# THE EFFECT OF DIETARY FLAXSEED 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 Fulfillment of the Requirements for the Degree of

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Department of Physiology

Faculty of Medicine

University of Manitoba

and the Division of Stroke and Vascular Disease

and the National Centre for Agri-food Research in Medicine

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

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## ABSTRACT

Atherosclerosis is the leading cause of ischemic heart disease. It is a chronic disease preceded by endothelial cell dysfunction and triggered by certain risk factors such as hypercholesterolemia. Omega-3 fatty acids from fish have been shown to reduce atherogenesis and improve vascular function in the presence of hypercholesterolemia. Flaxseed is a rich source of omega-3 fatty acids and lignans, which are reported to possess anti-atherogenic properties. It is unclear, however, if dietary supplementation with flaxseed would exhibit the same effect as fish oils. We hypothesized that daily dietary supplementation with flaxseed would preserve vascular function and reduce atherosclerotic plaque formation in the presence of hypercholesterolemia. We examined the effects of dietary flaxseed supplementation on aortic function and plaque formation in male New Zealand White rabbits ± a high cholesterol diet. The animals were divided into four groups based on diet: control, supplementation with 10% flaxseed, supplementation with 0.5% cholesterol, and supplementation with a mix of 0.5% cholesterol and 10% flaxseed. Rabbits were maintained on their deteary regime for 6 or 8 weeks. These durations for the dietary intervention were selected so that we would induce modest and more serve atherosclerotic plaque formation, respectively, in the cholesterol-fed rabbits. After 6 and 8 weeks of dietary intervention, the aortae were removed from the rabbits. The lumen of the aorta was exposed, digitally photographed and analyzed to quantify the percentage of the surface covered in plaque. As well, sections were stained with Oil Red O, digitally photographed and the lipid deposition was quantified. Vascular contractile function was measured with a force transducer as tension generation and relaxation in

aortic rings perfused in an organ bath. The contractile responses to 47 mM KCl and $1x10^{-9}M - 10^{-4}M$ norepinephrine were measured. The relaxation responses to $1x10^{-8}M - 10^{-5}M$ acetylcholine were also monitored.

Cholesterol supplementation of the diet for 8 weeks resulted in significant increases in plasma cholesterol concentrations. Flaxseed supplementation did not significantly reduce plasma cholesterol concentrations. Plasma triglyceride concentrations were also elevated in the cholesterol-fed animals. Again, flaxseed did not influence these increases. Supplementation of the diet with ground flaxseed alone resulted in a significant and selective increase in the concentration of alpha linoleic acid (ALA) levels (18:3) after both 6 and 8 weeks of dietary intervention. Surprisingly, cholesterol supplementation even in the absence of dietary flaxseed resulted in large increases in the plasma levels of ALA. The stimulatory effect of cholesterol on fatty acid absorption was not specific, however, to the 18:3 fatty acid species. Supplementation of the diet with cholesterol (in the presence of ground flaxseed or in its absence) dramatically altered almost the entire fatty acid profile of the blood. There were significant increases in the plasma concentrations of every fatty acid with the exception of the 12:0, 14:0, 20:1, 22:1 and 22:6 species after 8 weeks of feeding. The increase in plasma [ALA] was amplified many-fold by including flaxseed in the diet. [ALA] increased 6-8 fold in the cholesterol-flax supplemented group compared to the rabbits supplemented with cholesterol alone. Levels of ALA in tissue extracts obtained from aorta exhibited a similar qualitative increase in the four groups.

Aortae were removed from rabbits after 6 or 8 weeks of dietary intervention and contractile tension was measured in isolated organ baths. There were no significant

differences in the maximal contractile response to 47 mM KCl among the four groups in either the 6 or 8-week study. Similarly, there were no significant differences in the maximal contractile response to any concentration of norepinephrine examined among the four groups at either duration of dietary intervention. Vascular relaxation induced by acetylcholine was not significantly different in any of the groups as compared to control.

Atherogenesis was measured in the rabbit aorta and carotid arteries. There was a significant increase in the plaque formation in the aortae of both the cholesterol and cholesterol-flax fed groups after 6 and 8 weeks of feeding as compared to controls. A significant anti-atherogenic effect was observed in animals fed flax with the cholesterol. As detected by en face analysis of the tissue, significantly less plaque developed in the aortae and carotid arteries of animals fed the cholesterol-flax diet as compared to the cholesterol alone fed animals in the 8-week intervention.

After supplementing the diet with cholesterol and with ground flaxseed, we can make some important conclusions about the composition of the circulating blood, aortic contractile function and atherosclerotic plaque formation in two different vessels. Cholesterol supplementation of the rabbit diet results in a large increase in plasma cholesterol and stimulates the absorption of fatty acids from the gut. Relaxation of the aorta through an endothelial dependent mechanism is moderately impaired after supplementation of the diet with cholesterol. Flaxseed supplementation appears to attenuate this effect. Atherosclerosis is stimulated by a cholesterol-supplemented diet in both aorta and carotid arteries and this effect is strongly inhibited by including ground flaxseed in the diet. The mechanism is not entirely clear but does not involve a change in circulating cholesterol levels. Overall, we can conclude that supplementation of the diet

with flaxseed can protect the circulation from some of the deleterious effects associated with high cholesterol diets. It is suggested that ground flaxseed represents a potentially important modality to positively influence cardiovascular health.

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# I. INTRODUCTION

In the 1970's, Bang and Dyerberg observed the benefits of n-3 polyunsaturated fatty acids (PUFA) from marine fish on ischemic heart disease among the Greenland Inuits [1, 2]. They found that a high consumption of these fatty acids correlated with a low incidence of coronary occlusion and low plasma lipid levels in the Inuit people of Greenland. They also found increased bleeding times due to decreased platelet aggregation in the Inuit people as compared to the Danish people tested [1, 2]. They concluded that an increased intake of PUFA's, in particular eicosapentaenoic acid (EPA) has an anti-aggregatory effect and so reduces the risk of thrombosis and myocardial infarction. Since then, numerous studies have been attempted to determine the cardioprotective effects of these fatty acids. EPA and docosahexaenoic acid (DHA) are omega-3 fatty acid and belong to the long-chain fatty acid category of fatty acids (LCFA) that are found primarily in fish oils. An omega-3 fatty acid is a long-chain polyunsaturated fatty acid with its first double bond located at the third carbon from the methyl terminus. They have been found to be anti-arrhythmic [3], have potent anti-inflammatory effects[4] and inhibit atherogenesis[4, 5]. Atherosclerosis is a chronic disease involving the deposition of lipid in the intima of the vessel wall[6, 7]. It is the primary cause of ischemic heart disease [8], which is the leading cause of death in the industrialized world today [8]. Despite the important cardiovascular benefits that omega-3 fatty acids have proven to possess, the general public has not accepted fish oil as a common, regular dietary intervention. Its taste and aftertaste lead to long-term

compliancy problems and an alternative source of omega-3 PUFAs would be a welcome dietary supplement[9].

