentrations of HDL, which may be due to high levels of triglycerides, or due to the breakdown of HDL by adipose tissue (19). The effects of obesity on lipoprotein levels in the blood can be reversed with weight loss so long as there are no genetic factors present that may influence triglyceride, cholesterol, and lipoprotein levels (19).

E. Fatty Acids

Fatty acids are an integral part of the human diet because they are required for proper cell function and for maintaining membrane flexibility and fluidity. They are essential in reproductive functioning, for growth and development, and for cholesterol metabolism (35, 36). The basic structure of fatty acids consists of a carbon chain with a carboxylic acid moiety on one end and a methyl group on the other. The carboxylic acid and methyl ends are known as the delta (Δ) and omega (ω) end, respectively. Fatty acids can be divided into two groups – saturated and unsaturated fatty acids, based on the absence or presence of double bonds (37). Saturated fatty acids (SFA) do not contain any double bonds and are identified by the number of carbons that make up the chain (37). Dodecanoic acid (lauric acid) is illustrated in **Figure 10** as an example. Conversely, unsaturated fatty acids contain at least one double bond and are further sub-classified based on the number of double bonds present. Monounsaturated fatty acids (MUFAs) contain only one double bond and polyunsaturated fatty acids (PUFAs) contain more than one (**Figure 10**). By convention, the carbon atoms of a chain are numbered for identification purposes, starting with the methyl or omega (ω) end of the molecule. When one or more double bonds are present in the structure, the location of the double bond is given by the number of the first carbon of the double bond. Two isomers can be formed when a double bond is present in a molecule, known as *cis*- and *trans*- isomers. The two hydrogen atoms that remain at the site of the double bond can be in the *cis*- configuration (on the same side of the double bond) or in the *trans*- configuration (on opposite sides of the double bond). Natural plant

and fish oils exist in the *cis-* configuration which is known to be beneficial for the maintenance of membrane fluidity. In contrast, animal fats, which are solid at room temperature, typically exist in the *trans-* configuration (36) (38).

Trans- fatty acids, due to their configuration, allow for PUFAs present in cell membranes to pack tightly together and can thus impact on the fluidity of the membranes. Consequently, a change in membrane fluidity may negatively affect cell function. Studies have shown that diets high in *trans*-fatty acids result in increased lipoprotein(a) levels, triglycerides levels, endothelial dysfunction, risk of type 2 diabetes, and inhibition of delta-6 desaturase necessary for normal metabolism of fatty acids (39).

Polyunsaturated fatty acids can be further subdivided into non-essential and essential fatty acids. Non-essential fatty acids are those that can be produced by the body from other lipid precursors, and the majority of fatty acids fall into this category. Essential fatty acids, alternatively, must be obtained from external sources because the human body cannot synthesize them (40). The human body lacks the enzymes necessary to convert single bonds to double bonds at a position further than nine carbons from the delta end of the molecule (38). The only fatty acids that are essential are alpha-linolenic acid (omega-3) and linoleic acid (omega-6) PUFAs (**Figure 10**). These essential fatty acids are required for optimal functioning of the cell, the immune and organ systems, as well as for overall healthy growth and development (35) (38) (41). Once these essential fatty acids are ingested, they are metabolized through a series of desaturation and elongation reactions that produce

Saturated Fatty Acid (SFA)

$$CH_3\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}COOH$$

Lauric Acid

Monounsaturated Fatty Acid (MUFA)

$$CH_3\text{-}(CH_2)_7\text{-}CH{=}CH\text{-}(CH_2)_7\text{-}COOH$$

Oleic Acid – Omega-9

Polyunsaturated Fatty Acid (PUFA)

$$CH_3\text{-}(CH_2)_4\text{-}CH{=}CH\text{-}CH_2\text{-}CH{=}CH\text{-}(CH_2)_7\text{-}COOH$$

Linoleic Acid - Omega-6

$$CH_3\text{-}CH_2\text{-}CH{=}CH\text{-}CH_2\text{-}CH{=}CH\text{-}CH_2\text{-}CH{=}CH\text{--}(CH_2)_7\text{-}COOH$$

Alpha-Linolenic Acid - Omega-3

Figure 10: Chemical structures of saturated, monounsaturated, and polyunsaturated fatty acids
(Adapted from
http://www.library.csi.cuny.edu/~davis/Biochem_3521/lect21/lipids.html)

eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are in turn involved in eicosanoid metabolism (38).

The enzymes involved in the metabolism of both linoleic acid (LA) and alpha-linolenic acid (ALA) are elongase, Δ4-, Δ5-, and Δ6- desaturase (38). The same enzymes are involved in each set of reactions. As a result, competition exists between the two pathways (36, 42, 43). An optimal ratio of omega-3 and omega-6 PUFA has been extablished, at 4:1 (LA to ALA)which is very important for normal metabolism of ALA to EPA (35, 44). When the ratio is too high, due to increased consumption of omega-6 PUFA (LA),competition favors the omega-6 pathway, causing a deficiency of omega-3 PUFA (45).

1. Eicosanoid Metabolism

The metabolism of n-6 PUFA and n-3 PUFA produces AA and DPA, and EPA and DHA respectively, as illustrated in **Figure 11**. These substrates can in turn produce eicosanoids with the aid of cyclooxygenase (COX) and lipoxygenase (LOX) (46). These reactions form prostaglandins, thromboxanes, and leukotrienes, as illustrated in **Figure 12**. The COX pathway from AA, the major metabolite in the breakdown of n-6 PUFA, produces prostaglandins and thromboxanes from the 2-Series (i.e. TxA_2 and PGI_2) (46). These eicosanoids oppose each other, with the major effects of TxA_2 being aggregation and vasoconstriction, with PGI_2 exhibiting anti-aggregatory actions and vasodilation (46) (35). The same pathway when interacting with EPA or DHA, the metabolites from n-3 metabolism, produces 3-

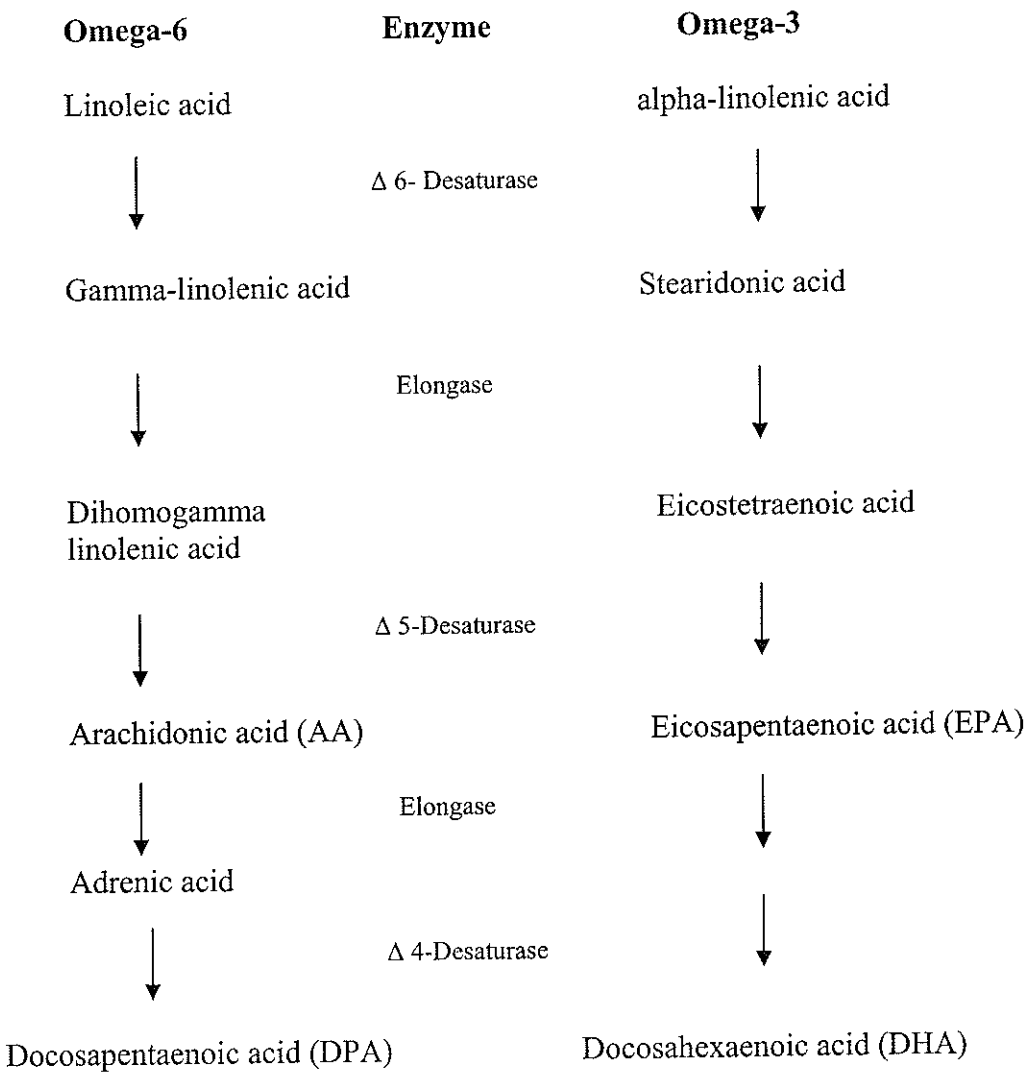


Figure 11: Polyunsaturated Fatty Acid Metabolism (38)

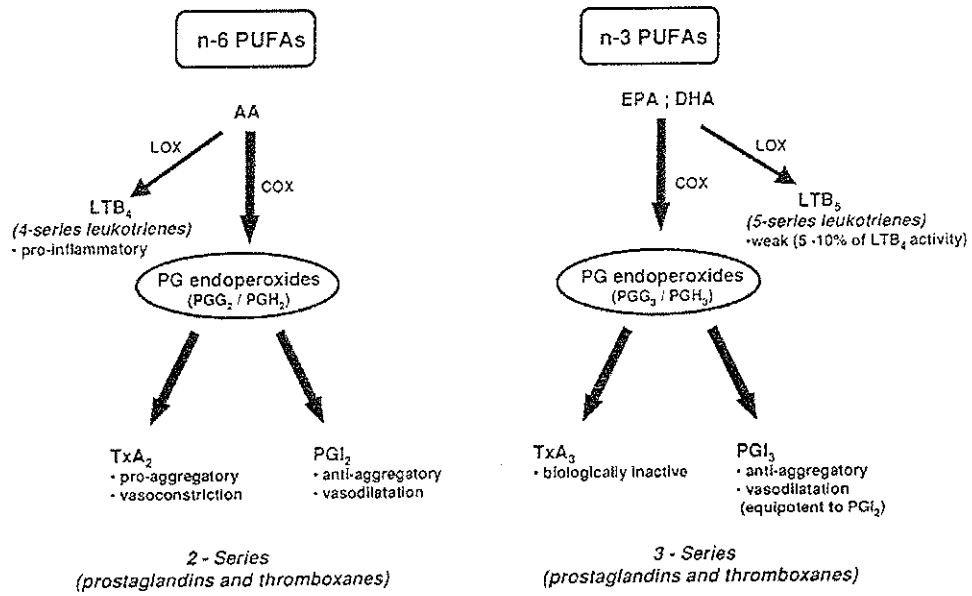


Figure 12: Eicosanoid synthesis (46)

series prostaglandins and thromboxanes (i.e. TxA_3 and PGI_3) (46). The TxA_3 produced from this pathway is not biologically active, and, therefore, does not promote aggregation. PGI_3 produced from EPA and DHA has actions similar to PGI_2. It inhibits aggregation, and promotes vasodilation (46).

2. Polyunsaturated Fatty Acids

Omega-3 (n-3) polyunsaturated fatty acids (PUFA) in the form of alpha-linolenic acid (ALA) have been the focus of a great deal of research due to the cardioprotective effects they exhibit (39, 44). The beneficial effects of n-3 PUFA, those obtained from fish oil in particular, include anti-thrombotic effects, anti-atherogenic effects, anti-arrhythmic effects, and vasoprotective effects (44, 46).