EPA and DHA can be synthesized endogenously in humans and animals from another omega-3 fatty acid, alpha-linolenic acid (ALA)[10]. Flaxseed, a grain common in the Canadian prairies, is the richest plant source of ALA. Approximately 50-70% of the lipid composition of flaxseed is ALA. Like EPA and DHA, it is possible that ALA may offer significant benefits in the prevention of heart disease [11]. However, this is not certain because different fatty acids are known to induce very different effects in the body. Therefore, the goal of our study was to determine if flaxseed could induce beneficial cardiovascular effects when introduced to the body in the form of a dietary supplement. Specifically, we wanted to test the capacity of flaxseed to inhibit the progression of atherosclerotic lesions and improve the contractile function in the aortae of hypercholesterolemic rabbits.

# II. REVIEW OF LITERATURE

## A. Aortic Structure and Function

### 1. Histology

The aorta is a large artery conducting blood away from the heart. It branches into smaller vessels that supply blood to all of the tissues of the body. The aorta has a relatively large diameter and thick walls as compared to smaller arteries. The human aorta has an internal diameter of 12.5 mm and a wall thickness of 2 mm [12]. The rabbit aorta is about $1/6^{th}$ the size. Arteries contain elastic, collagen and smooth muscle cells. The relative ratio of connective tissue to smooth muscle is greater in large conducting arteries like the aorta compared with smaller arteries [13]. This combination enables the aorta to be stiff and flexible. Collagen provides the vessel with tensile strength that provides the ability to withstand the high intraluminal pressures exerted by the blood after cardiac left ventricular contraction. The elasticity of the vessel allows the vessel to expand after blood has been ejected from the ventricle. This allows the vessel to act as a pressure reservoir. In this way, the aorta can store the kinetic energy of the ejected blood and transfer it slowly to the smaller branching vessels. The aorta can then recoil to its original shape acting to dampen the forces exerted by the blood on the smaller vessels. This also ensures that there is a continual flow of blood through the vasculature as the blood pressure rises and falls with each heartbeat [13].

Three distinct histological regions or layers exist within the vessel (Figure 1): the tunica intima, the tunica media, and the tunica adventitia [14]. The tunica intima is the inner most layer. The tunica media is the middle layer and the outer layer is the tunica adventitia. The boundary between these layers is created by the internal elastic lamina

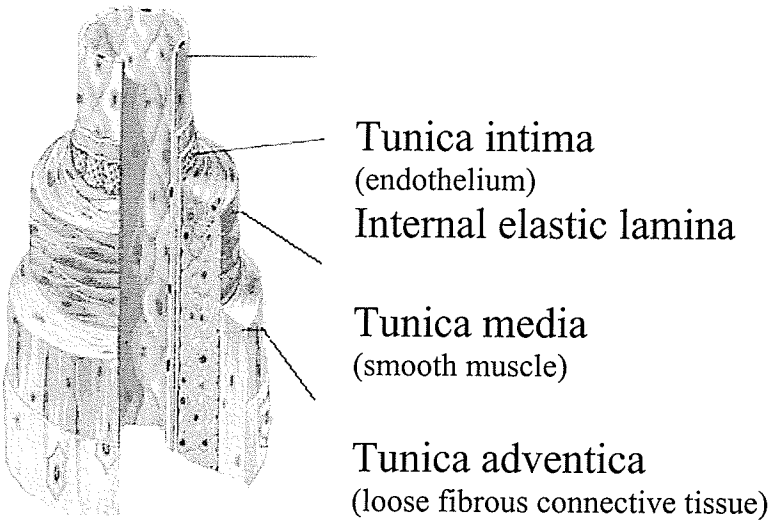


**Figure 1. The structure of the aortic vessel wall.** The innermost layer, tunica intima, contains a monolayer of endothelial cells attached to the basement membrane. This layer is separated from the middle muscular layer, tunica media by the internal elastic lamina. The middle layer contain the vascular smooth muscle cells. The external elastc lamina separates this layer from the most outer layer, the tunica adventicia. The adventicia is composed mainly of connective tissue. This layer offers protection and strength to the vessel wall and houses the nerve and blood supply to the vessel.
*Adapted from Fox, Stuart I. Human Physiology, 4th Ed. 1992, McGraw-Hill: Figure13.23*
http://www.sci.sdsu.edu/class/bio590/pictures/lect5/5.2.html

and the external elastic lamina, which are distinct layers of concentric elastic fibers separating the tunica intima from the tunica media and the tunica media from the tunica adventitia, respectively.

The tunica intima is the most medial layer of the aorta. The inner most layer of the tunica intima is the endothelium, a monolayer of cells lining the lumen of the vessel that plays a critical role in the homeostasis and function of the vessel [14]. The endothelium is attached to a basement membrane. Beneath the basement membrane is the sub-endothelial fibro-elastic space. The internal elastic lamina contains openings large enough to allow for cells, macromolecules, metabolites and growth factors to pass in either direction through the vessel wall [14].

The tunica media or muscular layer is the middle layer of the vessel. It consists predominantly of vascular smooth muscle cells, with a matrix of elastic and collagen fibers [14]. The vascular smooth muscle cells of this layer contract and relax to narrow and widen the diameter of the arterial wall. This ultimately controls lumen size and, therefore, blood flow. The matrix contains elastin and collagen fibers and functions to support and orientate the vascular smooth muscle cells. Small molecules can pass through this layer of the vessel wall to the outer most part of the media, the external elastic layer [14].

The adventitia is the external covering of the artery. This layer offers strength and protection to the artery [15]. It is predominately composed of fibroblast cells, collagen and elastin. The blood, lymph and nerve supply to the artery are found within this layer[14].

## 2. The Role of the Endothelium

Up until the 1960's, the vascular endothelium was viewed simply as a semi-permeable, non-thrombotic barrier between the blood and the interstitium [16]. In the 1970's, it was discovered that normal function of the endothelium is essential for the maintenance of vascular homeostasis [16]. In the 1980's, Furchgott and Zawadzki postulated the role of the endothelium in vascular tone. They showed the existence of a factor released by the endothelium that was responsible for vasodilation. The endothelial-derived relaxant factor, now known as nitric oxide (NO) caused acetylcholine-induced relaxation of the vascular smooth muscle cell only in the presence of an endothelium. Removal of the endothelium resulted in an acetylcholine-induced contraction of the vascular smooth muscle cells [17]. This proved that the endothelium could mediate vascular tone. Today, it is clear that the endothelium plays a pivotal role in many important regulatory processes of the vessel wall. The endothelium can release vasoactive substances and growth factors. Which can activate immune cells, smooth muscle cells and gene transcription of certain cellular proteins. It functions in the regulation of thrombosis, thrombolysis, coagulation, inflammation, lipid metabolism and the immune response. It is involved in cell signaling, vascular growth and cell migration [18].

The endothelium is able to sense changes in hemodynamic forces or blood borne signals via mechanoreceptors and ligand receptors located in the cell membrane of the endothelial cells [18]. In response to such physical or chemical stimuli, endothelial cells release substances either into the lumen or toward the vascular smooth muscle cell layer to exert important regulatory effects [18]. Such substances include the vasoactive

substances nitric oxide, endothelin, and eicosanoids such as prostacyclin and thromboxane, which also have thromboregulatory influences [19]. Other molecules include cell surface adhesion molecules like intracellular adhesion molecule (ICAM's) and vascular cell adhesion molecule (VCAM's), growth factors such as platelet derived growth factor or immune modulators such as cytokines, interleukins or tumor necrosis factor $\alpha$ [20].

### 3. Structure of Vascular Smooth Muscle Cells

Smooth muscle is the type of muscle found in the tunica media of our arteries. This muscle is responsible for the regulation of blood flow by causing contraction and relaxation of the vessel. A smooth muscle cell contains the common structural elements of all cells and a specialized arrangement of force-generating contractile components. It is named "smooth muscle" because it lacks the characteristic striations found in skeletal and cardiac muscle [13]. The organization of the contractile apparatus of smooth muscle is irregular, with the contractile units running in oblique directions throughout the cell (Figure 2a) [21]. Therefore, contraction occurs along several oblique axes (Figure 2b). The contractile apparatus of smooth muscle consists of a thin filament made up of actin and tropomyosin, and a thick filament composed of myosin [22]. A smooth muscle cell contains structures known as dense bands and dense bodies, which are attached to the cell membrane and dispersed throughout the cytoplasm respectively [13]. These dense bodies are made up of and held in place by a scaffolding of intermediate filaments comprised of the structural proteins vimentin and desmin [23]. Actin filaments are attached to these

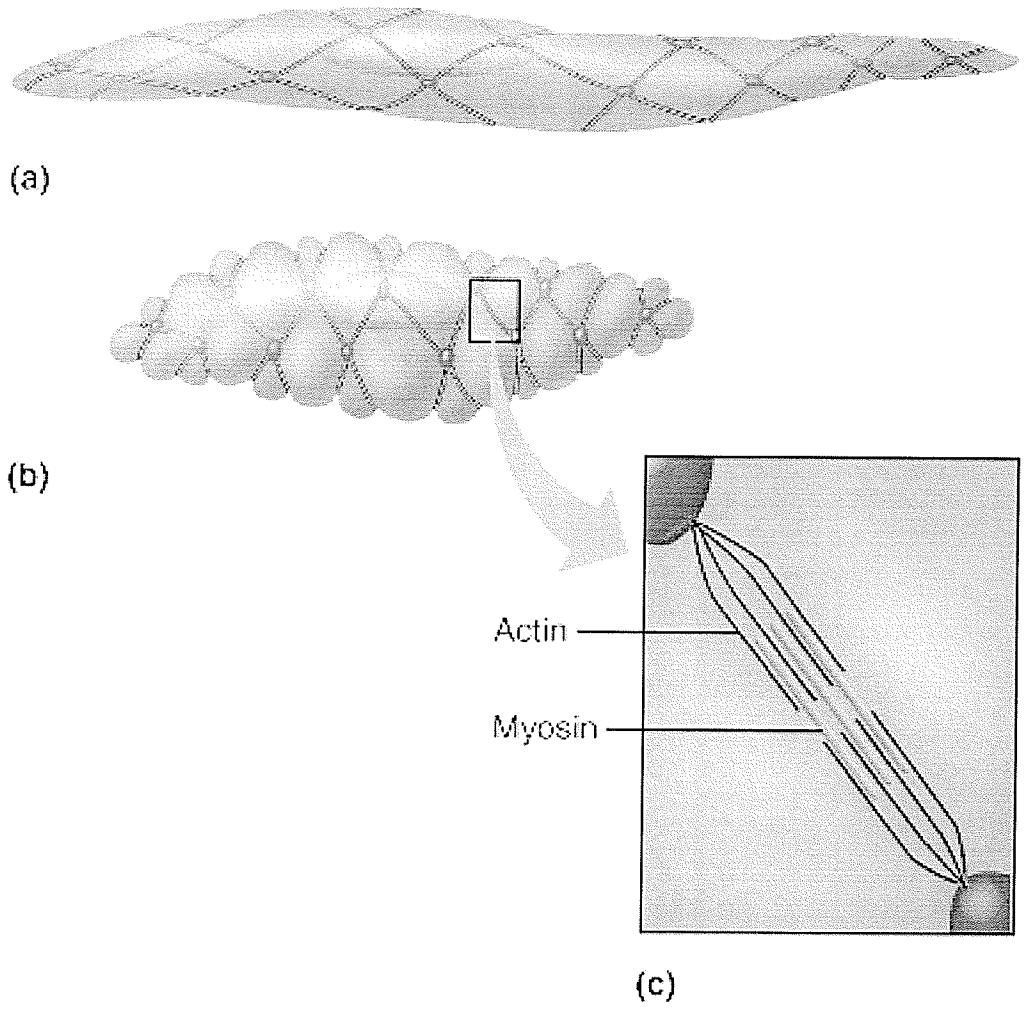


**Figure 2. The structure of a smooth muscle cell.** a) smooth muscle in a relaxed state b) contracted state c) contractile machinery of the cell: Actin and myosin filaments interdigitate at the midpoint between two dense bodies.
*2001 Benjamin Cummings, an imprint of Addison Wesely Longman Inc.*
http://www.bmb.psu.edu/courses/bisci004a/muscle/musc-img/smoocon2.jpg