A diet with n-3 PUFA has been shown to have a positive effect on high blood pressure, in both animals studies and human studies (46). The mechanism for this decrease in blood pressure is thought to involve vasodilation, and inhibition of contraction. Nitric oxide released from the endothelium is known to cause vasodilation, and studies with spontaneously hypertensive rats (SHR) have shown increased dilation and decreased contraction when the diet included omega-3 PUFA (46). Studies have shown that EPA and/or DHA stimulates endothelium dependant relaxation by increasing the concentration of NO, and by inducing the release of endothelium derived relaxation factor (EDRF) and vasodilator prostacyclins (46). A second mechanism, which has been studies in animals (Wistar-Kyoto Rat and SHR)

has shown that n-3 PUFA can induce relaxation by altering membrane ion channel activities (activation of K^+, and inhibition of Ca^{2+} and Na^+) (46).

The anti-thrombotic effects exhibited by n-3 PUFA is thought to be a result of eicosanoids produced from EPA and DHA (46). Omega-3 PUFA metabolism favors the formation of the beneficial prostacyclins and not thromboxanes, creating an anti-aggregatory environment (46). Additional studies have also shown that DHA and EPA block the TxA_2 and PGI_2 receptor on platelets, causing a decreased ability to aggregate (46).

Smooth muscle cell proliferation is a significant aspect of atherosclerosis, and n-3 PUFAs have been shown to modulate VSMC proliferation (46). The alteration of VSMC proliferation by n-3 fatty acids and n-6 fatty acids (24) appears to occur as a result of inhibition of DNA synthesis, and by interfering with PDGF, and other growth signals (46).

One stage of atherosclerosis involves the chemo-attractant properties of the endothelium when it has been activated, and fatty acids have been shown to impact the release of adhesion molecules from the activated endothelium (46). The effect of PUFA on the endothelium appears to be due to the presence of double bonds in the fatty acid structure (46). Any fatty acid with a double bond has the ability to affect the activation of the endothelium, but it has been shown that n-3 fatty acids are more effective than other unsaturated fatty acids because they possess the greatest number of double bonds (46) (8).

Unsaturated fatty acids, have double bonds by definition, and in addition to affecting endothelial activation, they are susceptible to oxidation. Studies have

shown that these fatty acids can also work as a free radical scavenger, and by removing circulating free radicals, limit the oxidation of LDL (46)

a. Omega-6 Polyunsaturated Fatty Acids

Linoleic acid is an omega-6 fatty acid that is important in healthy development and growth, but the benefit of adding omega-6 fatty acids to the diet is controversial. An optimal ratio of 4:1 (omega-6: omega-3) or less is recommended (35), given that the metabolism of these essential acids is competitive (43). When an excess of LA is present, ALA concentrations are decreased (47). When ALA is metabolized, it produces arachadonic acid (AA), which acts as a substrate for the COX and LOX pathways as illustrated previously in Figure 11. The eicosanoids produced from omega-6 fatty acids have a higher biological activity than those produced from omega-3 fatty acids(47), and when present in high concentrations, they are pro-inflammatory (47), stimulate thrombosis, stimulate vasoconstriction, and promote adhesion (46) (35, 48). While omega-6 PUFA appear to be atherogenic, beneficial effects of omega-6 PUFA and LA have been observed which contradict this initial observation.

Studies have shown that omega-6 (LA) in the diet caused a significant decrease in the concentration of LDL cholesterol (47) (44, 48). The Jerusalem nutritional study found that there was also an increase in LA hydroperoxides, indicating that oxidation occurred *in vitro* (47). These LA hydroperoxides, and LA were found to inhibit platelet aggregation by affecting prostacyclin release and thromboxane production (47). Some studies have shown that AA (from LA

metabolism) can cause changes in the normal rhythm of the heart and others have shown that fatal ventricular fibrillation in rats was reduced significantly when the rats were fed omega-6 fatty acid in the form of sunflower oil (44, 47). A study from Japan found that linoleic acid exhibited cardioprotective effects including decreased platelet aggregation and blood pressure, and enhanced erythrocyte deformability , resulting in decreased risk of stroke (49). Diets with a higher concentration of omega-6 PUFA, in some animal studies have shown increased insulin sensitivity and the Nurses'Health Study showed a significant decrease in type 2 diabetes when saturated fatty acids were replaced with omega-6 PUFA (44).

More clinical studies need to be done to illustrate the potential physiological benefits of omega-6 fatty acids in atherosclerosis and other diseases.

b. Hempseed

Hempseed is the seed portion of the *Cannabis sativa* plant and is a rich source of essential fatty acids, having an optimal ratio of 3:1 omega-6 (LA) : omega-3 (ALA) (50). The unique nutritional and lipid composition of hempseed is illustrated in **Figure 13** and **Table 1**. Hempseed is a complete source of protein, dietary fiber, minerals and vitamins, which is a source of omega-6 PUFA as linoleic acid (LA), omega-3 PUFA as alpha-linoleic acid (ALA), and gamma-linoleic acid (GLA) (50). Hempseed also has a high concentration of phytosterols, which have been shown to decrease cholesterol levels by inhibiting its absorption from the GI tract (51, 52). Because the optimal ratio of LA and ALA is present in hempseed, and due to the presence of phytosterols, it has a great deal of potential as a source of

cardioprotective lipid (50). Some limited studies have been done with rats, to show that when the diet is supplemented with hemp, a significant decrease in free cholesterol, and triglycerides was observed (53). Another animal study supplemented the rat chow diet with the whole hempseed and found decreased levels of serum cholesterol and triglycerides (53). Insulin levels were also decreased in the study diets, when compared to the control diets (53). Additional studies are needed to examine the impact of dietary supplementation with hempseed on a variety of disease conditions including atherosclerosis.

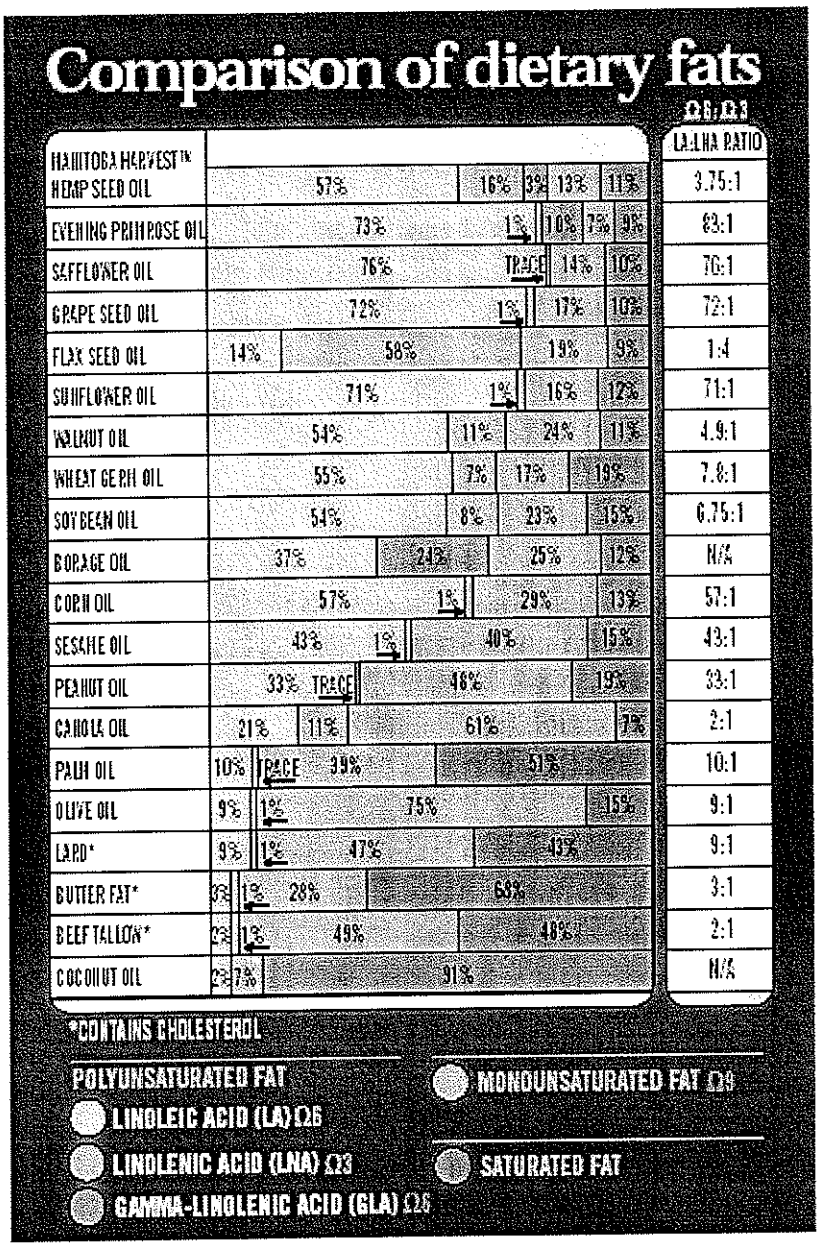


Figure 13: Breakdown of saturated fat, polyunsaturated fat, and monounsaturated fat in many types of fats (http://www.manitobaharvest.com/nutrition/index.asp?itemID=183)

Nutritional Facts Per 100g Serving	Whole Hemp Seed	Hulled Hemp Seed	Hemp Seed Oil	Hemp Flour
Energy	500kCal	560 kCal	725 kCal	385 kCal
Protein	23 g	33 g	0 g	33 g
Total Fat	31 g	44 g	99.9 g	7 g
Saturated	3 g	15 g	9.7 g	0.7 g
Unsaturated	28 g	39 g	90.2 g	6.3 g
Carbohydrates	34 g	12 g	0 g	44.5 g
Dietary Fiber	30 g	7 g	n/a	n/a
Sugars	2 g	3 g	n/a	n/a
Ash	6 g	6 g	0 g	7.3 g
Moisture	6 g	5 g	0.1 g	8.2 g
Cholesterol	0 g	0 g	0.0 g	0.0 g
Minerals				
Calcium	75 mg	12 mg	n/a	200 mg
Iron	5 mg	3 mg	n/a	23 mg
Sodium	8 mg	1.5 mg	n/a	15 mg
THC Content	None Detected	None Detected	None Detected	None Detected
Typical Specifications for Whole and Hulled hemp seeds, Hemp seed oil and Hemp Flour				

Table 1: Nutritional Composition of Hemp Seed
(http://www.hempoilcan.com/compo.html)

III. Materials and Methods

1. List of Materials

PRODUCT	SOURCE
Norepinephrine	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Acetylcholine	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Sodium nitroprusside	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Rabbit Chow	Federal Co-operatives Ltd. (Saskatoon, SK.)
Rabbit Chow + 2% cholesterol	Federal Co-operatives Ltd. (Saskatoon, SK.)
Hempseed	HempOil Canada (Winnipeg, MB)
Seed Cake	Hemp Oil Canada (Winnipeg, MB)
Coconut Oil	Kissan Int. (Canada) Inc. (Scarborough, ON)
Sodium chloride	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Potassium chloride	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Sodium Bicarbonate	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Magnesium sulfate	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Potassium dihydrophosphate	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Calcium chloride	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Dextrose	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Sucrose	Fisher Scientific (Nepean, ON)
Formalin	Fisher Scientific (Nepean, ON)

PRODUCT	SOURCE
HCl	Sigma-Aldrich Canada Ltd. (Oakville, ON)
New Zealand White Male Rabbits	Southern Rose Rabbitry Farm (St. Claude, MB)
Ethanol	Fisher Scientific (Nepean, ON)
Heparin	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Vacutainer Tubes (EDTA)	Becton Dickinson (Oakville, ON)
Vacutainer Tubes (heparin)	Becton Dickinson (Oakville, ON)
Vacutainer Tubes (sodium citrate)	Becton Dickinson (Oakville, ON)
Superfrost Plus Slides	Fisher Scientific (Nepean, ON)
Chloroform	Fisher Scientific (Nepean, ON)
Methanol	Fisher Scientific (Napean, ON)
Sodium sulfate	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Collagen	Sigma-Aldrich Canada Ltd. (Oakville, ON)
ADP	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Potassium carbonate	Fisher Scientific (Nepean, ON)
FAME standard GLC 462	Nu-Chek Prep, Inc.
O.C.T. Compound	Cedarlane Laboratories Ltd. (Hornby, ON)

B. Methods

1. Animal Groups and Dietary Interventions

Sixty male albino New Zealand white rabbits were used for the study. Each rabbit weighed between 2.5 kg and 3.0 kg at the time of arrival in the St. Boniface Hospital Research Centre. Once the animals arrived in the animal holding facility, they were observed over a one-week period to ensure health and ability to be included in the study.