dense bodies/bands. The contractile apparatus of smooth muscle is shown in Figure 2c. Actin filaments radiating from a dense body/band reach toward another group of actin filaments radiating from a second dense band/body. A bundle of myosin filaments is located midway between the two dense bodies and interdigitates with the actin filaments radiating from the two dense bodies [13]. The force of contraction, therefore, is transmitted from one dense body to another within the cell and to the dense bands attached to the cell membrane. As well, there is bonding of adjacent dense bands of one cell membrane to that of another so that the force of contraction is also transmitted between adjacent cells[13].

## B. Regulation of Vascular Smooth Muscle Cell Contraction and Relaxation

### 1. Excitation - Contraction Coupling (E-C coupling) in Vascular Smooth Muscle

Contraction of smooth muscle occurs by the sliding filament action of actin and myosin [13]. Stimulation of this cross bridge formation is induced by the release of calcium from sequestered stores in the sarcoplasmic reticulum (SR) via calcium induced calcium release or via a G-protein coupled second messenger system $IP_3$ [12]. The resting membrane potential of the cell is regulated by the permeability of four ions, $K^+$, $Cl^-$, $Na^+$, and $Ca^{2+}$. Various channels in the membrane are permeable to and regulate the passage of these ions into and out of the cell. As well, electrogenic transporters like the $Na^+/K^+$ pump and the Na/Ca exchanger also govern the movement of these ions and are important for maintaining the electrochemical gradiant across the cell membrane [13]. The voltage-operated $Ca^{2+}$ channel is the major $Ca^{2+}$-permeable channel in vascular

smooth muscle [21]. In addition, there are also intracellular calcium stores in the sarcoplasmic reticulum and the mitochondria. Calcium is released and sequestered from the cytosol via receptor-operated channels and active pumps located in the cell membrane and in the membrane of these organelles. The SR is considered to be the primary site of calcium release during E-C coupling [21].

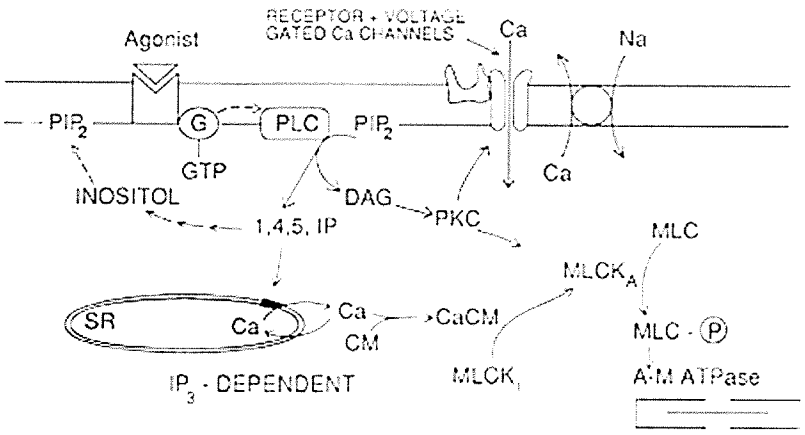


**Figure 3. Excitation-contraction coupling in smooth muscle.** Activation of a membrane receptor, a receptor-operated calcium channel or a voltage-operated calcium channel leads to signaling events resulting in an increased intracellular calcium concentration, the formation of the calcium-calmodulin comples and the activation of myosin light-chain kinase which triggers the cross bridge formation of the actin and myosin contractile proteins.
*Bers, D.M. Excitation-contraction coupling and cardiac contractile forces. 1991, Kluwer Academic Publishers.*

There are two E-C coupling mechanisms of smooth muscle (Figure 3). One involves the depolarization of the sarcolemma resulting in the opening of voltage-gated calcium channels [21]. With this, an influx of extracellular calcium ions occurs. This calcium activates the ryanodine-receptor (RyR), a calcium release channel on the surface of the SR. The SR contains an intracellular store of calcium that is released upon activation of the RyR. This is termed calcium-induced calcium release [23]. A second mechanism involves the activation of a receptor-operated channel in the cell membrane[21] The binding of an appropriate ligand to its specific receptor on the surface of the cell membrane will activate this receptor resulting in one of two outcomes [21] . This receptor can be a calcium channel itself and so activation of it will result in an influx of calcium, and calcium–induced calcium release from the SR. An additional pathway is that receptor activation would result in the triggering of signal transduction pathways [21] . A receptor binds an agonist, producing a change in the receptor. The receptor is coupled to a G-protein. This change results in the activation of the G-protein, which then activates an enzyme like, for example, phospholipase C (PLC). PLC will hydrolyse the membrane phospholipid, phosphatidylinositol, into two second messengers, inositol triphosphate ($IP_3$) and 1,2 diacylglycerol (DAG) [21]. $IP_3$ will then move to the SR and bind to the $IP_3$ specific receptors on the SR membrane, initiating the release of calcium from the SR [21]. DAG may also play a role in vascular smooth muscle contraction. DAG will increase the activity of protein kinase C. This enzyme is responsible for the phosphorylation of myosin light chain kinase and the actin binding proteins viniculin, filamin and caldesmon [23]. This phosphorylation triggers cross-bridge formation resulting in smooth muscle contraction [23]. However, the significance

of the effects of DAG are uncertain because its appearance in the membrane is very transient [23].

Upon activation of its membrane channels, the SR then releases its stored calcium into the cytosol of the cell [13]. The calcium binds with calmodulin in a 4:1 molar ratio. Calmodulin is a naturally occurring protein found in the cytosol of the smooth muscle cell. It functions in the regulation of smooth muscle contraction [12]. The conformational change from calmodulin to the calcium-calmodulin complex activates the myosin light-chain kinase. This enzyme phosphorylates the serine residue of the myosin light-chain protein molecule [23]. The phosphorylation of the myosin triggers the cross-bridge formation between the actin and myosin filaments resulting in contraction of the smooth muscle cell [22].

Relaxation of the vascular smooth muscle cell is induced by an efflux of calcium from the cytosol and by the action of another enzyme, myosin light-chain phosphatase [22]. Calcium is sequestered back into the SR and pumped out across the cell membrane by the action of the Na/Ca exchanger and by ATP dependent calcium pumps [22]. This results in the disassembly of the calcium-calmodulin complex and the deactivation of myosin light-chain kinase. As well, at this point, the myosin light-chain phosphatase is activated which removes the phosphate from the myosin ATPase and, therefore, terminates cross bridge formation [22].

## 2. Mechanisms of Humoral and Hormonal-Mediated Vasoconstriction in Vascular Smooth Muscle

Norepinephrine (NE) is a good example of a common and important vasoconstrictor that deserves additional attention. NE binds to the α1-adrenergic receptor on the surface of the vascular smooth muscle cell [19]. Phospholipase C then becomes activated, hydrolysing phosphatidylinositol into $IP_3$ and DAG. DAG activates protein kinase C. $IP_3$ will activate an $IP_3$ specific receptor on the membrane of the SR, activating release of this intracellular store of calcium into the cytosol. This increased cytosolic calcium concentration and increased protein kinase C activity is responsible for the contraction of the vascular smooth muscle cell (Figure 4) [19, 23].

The activation of voltage-operated calcium channels can be achieved by increasing the extracellular concentration of potassium ions ($K^+$) [13, 19, 23]. The resting membrane potential of the cell is near the equilibrium potential for $K^+$ [13]. The balance between the intracellular and extracellular concentrations of $K^+$, therefore, largely determines the resting membrane potential. An increased extracellular concentration of $K^+$ will reduce the leak of $K^+$ out of the cell via $K^+$ channels. This results in a long, slow depolarization of the cell [13]. As the membrane potential shifts, it will reach a level where voltage operated calcium channels (L-type and T-type calcium channels) will become activated. An influx of calcium will occur resulting in a calcium-induced calcium contraction of the vascular smooth muscle cell (Figure 4) [13, 19, 23].

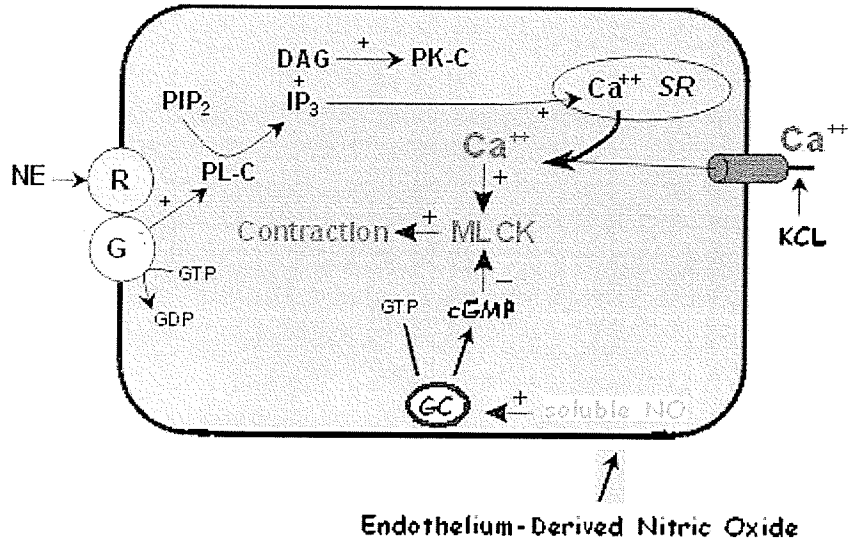


**Figure 4. Mechanisms of vasoconstriction and vasodilation in vascular smooth muscle.** Stimulation, by norepinephrine (NE), of a receptor (R) in the cell membrane coupled to a G-protein (G) leads to an increase in the activity of phospholipase C (PL-C), which splits phosphatidly inositol ($PIP_2$) into inositol trisphosphate ($IP_3$) and 1,2 diacylglycerol (DAG). $IP_3$ promotes the release of calcium from the SR. DAG activated protein kinase C (PKC). Transmembrane receptor operated or voltage operated calcium channels can also induce a contraction when stimulated by the appropriate receptor or by depolarization of the cell as would occur with an increased concentration of extracellular potassium ions (KCL). The increased intracellular calcium concentration results in the activation of the myosin light chain kinase triggering contraction of the vessel. Vasodilatory cGMP is formed by the stimulation of gyanylate cyclase (GC) in response to the diffusion of nitric oxide (NO) from adjacent endothelial cells. cGMP inhibits the action of the MLCK therefore promoting relaxation of the vessel.
*Adapted from* © 1999-2002 Richard E. Klabunde
*http://www.oucom.ohiou.edu/cvphysiology/BP011_sig_trans.gif*

### 3. Mechanisms of Vasodilation in Vascular Smooth Muscle

Most agents that cause vasodilation of vascular smooth muscle cells act via the formation of cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP) [19]. For example, nitric oxide (NO), an endogenously produced vasodilator, activates the enzyme guanylate cyclase resulting in the production of cGMP. cGMP causes vasodilation by inhibiting the myosin light-chain kinase enzyme and the L-type calcium channels. On the contrary, adenosine, β2-adrenergic stimulation or prostacyclin will activate adenylate cyclase resulting in the production of cAMP. cAMP may inhibit myosin light-chain kinase and stimulate $Ca^{2+}$ pumps to remove calcium from the cell resulting in relaxation of the vascular smooth muscle cell (Figure 4) [19].