The study involved an eight-week dietary intervention. Based on previous studies performed in our lab, this time frame was chosen because it allowed for the ideal amount of atherosclerotic plaque formation in the high cholesterol diet group (54). Studies performed for a shorter duration were found to induce modest plaque formation, and studies for a longer duration were found to induce plaque formation that was too severe. The rabbits involved in the study were randomly divided into one of six groups; one control group and five experimental groups. The diets were organised in the following manner: the control diet (RG) consisted of regular rabbit chow (CO-OP Complete Rabbit Ration, Federal Co-operatives Limited, Saskatoon, SK, Canada), this diet was then supplemented with 5% coconut oil (CO), with 10% hempseed (HP), with 0.5% cholesterol (OL), with both 10% hempseed and 0.5 % cholesterol (OLHP) or with 10 % seed cake (SC). The fat content of hempseed is 60 % omega-6 polyunsaturated fat (PUFA), 20 % omega-3 PUFA, 10 % monounsaturated fatty acids, 9 % saturated fatty acids, with 46.5% total fat. Seedcake is hempseed that has been modified to remove the lipid and has only 8.5 %

fat. This group was included in the experiment as a hempseed control. That is, an effect observed in the hempseed diet and not in the seed cake diet is most likely attributed to the fats that are present in the hempseed and not in the seed cake. All of the concentrations were added to the regular rabbit chow diet based on weight. Each day the rabbits were fed 125 grams of the appropriate diet, based on their nutritional requirements.

All diets, with the exception of the control diet, were made by mixing pre-weighed amounts of each appropriate supplement (cholesterol, hempseed, seed cake or coconut oil) and regular rabbit chow, moistened, and then ground. The diet was then re-formed into pellets, fan dried, and stored in a 4°C refrigerator in containers to aid in preservation, and protect them from light.

2. Experimental Protocol for Animal Sacrifice

After the 8-week study duration was completed, the animals were anaesthetized with CO_2 and ventilated. The neck and chest of the animal was shaved. The skin was cut back on the neck to expose the jugular veins, and blood was taken for platelet aggregation and for lipid analysis. Following blood collection, the rabbit was given heparin to prevent clotting at the time of termination. The chest was cut open, and the heart removed. The heart was used for another set of experiments by a colleague in our lab, and then was immediately flash frozen in liquid nitrogen and stored at -80°C. The lungs, one kidney, and one lobe of the liver were removed, rinsed in cold 1x PBS to clean the tissue, and flash frozen in liquid nitrogen. The

aorta was completely exposed and was cut at the distal end of the abdominal aorta. Forceps were used to gently lift the abdominal aorta as it was grossly dissected in a superior direction to the cut from the removal of the heart. The aorta was removed with caution not to stretch the fibres and smooth muscle cells, and immediately placed in cold Krebs Henseleit solution containing 118mM NaCl, 22mM $NaHCO_3$, 1.4 mM NaH_2PO_4, 5 mM KCl, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 6 mM Glucose, pH 7.4. The aorta was cleaned to the adventitia, and prepared for vascular function testing, sectioning, enface staining, and lipid content analysis.

3. Experimental Protocol for Assessing Vascular Function

The ability of the vessel to respond to contraction and relaxation was assessed using a tissue bath system (Experimentria, Ltd., Budapest, Hungary), tension was measured with a FSG-01/50 force transducer (Experimentria Ltd., Budapest, Hungary), connected to a SG-Type DC-Bridge amplifier (Experimentria Ltd., Budapest, Hungary), and recorded in an Isosystem 1.0 chart, with the data captured on the S.P.E.L. (Solution Pack for Experimentria Ltd.) software system. Prior to the start of the experiment, the instrument was calibrated to 0.2V/1g, and further confirmed using 1g, 10g, and 20g weights. Following the calibration procedure, the aorta was cut at the superior end just distal to the cut made when the heart was removed. A 3mm ring section was cut and mounted in the tissue bath system using surgical wire. The wire was fastened to the bath and to the force transducer, with the aortic ring mounted between the two. The bath was perfused with Krebs Henseleit

solution, at a temperature of 37°C, and aerated with 95%O_2 and 5% CO_2 to maintain the pH of the solution at 7.4. Once the tissue was hung in the bath, the rings were stretched to a baseline tension of between 5.5 and 6.0 grams. This basal tension was based on previous studies in our laboratory, which showed that when tissue was brought to a baseline tension of between 5.5 and 6.5, optimal contractile response of the aorta rings was observed. The contractile response was expressed as grams of tension per gram of tissue. The weight of the tissue was taken at the termination of the tension experiments, by removing any excess buffer from the ring, and recording the weight to10^{-4} grams.

In order to assess the response of the tissue to both contraction and relaxation stimuli, agonists such as KCl, norepinephrine (NE), acetylcholine (Ach), and sodium nitroprusside (SNP) were used. The first step involved the use of KCl to equilibrate the tissue, by contracting it to a plateau and then washing it out with Kreb's buffer until the tissue returned to its baseline level. The equilibration step was performed a total of 3 times, to confirm that the tissue had reached full contraction. At the completion of this step, the tissue was exposed to increasing concentrations of NE. A dose response curve was performed, using the following concentrations 10^{-8} M, $5x10^{-8}$ M, 10^{-7} M, $5x10^{-7}$ M, 10^{-6} M, 10^{-5} M, 10^{-4} M. The tissue bath was filled to a known volume of Kreb's solution, and each concentration of NE was added, allowing for a plateau to be reached before the next dose was added. Once the plateau was attained for the final concentration, the tissue bath was drained, and washed out with Kreb's solution allowing the tissue to relax and return to the baseline tension level.

The ability of the tissue to relax in response to drugs that are known to elicit vascular relaxation was the next step in the experiment. The tissue was contracted a second time using 10^{-6} NE, and was allowed to reach a stable plateau. Acetylcholine (ACh) was used to measure endothelium dependant response. ACh was added to a bath filled with a known volume of Kreb's solution in concentrations of 10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M. The tissue was allowed to plateau following each addition, and prior to the next, which usually occurred after 5 minutes. The tissue bath was washed out with Kreb's solution after the final concentration of ACh was added, until the baseline tension was reached. The final step in the experiment was to use SNP, and assess the relaxation response of the tissue, following contraction. As with the ACh relaxation step, the tissue was first brought to a steady state contraction using 10^{-6} NE, followed with the addition of increasing concentrations of SNP. The concentrations used were the same as those used in the ACh relaxation step (10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M), and approximately 5 minutes passed between each addition. At the completion of the experiment, the tissue was rinsed with Kreb's solution, weighed, and prepared for tissue sectioning.

4. Quantification of the Atherosclerotic Plaque

a. Sectioning and Staining

The 3mm rings that were used for contractility testing were prepared for sectioning at the completion of the vascular function experiments. The tissues were

fixed in 10% formalin at 4°C overnight, rinsed with 1X PBS three times, and stored in a 30% sucrose solution.

The fixed tissues were submerged in OCT and frozen in preparation for sectioning. The rings were sectioned to 9 μm widths, and were mounted on Superfrost Plus slides. Duplicate sections were taken from the upper, middle, and lower portions of each 3mm section. The slides were stored at -20°C until staining could be performed. The presence of lipids was measured using a solution of Oil Red O (60% isopropanol) which stains lipids in tissue sections.

The quantification of stained lipids on the tissue sections was completed using a digital photograph (Nikon Cool Pics 990 digital camera) of the section through a microscope, and this digital image was visualised using Corel Photo-Paint. Using the Silicon Graphics Imaging (SGI) computer software system, the total area of the section and the area covered in atherosclerotic plaque was quantified. The plaque area was calculated as a percentage of the total area.

b. Enface Analysis

The length of aortic tissue that remained following the removal of sections for vascular function analysis was used for enface analysis. The tissue was opened longitudinally to expose the plaque formation on the intimal region of the aorta. The opened tissue was pinned to a culture dish, and photographs were taken using a Nikon Cool Pics 990 digital camera. The digital image was opened into Corel Photo-Paint, and the image was adjusted to improve the visual differentiation between the plaque

and non-plaque areas. The magic wand function was used to select the area to be analysed, and the image was converted to greyscale. The plaque lesions were quantified as a percentage of the total tissue area, using the SGI computer software system.

5. Platelet Aggregation

Blood collected for the purpose of the analysis of platelet aggregation was drawn from the jugular vein as previously outlined in Section B. The blood was collected in sodium citrate tubes, gently mixed, and left at room temperature for 30 minutes. The sample was then centrifuged at 22°C at 100g for 15 minutes, producing the platelet rich plasma (PRP) upper layer. This layer was removed (approximately 600μl) and stored in a labelled eppendorf tube at room temperature. The blood was spun in the centrifuge again, at 2400g for 15 minutes, to yield the platelet poor plasma (PPP) as the upper layer. A volume of 600μl was removed and transferred to a labelled eppendorf tube and kept at room temperature.

For testing, 500μl of PRP was transferred to 2 separate glass cuvettes, with magnetic stir bars, and they were each placed in the wells labelled with "PRP". The stir bars were turned on. A volume of 500μl of PPP was transferred to another glass cuvette, and placed in the well labelled "PPP". On the computer, the AGGLINK program was opened, and the pre-set test procedures were opened for the aggregometer. The procedure was set for collagen (4μg/mL) and ADP (10μM) as the clotting agents. To achieve these concentrations, 2μl of collagen and 5μl of ADP

were prepared for addition to the 500µl samples. On the Aggregometer program, "Run Test" was selected, sample information was added, and the "OK" was selected which initiated sample monitoring by the aggregometer. Both "Set Baseline" buttons were depressed at the same time, and when the signals stabilized, both were let go, and the machine set itself to zero. The two volumes of collagen and ADP were dispensed at the exact same time to separate tubes, and the platelet aggregation was observed. The test was stopped when the aggregation was complete, the slope and amplitude were recorded, and the graph was printed. The slope represents the rate of aggregation (% change / minute) and the amplitude represents the % aggregation.