### 4. Nitric Oxide: A Key Player in the Regulation of the Vessel Wall

NO acts on platelets, monocytes, and vascular smooth muscle cells and, therefore, is a key player in the maintenance of the normal health as well as the pathology of arteries [18]. NO is synthesized in the endothelial cell from the amino acid L-arginine upon activation of the endothelial cell by the appropriate stimulus [19]. NO release can be triggered by the binding of ACh, bradykinin or thrombin to a receptor on the surface of the cell. These mechanoreceptors are sensitive to stretch or pressure [24]. Therefore, the production of NO can be triggered by changes in the shear force of blood flow, which activates these mechanoreceptors in the membrane of endothelial cells.

NO is a gas and, therefore, it can diffuse immediately through the membrane of a cell. Its action is very local because of its very short half-life. Once produced, NO

diffuses from the endothelial cell through the internal elastic lamina to the adjacent vascular smooth muscle cell of the tunica media [19]. Once inside the vascular smooth muscle cell, NO activates guanylate cyclase triggering the synthesis of cGMP. cGMP is a second messenger that is believed to activate a cGMP dependent protein kinase. This protein kinase acts to decrease intracellular calcium thereby affecting relaxation of the vascular smooth muscle cell [19]. NO functions not only to cause local dilation in the vessel and inhibit vasoconstriction but it also inhibits platelet aggregation and adherence, vascular smooth muscle cell proliferation, and endothelial-leukocyte interaction [18, 24]. Therefore, a reduction in arterial NO may predispose the vessel to the processes of atherogenesis [24]. Reduced NO production will limit vasodilation, augment leukocyte and platelet adhesion to the vessel wall, induce abnormal vasoconstriction and vascular smooth muscle cell proliferation[18].

## C. Pathogenesis of Atherosclerosis

### 1. Theories of Atherogenesis

Atherosclerosis is one type of cardiovascular disease. It is the principal contributor to the incidence of myocardial infarction, cerebral infarction and peripheral vascular disease [7]. It is a chronic disease involving the deposition of layers of lipid-laden macrophages, T-lymphocytes, smooth muscle cells and fibrous connective tissue in the intima of the vessel wall [7]. Atherosclerosis is preceded by endothelial cell dysfunction and triggered by certain risk factors such hypercholesterolemia and hyperlipidemia [25].

Theories of the pathogenesis of atherosclerosis began with Virchow in the 1800's. There is still controversy about the mechanisms involved. Prominent theories include the lipid infiltration theory, the response to injury hypothesis, fibrin encrustation and monotypic origin. The lipid infiltration theory and the response to injury hypothesis are the two most prominent theories [26].

The response to injury theory was first intoduced by Virchow (1856) and von Rokitansky (1852) and later modified by French (1966) and Ross (1973, 1986). This theory argues that an endothelial lesion would trigger an inflammatory response resulting in the cellular interactions leading to atherosclerotic plaque formation. The response to injury theory (Figure 5) is now better understood and it is accepted that physical damage to the endothelium such as denudation or necrosis is not required to initiate this process [6]. Endothelial dysfunction alone is now believed to be enough to initiate the atherosclerotic process [20]. Activation of the endothelium is triggered by risk factors that alter the normal environment surrounding endothelial cells resulting in abnormal cellular processes that initiate atherosclerosis [20]. Risk factors include both primary and secondary factors. Primary risk factors include elevated serum levels of cholesterol, triglycerides and low-density lipoprotein, reduced serum high-density lipoprotein, diabetes mellitus, hypertension and cigarette smoking. Secondary risk factors include obesity, age, genetics, sex, diet, lack of physical exercise, mental stress, and personality type [13, 26-28]. These risk factors contribute to the development of atherosclerosis by increasing circulating cholesterol, free radicals, immune complexes and catecholamines, and by altering the shear forces of blood flow or causing hypoxia [25]. All of these factors ultimately alter the normal functioning of the endothelium.