6. Analysis of Blood Samples

a. Lipid Analysis

The blood samples were taken as outlined in section B and were collected in vacutainer tubes containing EDTA, and stored on ice. The samples were centrifuged at 4500 x g for 10 minutes at room temperature. The upper plasma layer was removed and stored at -80°C. The experimental procedure for the extraction and derivatization of fatty acids was from the method of Lepage and Roy (55). In a test tube, 100 µl of plasma was added to 2 ml of methanol-benzene (4:1), and vortexed to mix. Once mixed, 200 µl of acetyl chloride was added and the tubes were sealed and heated to 90°C for one hour. To neutralize the solution, 5 ml of 6% K_2CO_2 was added, followed by the removal of a 0.5 µl aliquot of the upper benzene layer to be

analysed by gas chromatography. A Varian CP-3800 gas chromatograph, equipped with a flame ionisation detector and a Varian CP-Sil 88 capillary column (50m x 0.25mm x 0.20μm) was used. The aliquot obtained was injected with the CP-8400 auto sampler at a split ratio of 1:100 and the flow rate of the helium carrier gas was 1 ml/min. The initial oven temperature was 80°C. It was held at this temperature for 1 minute, raised at a rate of 30°C per minute to 140°C, and then raised again at a rate of 5°C per minute to a final temperature of 225°C, where it was held for 10 minutes. The total time to run each sample was 30 minutes, and the fatty acid contents of the sample were identified by comparison with reference standards.

b. Triglyceride (TG) Assay

Triglyceride levels in the plasma was measured using an enzymatic assay. Triglycerides Liquid Incorporating Dynamic Stabilization Technology reagent was added to a 96 well micro-plate and warmed to 36°C. To the warmed reagent, 0.9% NaCl, standard (250 mg/dl TG), and plasma sample was added in a 1:100 ratio, and mixed with gentle agitation. The samples were incubated at 37°C for 5 minutes. The absorbance of each well was measured at 500 nm. The concentration of TG was determined by multiplying the absorbance of the unknown by the standard concentration (mg/dl), and dividing this product by the absorbance of the standard. Plasma samples with a high concentration of lipid were diluted with 0.9% NaCl (v/v) to prevent interference.

c. Cholesterol Ester (CE) Assay

Cholesterol levels in the plasma was measured using an enzymatic assay. Infinity™ Cholesterol Liquid Stable reagent was added to a 96 well micro-plate and warmed to 36°C. To the warmed reagent, 0.9% NaCl, standard (200 mg/dl CE), and plasma sample was added in a 1:100 ratio, and mixed with gentle agitation. The samples were incubated at 37°C for 5 minutes. The absorbance of each well was measured at 500 nm. The concentration of CE was determined by multiplying the absorbance of the unknown by the standard concentration (mg/dl), and dividing this product by the absorbance of the standard. Plasma samples with a high concentration of lipid were diluted with 0.9% NaCl (v/v) to prevent interference.

7. Statistical Analysis

Data are expressed as the mean ± standard error (S.E.). A one-way analysis of variance (ANOVA) was used to make statistical comparisons, with the Duncan's Multiple Range Test for multiple comparison, performed on Sigma Stat 2.0. The difference between means was considered significant when $p<0.05$.

IV. Results

1. Animal Weights

The rabbits were weighed at the start of the study, prior to the administration of dietary change, and at the end of the study, prior to sacrifice. The animal weights in each dietary group increased by 68% to 70% following the 8 week study (**Figure 14**). No significant differences were observed for the initial animal weights, however significant differences were found for the final animal weights. The cholesterol with hempseed diet (OLHP) were significantly heavier than the control (RG) fed animals, and the hempseed (HP) diet was also significantly heavier than the control diet fed animals.

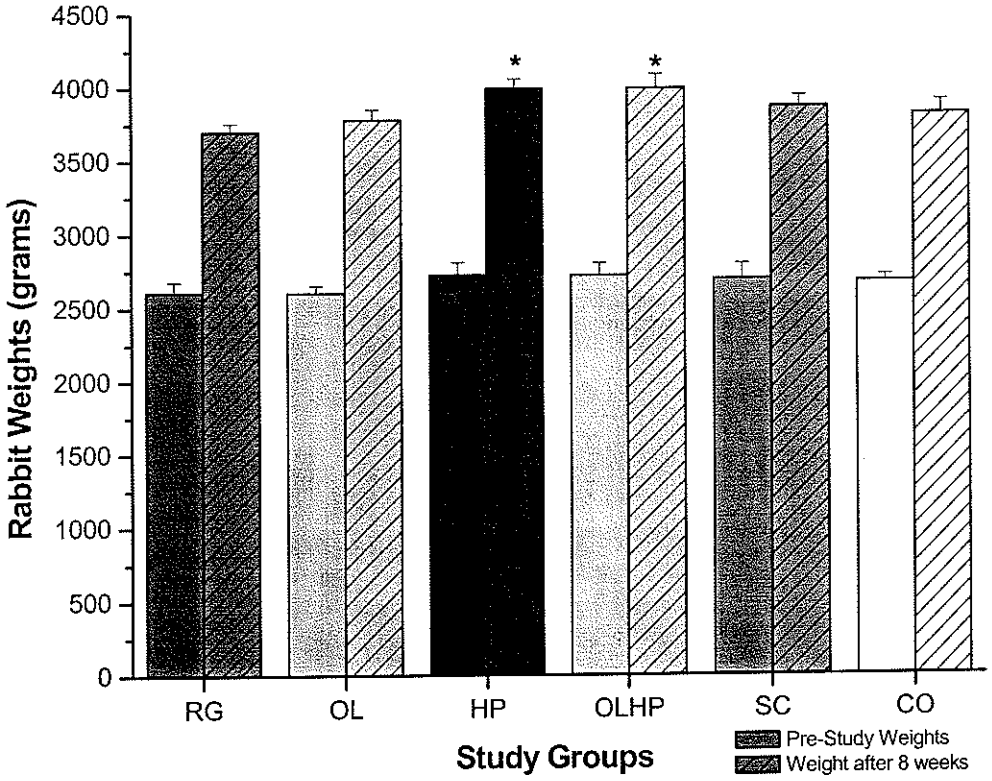


Figure 14: Comparison of Rabbit Weights taken at the start of the study and at the end of the 8 week study. (*) denotes significant difference using Duncan's post hoc analysis. N = 10.
(**Legend**: **RG** = Control Diet, **OL** = Cholesterol Supplementation, **HP** = Hempseed Supplementation, **OLHP** = Cholesterol/Hempseed Supplementation, **SC** = Seed cake supplementation, **CO** = Coconut Oil Supplementation)

2. Plasma Analysis

Plasma was sampled following the 8 week study, prior to sacrifice, and was analysed for cholesterol, triglycerides, and fatty acid composition. **Table 2** outlines the data obtained for circulating cholesterol and triglycerides. The two study groups that showed a significant increase in both cholesterol and triglycerides, when compared with the control group, were the cholesterol and cholesterol with hempseed groups. There was no significant differences observed between the cholesterol diet and the cholesterol diet supplemented with hempseed.

Plasma was also analysed for fatty acid composition using FAME analysis as described in the Methods section. The data obtained are included in **Figure 15**. The OL and OLHP diets induced a significantly greater concentration of most lipids, including linoleic acid, and alpha-linolenic acid. Interestingly, supplementation of the diet with hempseed did not result in a significant increase in any of the fatty acids detected.

Diet Groups	Cholesterol (mg/dL)	Triglyceride (mg/dL)
Regular	27.42 ± 2.20	62.32 ± 10.40
Hempseed	30.88 ±1.91	67.54 ± 23.10
Cholesterol	1426.76 ± 250.23 (*)	260.33 ± 55.25 (*)
Cholesterol/Hempseed	2013.47 ± 161.22 (*)	259.46 ± 41.89 (*)
Seedcake	35.51 ± 4.39	67.02 ± 16.71
Coconut Oil	54.70 ± 11.85	38.44 ± 2.42

Table 2: Plasma cholesterol and triglyceride levels following 8 week dietary intervention. (*) denotes significant difference from control according to Duncan's Multiple Range Analysis. N = 4.

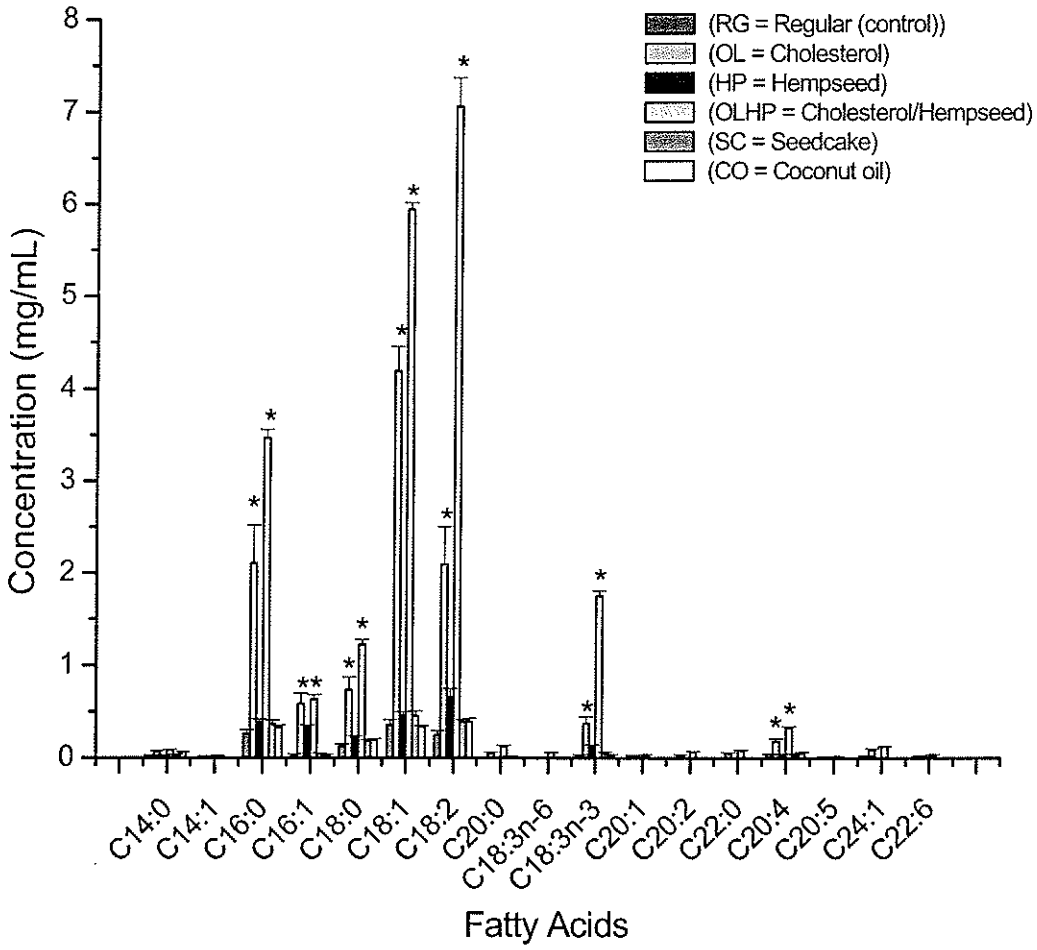


Figure 15: Fatty acid profile of plasma samples taken from 8 week rabbits. (*) Indicates significant differences according to Duncan's Multiple Range Analysis. N=8.

3. Platelet Aggregation

At the point of animal sacrifice, blood was taken and processed immediately for platelet aggregation analysis, according to the procedure summarized in the Methods section. Samples were treated with two aggregating agents: adenosine diphosphate (ADP) and collagen. Two parameters of platelet aggregation were analysed. The first parameter is percent aggregation, which illustrates how much aggregation occurred in the samples. The second parameter is the rate of aggregation, which represents the percent change per minute, i.e. how quickly aggregation occurred when either ADP or collagen was added to the samples. When ADP was used as the aggregation agent, the percent aggregation is presented in **Figure 16**. The cholesterol and seed cake diets have 44% and 39% aggregation respectively, which were significantly greater than with the four remaining dietary groups. The other dietary interventions had similar percent aggregations to that of the control fed animals.

When the platelet rich plasma samples were exposed to collagen, similar qualitative results were observed to those obtained for the ADP results (**Figure 17**). Two dietary groups demonstrated a significantly greater percent aggregation when exposed to collagen. Both cholesterol-supplementation (53%) and the seed cake (52%) diet had significantly greater aggregation than control. The dietary intervention with the lowest percentage of platelet aggregation was the cholesterol/hempseed diet, with a mean percent aggregation of 39%.

The rate of aggregation illustrates the percentage of change observed over a one minute time period. **Figure 18** presents the impact of ADP on the rate of aggregation of the samples. Once again, the two dietary groups that demonstrated a significantly increased rate of aggregation when ADP was added to the samples include the cholesterol-supplemented dietary group with a rate of aggregation of 105%, and the seed cake group with a rate of aggregation of 101 % ($P< 0.05$). The dietary intervention that had the lowest rate of aggregation was the cholesterol/hempseed diet group with a measured value of 80 %.

When collagen was used to promote aggregation, the results that were obtained are shown in **Figure 19**. The cholesterol-supplemented diet group was the only one that had a significantly greater rate of aggregation as compared with the other dietary groups, with an average value of 84%. All of the other diet groups had values for the rate of platelet aggregation between 51% and 60%.

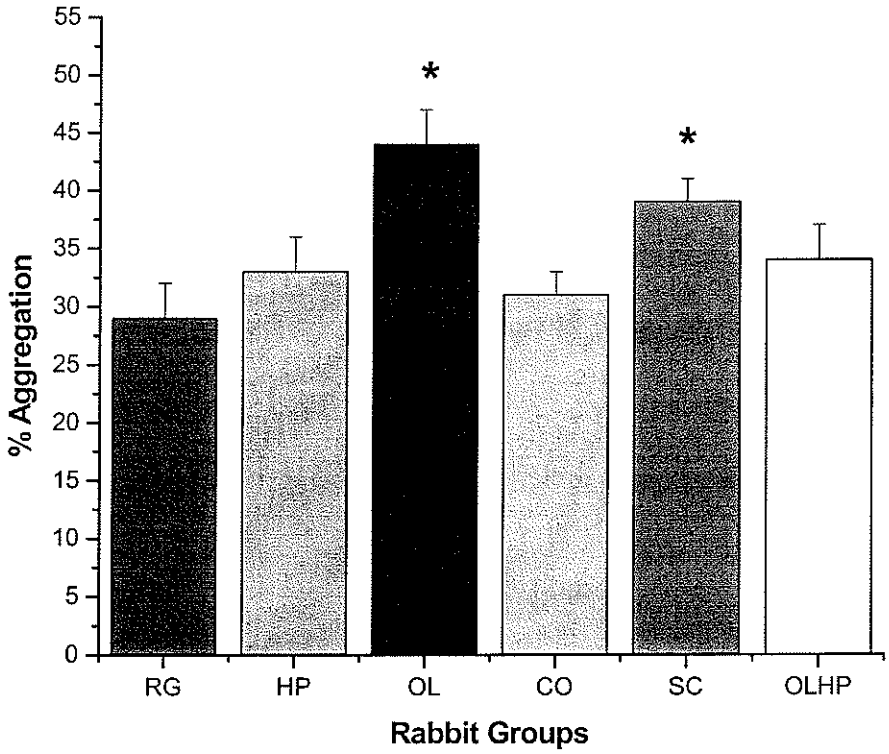


Figure 16: Percent Aggregation upon addition of ADP in platelet rich plasma in treated and non-treated rabbits at the 8 week study termination. (*) denotes significant difference according to Duncan's *post hoc* analysis. N = 8(RG), 8(HP), 8(OL), 10(CO), 7(SC), 9(OLHP)
(**Legend**: **RG** = Control Diet, **OL** = Cholesterol Supplementation, **HP** = Hempseed Supplementation, **OLHP** = Cholesterol/Hempseed Supplementation, **SC** = Seed cake supplementation, **CO** = Coconut Oil Supplementation)

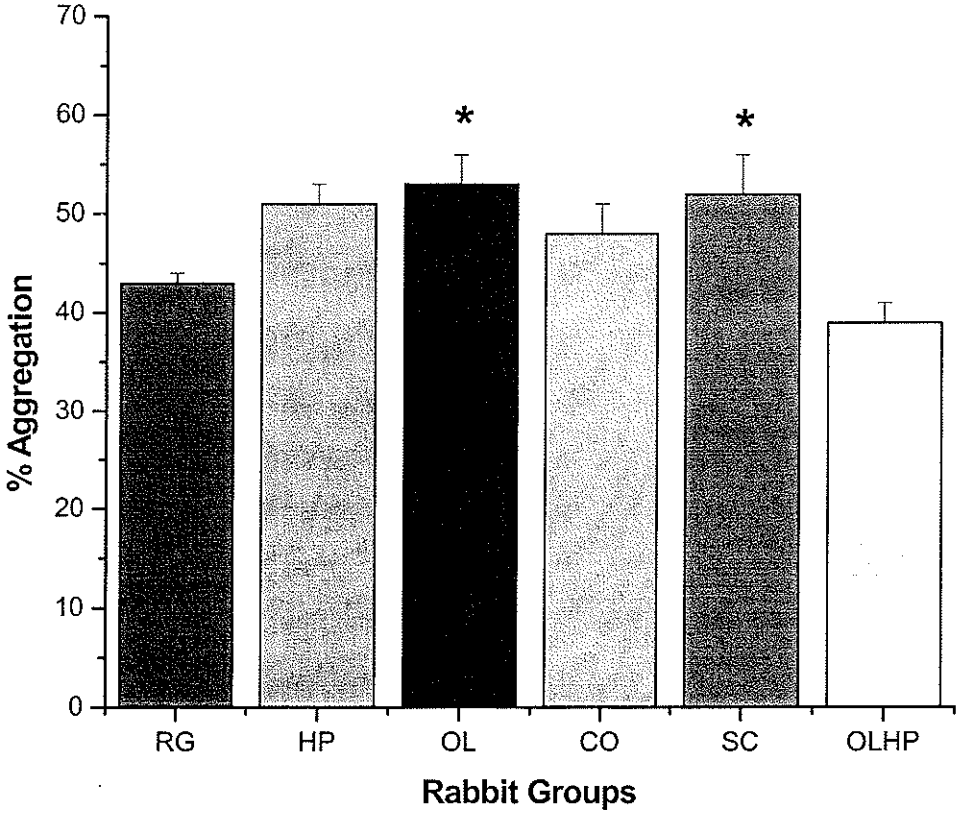


Figure 17: Percent Aggregation upon addition of Collagen to platelet rich plasma in Treated and Non-Treated Rabbits. (*) denotes significant difference according to Duncan's *post hoc* analysis. N = 8(RG), 7(HP), 8(OL), 10(CO), 7(SC), 9(OLHP)
(**Legend**: **RG** = Control Diet, **OL** = Cholesterol Supplementation, **HP** = Hempseed Supplementation, **OLHP** = Cholesterol/Hempseed Supplementation, **SC** = Seed cake supplementation, **CO** = Coconut Oil Supplementation)

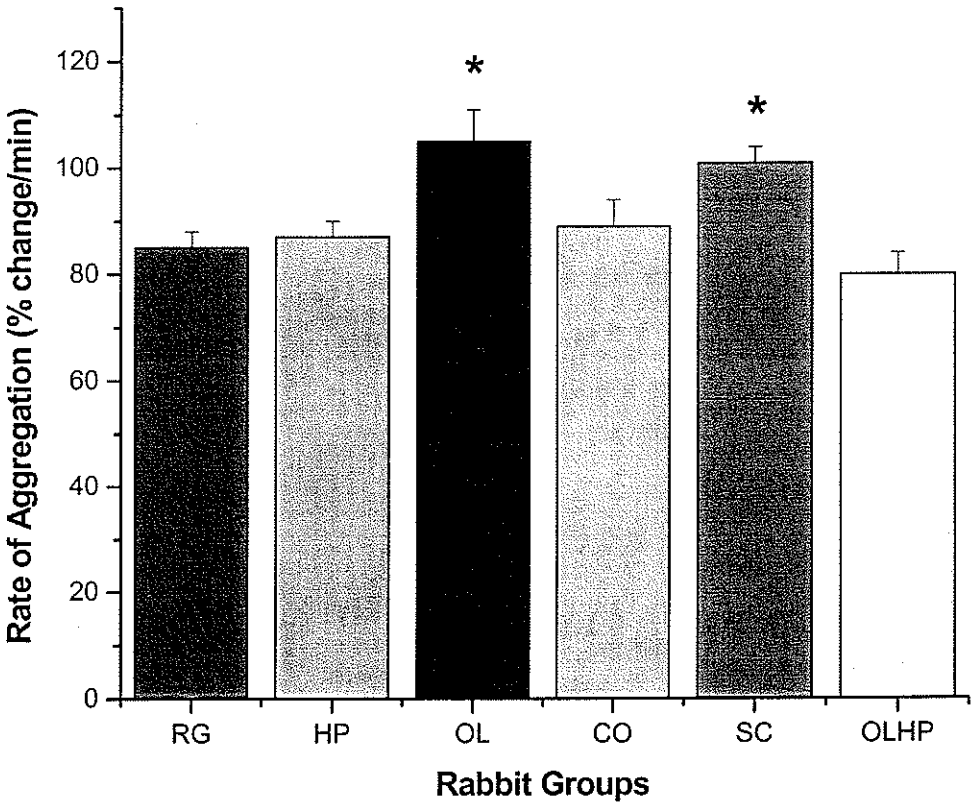


Figure 18: Change in Platelet Aggregation upon addition of ADP to platelet rich plasma in Treated and Non-Treated Rabbits. (*) denotes significant difference according to Duncan's *post hoc* analysis. N = 8(RG), 8(HP), 8(OL), 10(CO), 10(SC), 9(OLHP)
(**Legend**: **RG** = Control Diet, **OL** = Cholesterol Supplementation, **HP** = Hempseed Supplementation, **OLHP** = Cholesterol/Hempseed Supplementation, **SC** = Seed cake supplementation, **CO** = Coconut Oil Supplementation)

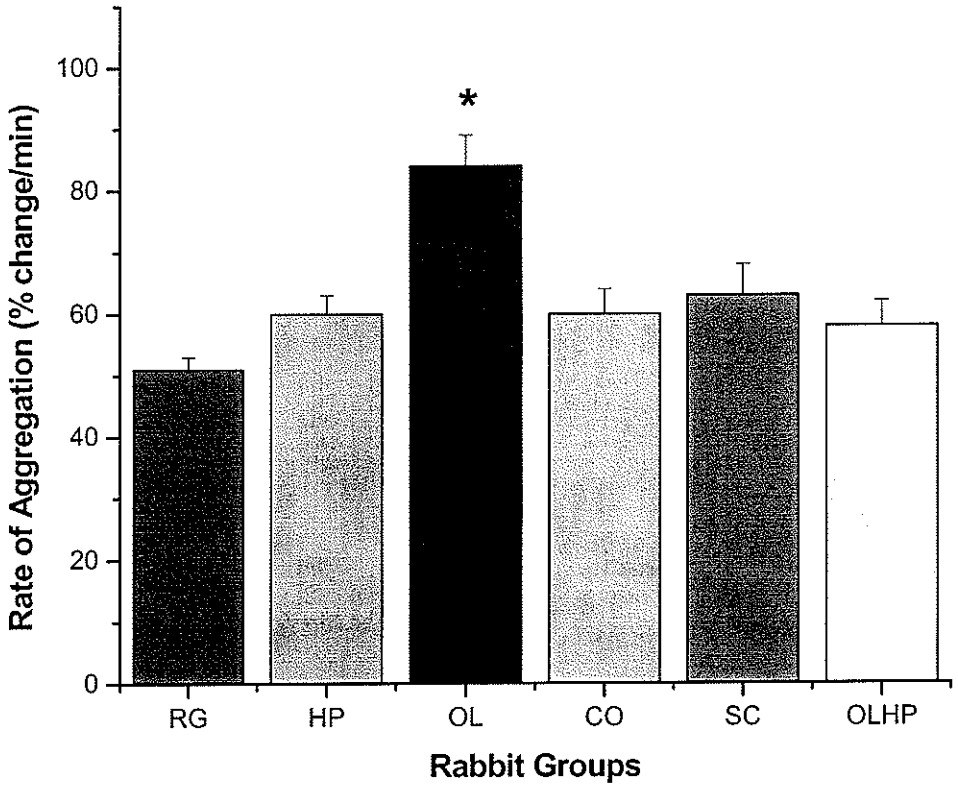
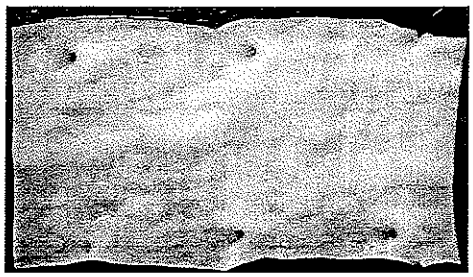