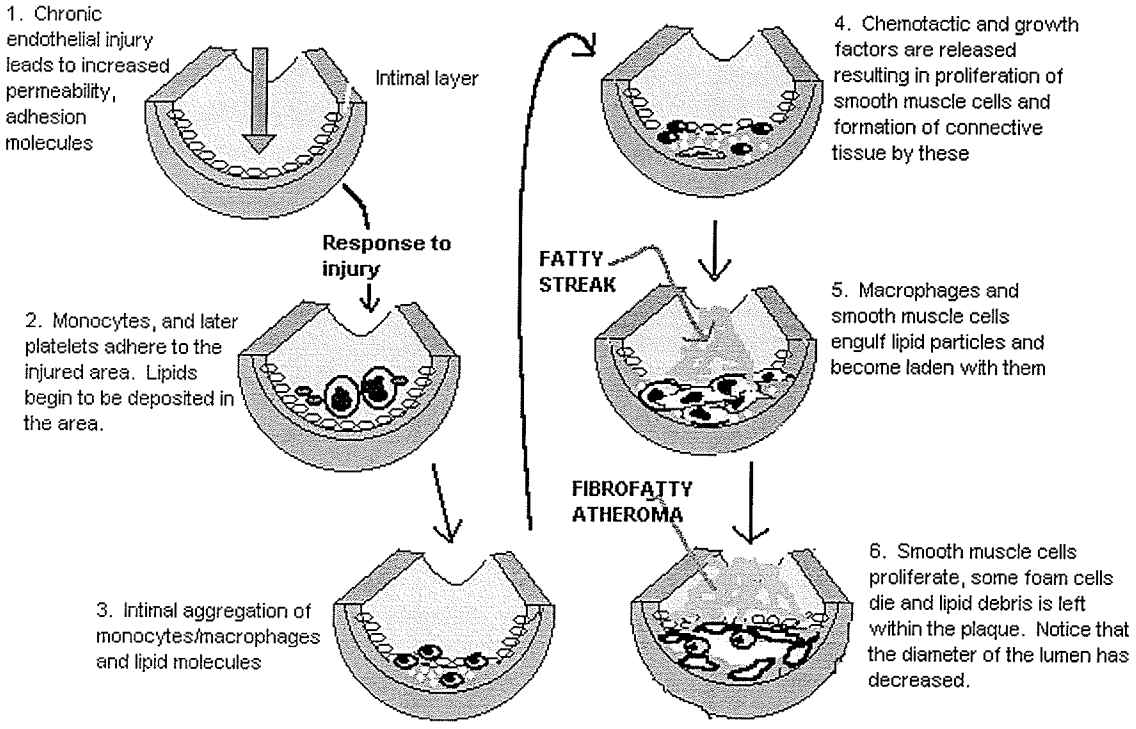


**Figure 5. The response to injury theory of atherogenesis.**
http://hsc.usf.edu/CLASS/jennifer/atherogenesis.JPG

The lipid hypothesis theory simply states that elevated levels of circulating serum cholesterol, specifically low-density lipoprotein (LDL) can promote atherogenesis by accumulating in endothelial cells and triggering endothelial dysfunction [26]. The oxidation of LDL can initiate the atherosclerotic process. Oxidized LDL (OxLDL) has cytotoxic and chemotactic properties and induces cell proliferation, which may initiate and propagate the cellular processes of atherogenesis [26, 29]. OxLDL is more readily absorbed than native LDL through its own receptor pathway, the scavenger receptor of the macrophages and vascular smooth muscle cells. The resultant excessive lipid accumulation in these cells promotes plaque formation [26].

The development of fatty streaks is the earliest stage of atherogenesis [6]. A fatty streak is an aggregation of foam cells and vascular smooth muscle cell in the intima of the artery. In a healthy vessel, the endothelium plays a key role in inhibiting this process by inhibiting coagulation, inflammation and promoting vasodilation through the release of vasoactive substances, such as NO and prostacyclin [6, 19, 20, 25]. These vasoactive substances act to maintain an appropriate vessel tone and to inhibit key processes in atherosclerosis [6, 19, 20, 25, 26].

Healthy endothelial cells do not express (or do not express in excess) adhesion molecules, growth factors, chemotactic molecules and cytokines. Examples include vascular cell adhesion molecule-1 (VCAM-1), intracellular cell adhesion molecule-1 (ICAM-1), fibroblast growth factor (FGF), platelet derived growth factor (PDGF), tumor necrosis factor alpha (TNF$\alpha$), or interleukin-1 (IL-1) [25]. In situations of abnormal mechanical, chemical, viral or immunological stimuli, the response of the endothelium is altered. This is what occurs in the response to injury theory or the lipid infiltration

theory. The endothelium response is disrupted, resulting in a decrease in the production and release of NO and prostacyclin, and an increase in the expression of substances that promote vasoconstriction, coagulation, platelet aggregation and adherence of leukocytes. The passive permeability properties of the endothelium are also increased, growth factors are liberated, and proliferation of smooth muscle cells is amplified [25]. This altered endothelial function results in the attraction and adhesion of leukocytes to the vascular wall. Ultimately, these cells will penetrate into the subendothelial space. Monocytes will engulf oxLDL and become macrophages and access the sub-endothelial space as well. Vascular smooth muscle cells and lymphocytes migrate and proliferate along with the foam cells surrounded by an excess of extracellular matrix and glycoproteins. Ultimately, this will result in the formation of a fatty streak in the intima of the artery [20, 25]. Progression of the lesion occurs with smooth muscle cells laying down a connective tissue fibrous cap of elastin, collagen and proteoglycans over the lesion and continued proliferation of the smooth muscle cells and macrophages until it develops into a more advanced atheromatous plaque [6]. As the lesion progresses to an advanced lesion, it can penetrate back through the endothelium into the lumen causing narrowing of the vessel lumen, ischemia and a risk of thrombolysis [6].

The lesions of atherosclerosis are prone to develop in conduit arteries at the bends, bifurcates and branches of the vessel [24]. This is due to the turbulent flow of blood that occurs at these points in the vessel. This increases the likelihood of endothelial activation or damage by increasing the stress on the endothelium at the point of turbulent flow and increasing the residence time of blood at this particular point in the vessel. This increases the contact time of lipoproteins and monocytes with the vessel

wall [24]. Adhesion of circulating leukocytes to the vascular endothelium will then occur to initiate the atherosclerotic process.

## D. Fatty Acids

### 1. Saturated, Unsaturated and Polyunsaturated Fatty Acids

A fatty acid is a hydrocarbon chain of 4-36 carbon atoms in length with a carboxyl terminus and a methyl terminus. Fatty acids differ in the chain length and the number and arrangement of double bonds within the chain [10]. Fatty acids are found embedded in cell membranes of all cells as components within phospholipids and glycolipids [30]. Fatty acids also circulate in our blood within lipoproteins, as triglycerides or cholesterol esters [31]. Fatty acids can be divided into three classes – saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) (Figure 6) [31]. The difference between saturated and unsaturated fatty acids is the presence of a double bond in the hydrocarbon chain. SFA have no double bonds while MUFA have one double bond and PUFA have greater than one double bond. The recommended daily fat intake is 30% of the daily caloric intake, with equal amounts of SFA, MUFA and PUFA [32].

### 2. Polyunsturated Fatty Acids: Structure and Function in the Body

PUFA can be further subdivided into non-essential and essential fatty acid. The non-essential PUFA include the n-9 and n-7 families of PUFA. These fatty acids can be synthesized in the body from MUFA precursors. Essential fatty acids, the n-3 and n-6

families of PUFA are termed so because they cannot be synthesized endogenously [32]. The location of the first double bond from the methyl terminus is what distinguishes these molecules from one another. For example, the n-9 families of PUFA have their first double bond located between the $9^{th}$ and $10^{th}$ carbon atoms from the methyl terminus. N-6 PUFAs have their first double bond between the $6^{th}$ and $7^{th}$ carbon atoms from the methyl terminus. The human body does not contain the enzymes necessary to insert a double bond in the hydrocarbon chain at a point closer to the methyl terminus than between the $9^{th}$ and $10^{th}$ carbon atoms [10]. Therefore, n-6 and n-3 PUFAs must be consumed in our diets. These two families of essential fatty acids are commonly referred to as omega-6 and omega-3 fatty acids. Omega-6 fatty acids are generally found only in vegetable fats while omega-3 fatty acids are found in fish oils and plant oils.