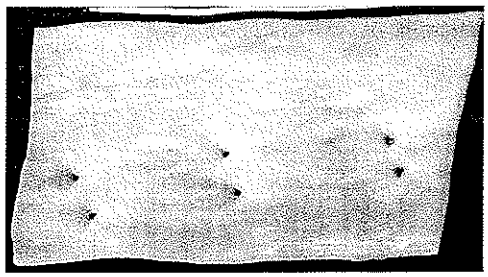
Figure 19: Change in Platelet Aggregation upon addition of Collagen to platelet rich plasma in Treated and Non-Treated Rabbits. (*) denotes significant difference according to Duncan's *post hoc* analysis. N = 8(RG), 7(HP), 8(OL), 10(CO), 7(SC), 9(OLHP)
(**Legend**: **RG** = Control Diet, **OL** = Cholesterol Supplementation, **HP** = Hempseed Supplementation, **OLHP** = Cholesterol/Hempseed Supplementation, **SC** = Seed cake supplementation, **CO** = Coconut Oil Supplementation)

4. Atherosclerotic Plaque Deposition: Enface Analysis

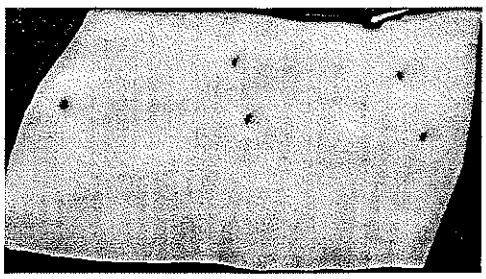
The aortic tissue was prepared for enface analysis of atherosclerotic plaque development as described in the Materials and Methods section. Atherosclerotic plaques were detected only in the cholesterol-supplemented dietary groups. Representative images of enface plaque in the aorta from the control (RG), the OL and OLHP groups are shown in **Figure 20**. The ratio of plaque area presented as a percentage of total luminal area in a number of samples is shown in **Figure 21**. There was evidence of plaque formation on the aortic tissue of rabbits in the 0.5% cholesterol (OL) diet and the 10% hemp seed with 0.5% cholesterol (OLHP) diet groups. The amount of plaque found on both of the tissues was not significantly different from each other but significantly elevated in comparison to the other groups.



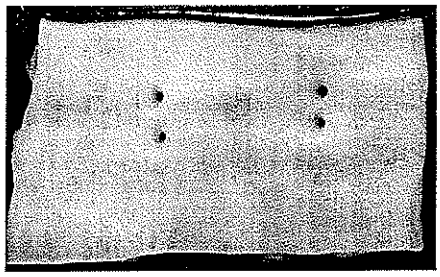
Cholesterol



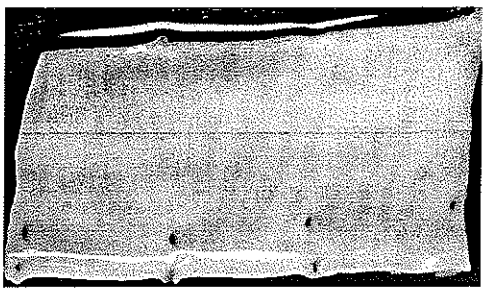
Cholesterol / Hempseed



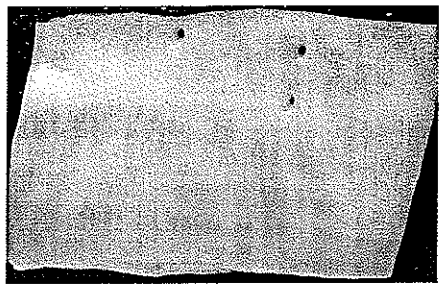
Coconut Oil



Hempseed



Seedcake



Regular (control)

Figure 20: Representative digital enface images from the aorta of rabbits following 8 week dietary intervention

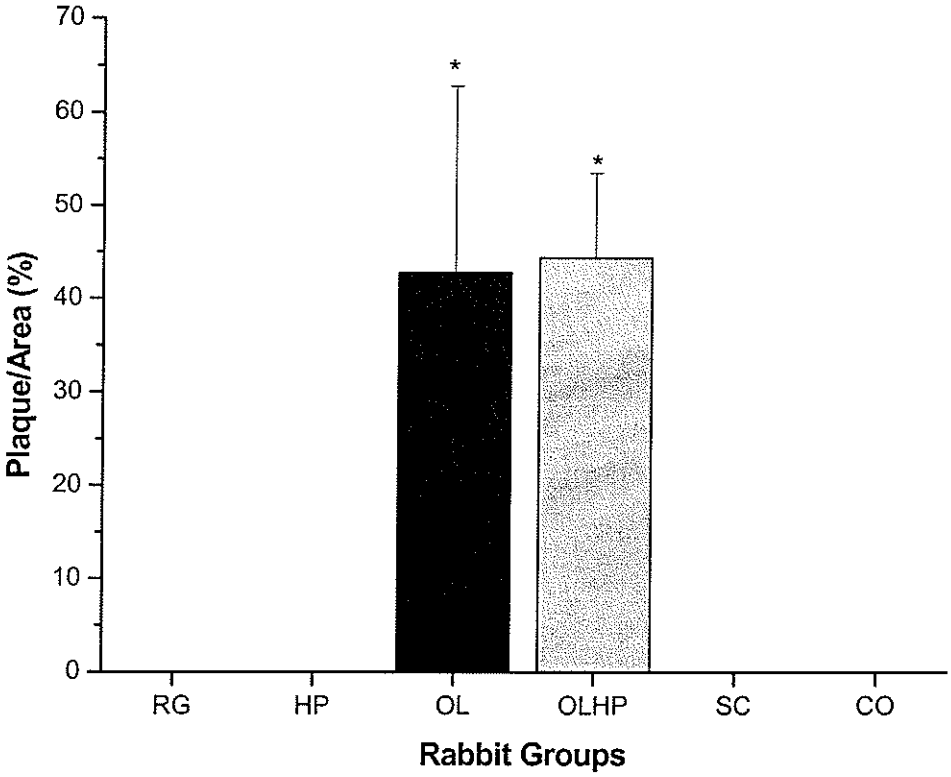
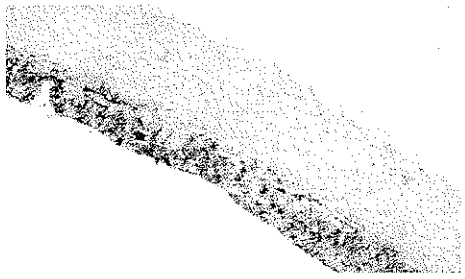


Figure 21: Percentage of plaque found on the luminal surface of rabbit aorta tissue as a function of dietary interventions. (*) Indicates significant difference from control values (n=4,5).
(**Legend**: **RG** = Control Diet, **OL** = Cholesterol Supplementation, **HP** = Hempseed Supplementation, **OLHP** = Cholesterol/Hempseed Supplementation, **SC** = Seed cake supplementation, **CO** = Coconut Oil Supplementation)

5. Atherosclerotic Plaque Deposition: Histology

Although the area that the plaque covered on the aortic luminal surface was not significantly different between the two groups fed cholesterol in their diet, it was possible that the thickness of the plaque was different. Sections of the aortic tissue were created, stored, and prepared as detailed in the Methods section. The sections were stained with Oil Red O for lipid determination within the plaque. The OL and OLHP dietary interventions were the only two groups with plaque formation identified as the presence of lipid on the aortic cross-sections. Representative images illustrate the result of the staining **(Figure 22)**. The data from a number of animals were analysed and presented as the percentage of lipid found in the plaque (**Figure 23**). Statistically, there was no significant difference between the two cholesterol-supplemented groups.



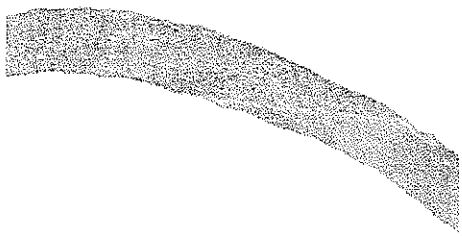
Cholesterol

Cholesterol/Hempseed

Coconut Oil

Hempseed

Seedcake



Regular (control)

Figure 22: Representative digital cross sectional images from the aorta of rabbits following 8 week dietary intervention. Tissues were stained with Oil Red O for the presence of lipids, and the red color indicates that lipids are present in the tissue.

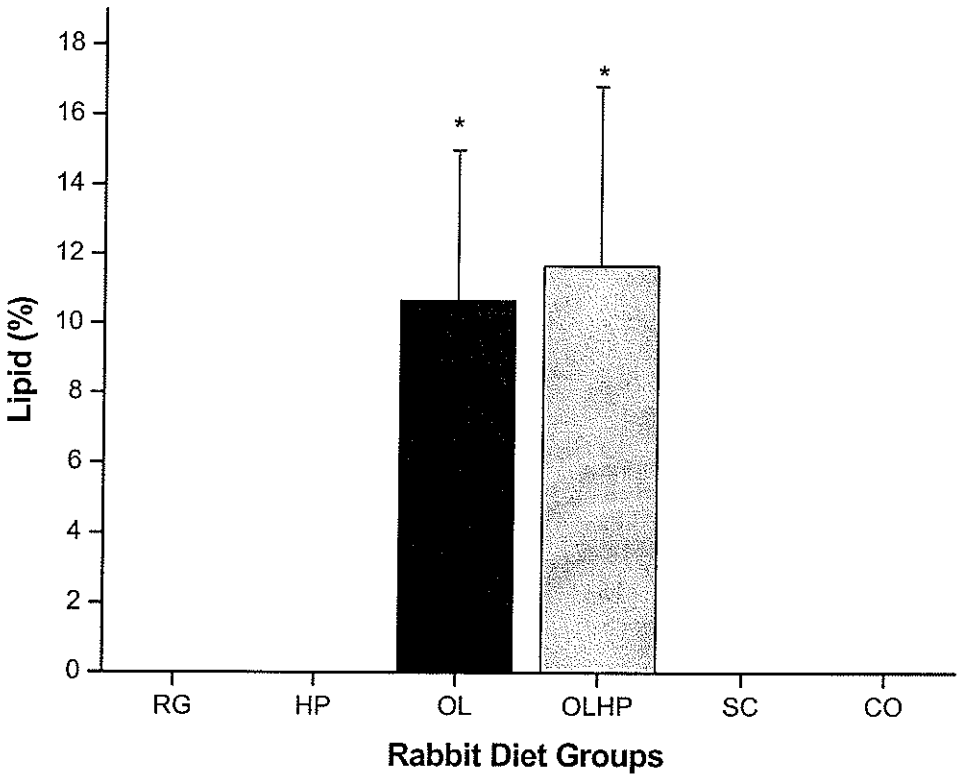


Figure 23: Percentage of lipid in aortic cross-sections identified by Oil Red O staining. (*) denotes significant difference from control ($P < 0.05$ vs RG) (n=4)
(**Legend**: **RG** = Control Diet, **OL** = Cholesterol Supplementation, **HP** = Hempseed Supplementation, **OLHP** = Cholesterol/Hempseed Supplementation, **SC** = Seed cake supplementation, **CO** = Coconut Oil Supplementation)

6. Vascular Tone: Contraction with KCl

The tissues were prepared for the analysis of contractile reactivity as described in the Methods section. The tissues were exposed to 47mM KCl, causing hyperpolarization, and contraction. The maximum contraction levels were recorded, and the data were plotted, with grams of tension expressed relative to tissue weight (**Figure 24**). The cholesterol-supplemented diet (OL), the hempseed diet (HP), and the cholesterol with hempseed diet (OLHP) generated significantly attenuated contractile tension in response to KCl relative to the control (RG) samples.

7. Vascular Tone: Contraction to Norepinephrine (NE)

Increasing concentrations of norepinephrine (NE) were added to the tissue bath, and the resulting aortic tension was observed and recorded. Tension was normalized relative to the tissue weight and plotted as a function of norepinephrine concentration (**Figure 25**). Tissue tension increased as a function of NE level. Statistical analysis revealed some significant differences between dietary groups. At all concentration levels, with the exception of the lowest concentration, the tensions developed by cholesterol (OL) and cholesterol plus hempseed (OLHP) were significantly lower than from the control diet group. The response observed with the OL and OLHP groups appears to be nearly identical at all NE concentration levels.

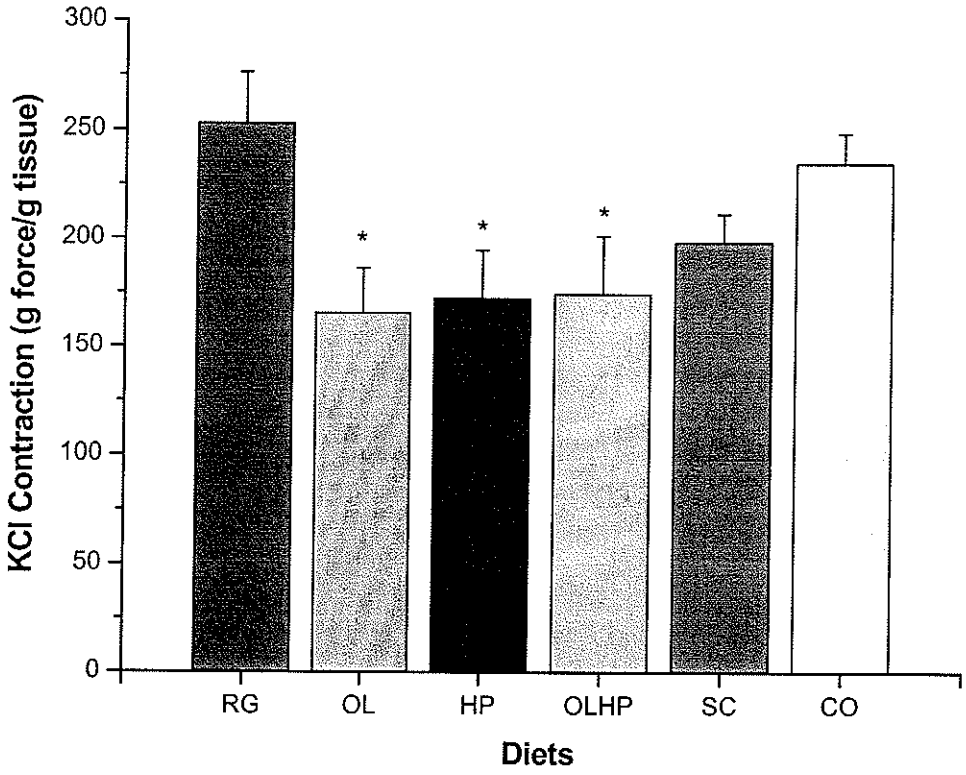


Figure 24: Aortic Tissue Contraction Induced by KCl. (*) denotes significant difference from control ($p<0.05$). N = 9,10
(**Legend**: **RG** = Control Diet, **OL** = Cholesterol Supplementation, **HP** = Hempseed Supplementation, **OLHP** = Cholesterol/Hempseed Supplementation, **SC** = Seed cake supplementation, **CO** = Coconut Oil Supplementation)

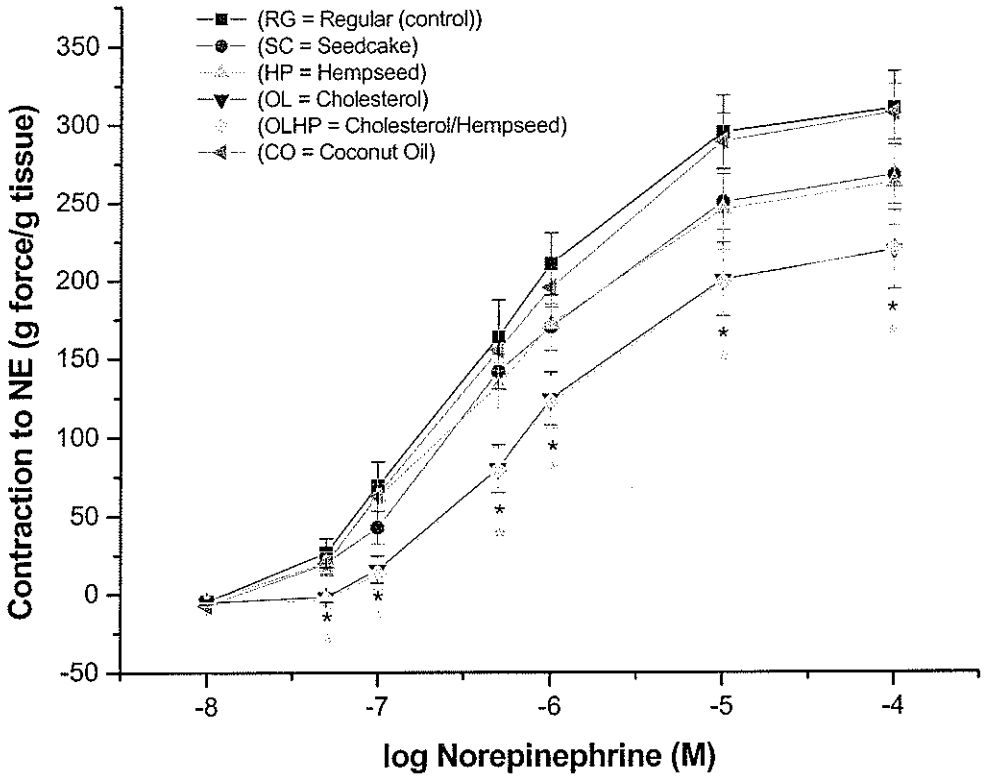


Figure 25: Tissue contraction as a function of norepinephrine concentration. (*) denotes significant difference from control ($p<0.05$). N = 9,10

8. Vascular Tone: Relaxation with acetylcholine

In preparation for analysis of tissue relaxation in response to acetylcholine, the tissue was exposed to norepinephrine, at $1x10^{-6}$ M, and the tissue was allowed to achieve a steady state of contraction. Acetylcholine was then added in increasing concentrations. The results shown in **Figure 26** illustrate the percent relaxation attained by the tissues after exposure to increasing concentrations of acetylcholine. All tissues relaxed to acetylcholine, with a large initial response at the lowest concentration followed by increasing tissue relaxation with increased concentration. No statistically significant differences were observed between the tissues and the control tissue.

9. Relaxation to sodium nitroprusside (SNP)

In order to assess the tissue's ability to respond to an exogenous source of nitric oxide, sodium nitroprusside (SNP) was used as a dilating agent. As with acetylcholine relaxation, the tissues were contracted initially, with $1x10^{-6}$ M NE. **Figure 27** shows the percent relaxation observed following the addition of SNP to the tissue bath. All tissues relaxed with increased concentrations of SNP. Significant differences were observed between the cholesterol diet group and the control diet group at the two highest concentration points.

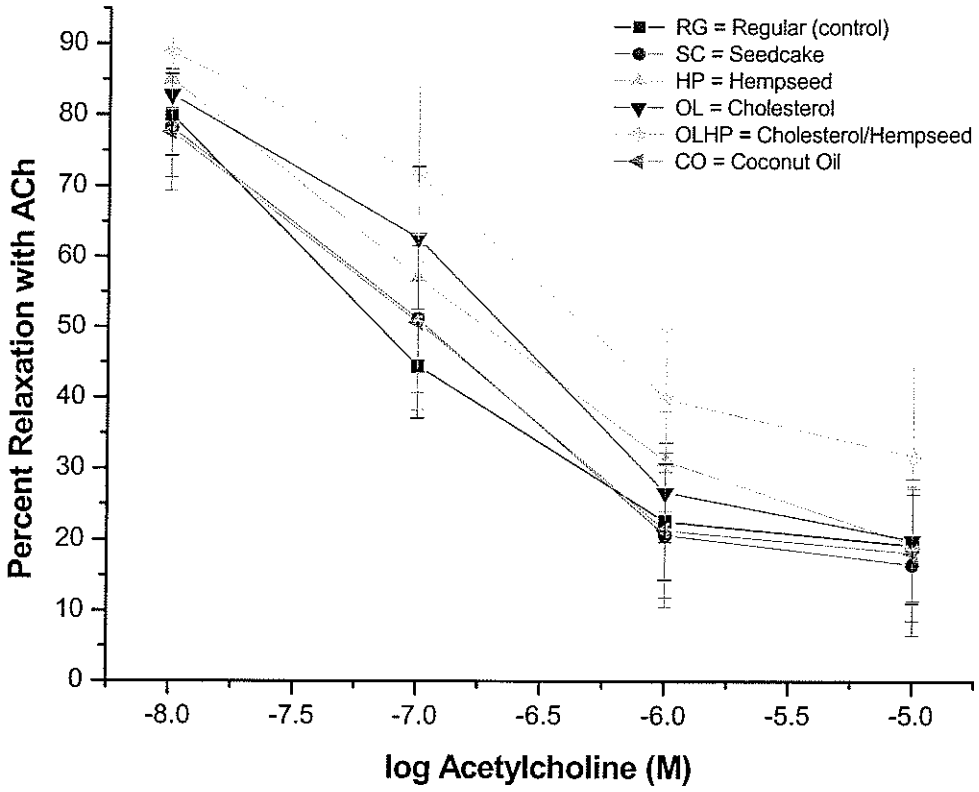


Figure 26: Tissue relaxation as a function of acetylcholine concentration.
N = 9,10.

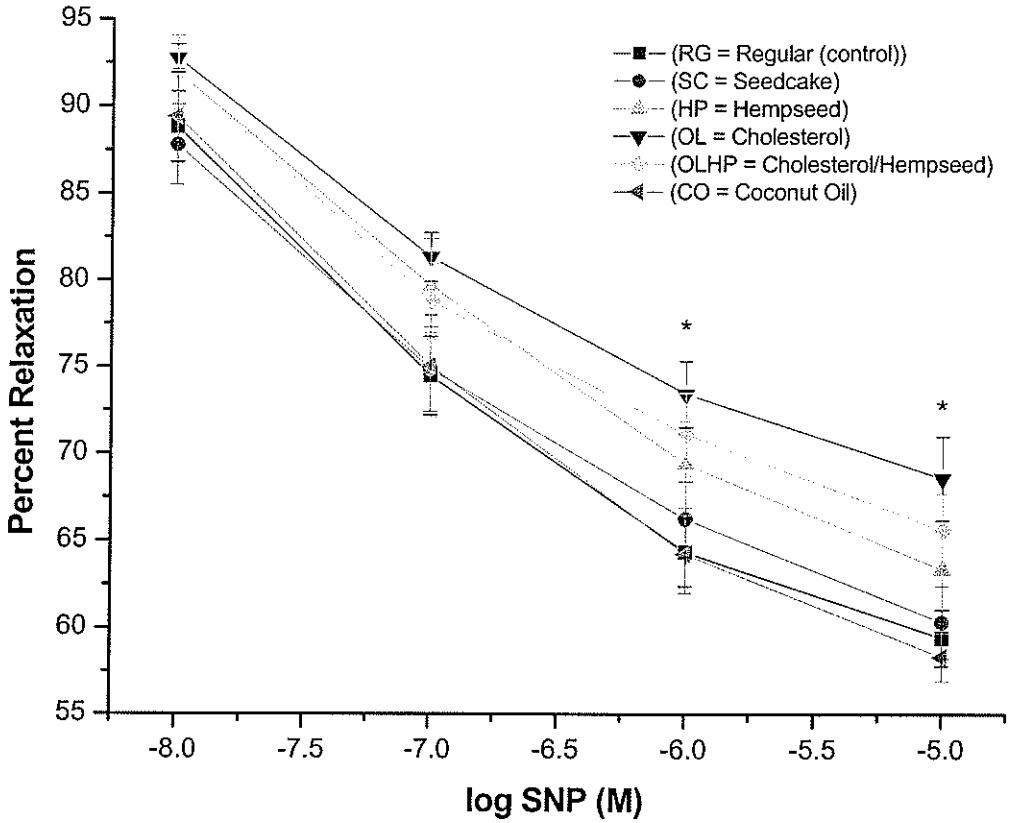


Figure 27: Tissue relaxation as a function of sodium nitroprusside (SNP) concentration. (*) denotes significant difference from control ($p<0.05$). N = 9,10.

V. Discussion

The purpose of this study was to examine the physiological effects that a diet supplemented with hempseed, with and without added cholesterol, would have on the cardiovascular health of rabbits. We hypothesized that this 8 week dietary intervention would induce significant differences in the clotting characteristics of the plasma, produce beneficial effects on the ability of the aortic tissue to respond to contractile and relaxation stimuli, and inhibit plaque formation when diets were supplemented with hempseed plus cholesterol when compared with diets supplemented with cholesterol alone.

The rabbits were administered a set amount of rabbit chow with one control group and five groups having supplements added to the regular food. The rabbits were weighed before the start of the study and throughout the study until the 8 week termination point. The environment was controlled and maintained for all six study groups in an effort to minimize the impact of external differences altering the results. A significant difference in body weight was observed between the OL and OLHP diet groups when compared to the control group. The additional weight gain observed can be attributed to the higher cholesterol content of the OL and OLHP diet groups. It is worth noting that the coconut oil diet group, having increased saturated fat in the diet, did not significantly impact the amount of weight gained when compared to the control group. Thus, the fat content of the diet was not a factor itself in causing the increase in body weight. The effects of cholesterol are in agreement with a study by

Thomson *et al.* (56) which demonstrated that cholesterol in the diet increased the absorption of lipids by the gut (56, 57). Our animal weight results appear to support the results reported by Thomson *et al.*, in that the cholesterol in the OL and OLHP diet groups seems to have enabled increased absorption of lipids across the jejunum of the rabbits (56), resulting in increased weight gain. The FAME data support this work of Thomson. We found that nearly all of the fatty acid levels were higher in plasma when cholesterol was included in the diet. Surprisingly, even when the diet was not supplemented with hemp, the fatty acids were elevated when cholesterol was included in the diet. Again, this would suggest that cholesterol had the capacity to increase the absorption of even the endogenous fatty acids in the control diet. There was another unexpected finding that resulted from our dietary interventions. The hempseed supplemented dietary group did not demonstrate a significant increase in ALA or LA concentrations when compared to the control group, as we would have expected. We may conclude that the polyunsaturated fatty acids found in hempseed are poorly absorbed when ingested without cholesterol. These results are consistent with those found in human studies where subjects' diets were supplemented with hempseed oil capsules (Kaul and Pierce, unpublished results).

The development and progression of atherosclerosis is characterised in part by increased platelet aggregation. Cholesterol supplementation of the diet not only generated significant atherosclerotic plaque development but also stimulated platelet aggregation. We did not examine the mechanism responsible for this effect. However, it may be due to increased incorporation of cholesterol in the platelet membrane, decreasing fluidity and causing increased platelet adherence.

Hempseed supplementation to the diet inhibited the stimulatory effects of cholesterol on platelet aggregation. Considering that rabbits fed the seedcake supplemented diet, which is hempseed that has had most of the lipids removed, exhibits increased aggregation, we can conclude that the decrease in aggregation in the OLHP and HP groups is due to the polyunsaturated fatty acids present in the HP diet. This may be surprising. It has been postulated that a diet rich in omega-6 fatty acids would result in an increase in platelet aggregation due to the eicosanoid generation induced by the metabolism of omega-6 polyunsaturated fatty acids. However, hempseed is unique in its ratio of omega-6 and omega-3 polyunsaturated fatty acids. Omega-3 polyunsaturated fatty acids inhibit platelet aggregation (58). It is possible, therefore, that the inhibitory action on platelet aggregation by dietary hempseed supplementation was due to its concomitant content of omega-3 fatty acids. The hempseed supplementation to the diet may induce very different changes in the lipid composition of the platelet membranes than cholesterol. Cholesterol is known to decrease platelet membrane fluidity and polyunsaturated fatty acids increase its fluidity (59). It is possible that hempseed counteracted the platelet stimulatory effects of cholesterol through an increase in membrane fluidity when hempseed is supplemented in the diet.

There are three other mechanistic possibilities to explain the anti-aggregatory effects of hempseed that may involve a direct action of omega-6 fatty acids. For example, the binding of fibrinogen to receptor complexes on the platelet membrane is a key component of platelet aggregation (60). One study demonstrated that a diet rich in omega-6 fatty acids (sunflower oil supplementation) had significantly reduced

platelet activity and a reduced fibrinogen load (61). A second mechanism involves decreased production of prostacyclin and thromboxane, from arachidonic acid, in diets supplemented with omega-3 polyunsaturated fatty acids (58). Some studies have shown that EPA and DHA from omega-3 polyunsaturated fatty acids block the receptors for TxA_2 and PGH_2 (46) resulting in an anti-aggregatory environment. Finally, a third possibility involves the endothelium. The endothelium plays an important role in the adherence of platelets and clot formation. The supplementation of hempseed in the diet may play a role in the formation of nitric oxide by the endothelium, increasing the concentration of nitric oxide and thereby reducing platelet aggregation (24). Although all of these proposed mechanisms are possible, the last possibility is the least likely choice. Our data with acetylcholine reactivity would suggest that NO reactivity was similar amongst all of the groups. However, additional experimentation is required to be certain about the mechanism of action.

The hallmark characteristic of atherosclerosis is the formation of plaque deposits on the luminal walls of arteries. Enface analysis and tissue sectioning of the aorta tissue was performed to quantify plaque area and thickness. There was evidence of plaque on the OL tissue and the OLHP tissue but no plaque was observed in any other tissues. When the aortic rings were sectioned and stained with Oil Red O to expose lipids on the vessel wall, lipids were observed only in the OL and OLHP groups. A diet having a high concentration of cholesterol is well known to induce altherosclerosis. However, it would appear that hempseed is not able to protect the vessel from this atherogenesis. This is in contrast to the anti-atherogenic action of flaxseed in the diet (62, 63). This may result from the poor absorption of

polyunsaturated fatty acids that are thought to have an anti-atherogenic action (Dupasquier and Pierce, unpublished results). Alternatively, the lignan content of hempseed may not be as high as flaxseed. Lignans found in flaxseed are thought to be strongly anti-atherogenic (62, 64).

Alterations in vascular tone are another characteristic of atherosclerosis. Our study examined the response of aortic tissues to KCl, norepinephrine, acetylcholine and sodium nitroprusside. All tissues contracted in response to KCl, as expected. However, the OL, OLHP, and HP groups all had an attenuated contractile response in comparison to control. KCl induced vascular contraction through a depolarization of the membrane and opening of Ca^{2+} channels. The attenuated response may be due to one of three possibilities: i) an inability of the KCl to induce membrane depolarization, or, ii) an alteration in the opening of the Ca^{2+} channels, or, iii) a post Ca^{2+} channel, intracellular defect in Ca^{2+} handling or contractile protein function. The concentration of KCl used was much higher than needed to achieve membrane depolarization. Thus, a major change in KCl-induced depolarization would be needed to cause the changes observed. It is unlikely that this was the cause of the defect in KCl-induced contraction. However, this was not directly evaluated. It is also possible that the tissues from these dietary groups may have a defect in the movement of Ca^{2+} from the extracellular environment into the intracellular space or changes in the intracellular handling of Ca^{2+}. This may have occurred due to alterations in the membrane composition. It is known that cholesterol and fatty acids can alter Ca^{2+} movements (5, 65). However, we have no direct evidence in support of this

hypothesis. Further work is required to identify more precisely the mechanism that may be responsible for these changes in contractile response to KCl.

The response of the tissues to norepinephrine may provide some further insight into this problem. Norepinephrine exposure caused all tissues to contract. However, the OL and OLHP groups had a significantly decreased response to NE at each concentration level when compared to the response from the control tissues. The attenuated response may be due to one of three possible defects: i) an inability of the norepinephrine to interact with norepinephrine receptors, or, ii) an alteration in the opening of the Ca^{2+} channels, or, iii) a post Ca^{2+} channel, intracellular defect in Ca^{2+} handling or contractile protein function. All three may be valid options. The norepinephrine receptors may have a decreased sensitivity to NE in tissues from OL and OLHP diet groups. This has been observed previously in studies on hypercholesterolemic rabbits (66). This may be the result of the presence of plaque on the interior wall of the aorta, affecting the receptor activity. However, because these same groups (OL and OLHP) had a decreased response to KCl as well as NE, we cannot exclude the possibility that a defective movement of Ca^{2+} either in the intracellular space or from the extracellular space may be responsible.

Tissue relaxation was investigated by examining the response of tissues to acetylcholine and SNP. In response to acetylcholine, tissues relaxed in a consistent fashion. The tissues did not contract in response to acetylcholine which would indicate that the endothelium was not damaged. There were no statistically significant differences between groups. Research has shown that plaques on the aorta have caused a decreased relaxation response to acetylcholine (67). However, the

present study does not appear to support these data. It is possible that the extent of plaque development was insufficient to induce defects in relaxation. Interestingly, when the tissues were exposed to sodium nitroprusside (SNP), a significant attenuation in the degree of relaxation was observed for the OL group in comparison to the control group. SNP acts as an external source of nitric oxide in order to induce non-endothelium dependant relaxation. The ACh response suggests that there was no defect in NO generation by the endothelium but the SNP response suggests that the relaxation defects may be attributed to a defect in intracellular Ca^{2+} handling or contractile protein function. These data would be consistent with the KCl and norepinephrine data as well.

A. Conclusions and Physiological Implications of the Data

Based on the results of this study, we can conclude that dietary hempseed supplementation has cardioprotective effects in terms of platelet activation. This is a significant finding and suggests that hempseed supplementation may be helpful for humans at risk for atherosclerosis. However, this beneficial effect is tempered by the lack of effects observed for hempseed supplementation in terms of atherogenesis and vascular contractile responses. Furthermore, it is clear that hempseed supplementation does not result in significant increases in plasma levels of unsaturated fatty acids. Only cholesterol supplementation caused a marked increase in the absorption of these fatty acids. Considering the negative cardiovascular effects of dietary cholesterol, this dietary approach could hardly be recommended in humans as a cardioprotective strategy. Alternatively, the amount of hempseed supplementation could be increased to achieve therapeutic levels of beneficial fatty acids. In the present study, the diet was supplemented with 10% hempseed. This amounts to ~13% of the caloric intake for the rabbits in the study. This percentage could be related indirectly to human diets. For example, a clinical trial that supplemented with 50g of flaxseed (13% of daily caloric intake) showed some significant improvement in the plasma lipid profile (68). Although one may consider 50g of hempseed per day to be a rather large consumption of the product, it has a relatively good, nutty taste and, if incorporated into foods in a cost efficient manner, could be comfortably ingested at these levels. However, considering the poor absorption of fatty acids from hempseed, it is unclear if even these amounts of hempseed ingestion would be sufficient to achieve a

significant increase in unsaturated fatty acid levels. In addition, the beneficial effects are tempered by the knowledge that although the unique balance of omega-6 and omega-3 fatty acids is an important reason for the benefits observed, if this ratio was increased and the diet included more omega-6 fatty acids, studies have shown negative effects (39, 47, 69).

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