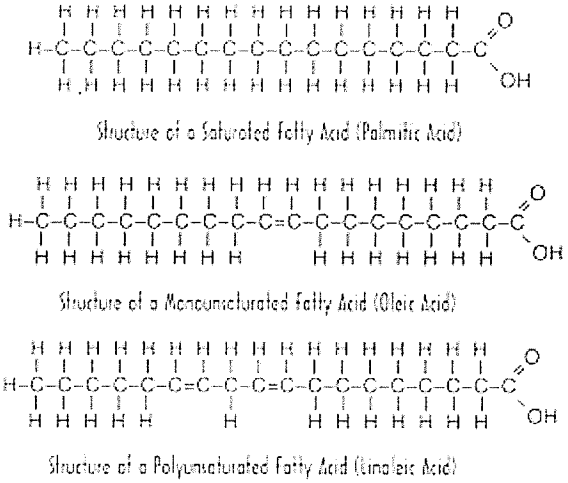


**Figure 6. Structure of a saturated, monounsaturated and polyunsaturated fatty acid**
*www.acsh.org/parentspage/ booklets/fattyacids*

Figure 7 shows the two families of essential fatty acids. The two parent fatty acids are linoleic acid (LA, C18:2 n-6) and alpha linolenic acid (ALA, C18:3 n-3). Once consumed, our body can alter these fatty acids by a process of desaturation and elongation through a series of specific enzymes. LA can be metabolized to arachidonic acid (AA, C20:4 n-6) and ALA is metabolized to eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic (DHA, C22:6 n-3). The same enzymes, elongase and Δ4-, Δ5-, Δ6-desaturase are utilized in the metabolism of LA and ALA to their longer chain daughters [10]. Therefore, these two families of PUFA compete for the same enzymes, especially the rate-limiting enzyme Δ6-desaturase, in their metabolism.

ALA is the chief fatty acid found in flaxseed and EPA and DHA are the principal fatty acids found in marine fish oils. LA is the primary fatty acid found in canola oil. The order of preference of the Δ6-desaturase enzyme for metabolism of these fatty acids is the n-3 family > n-6 family [10]. Therefore, the recommended ratio of n-6 to n-3 PUFA in the diet is at least 4:1 [33].

Ingestion of PUFA results in the distribution of PUFAs to virtually every cell in the body [30]. High concentrations of PUFA are found as membrane phospholipids. AA (20:4 n6), EPA (20:5 n3) and DHA (22:6 n3) are the most common PUFAs found in cell membranes as phospholipids [10]. Upon the release and metabolism of these PUFAs from the cell membrane, their metabolites play a role in intracellular and extracellular cell-signaling processes. AA and EPA upon release from the bilayer can be transformed into the intracellular metabolites $IP_3$, DAG, or the extracellular metabolites platelet-activating factor or eicosanoids. Therefore, the relative ratio of these fatty acids in our cell membranes influences cellular processes. This balance of n-6 and n-3 PUFA in the

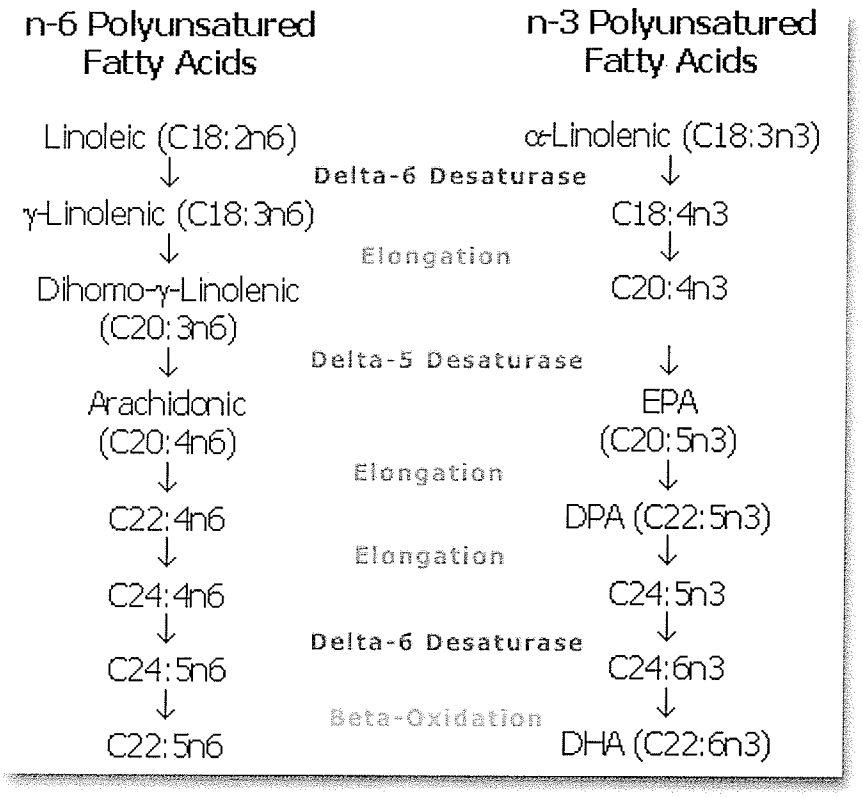


**Figure 7. Metabolism of n-6 and n-3 fatty acids by desaturase and elongase enzymes.**
*http://www.agsci.ubc.ca/courses/fnh/301/lipid/PUFA.gif*

cell is strongly influenced by diet. Excess intake of one kind of PUFA over another results in a greater phospholipid fraction of that fatty acid in the cell membrane. As well, each family interferes with the metabolism of the other when in competition for the desaturase and elongase enzymes. Therefore, different cell-signaling events can be affected by the PUFA consumption of our diet [30].

**E. Eicosanoids**

Eicosanoids are fatty acid cell-signaling molecules derived from long-chain n-3 and n-6 PUFA. They are synthesized in platelet and endothelial cells from EPA, or AA found in the phospholipids of the cell membrane. Eicosanoids play an important role in vascular tone, platelet aggregation and thrombogenesis. The generation of eicosanoids is thought to occur through the following sequence of events. First, the fatty acid is released from the membrane phospholipid by the action of phospholipase $A_2$. Once released, the fatty acid is a substrate for one of two enzymatic pathways, (Figure 8) the cyclooxygenase pathway or the lipoxygenase pathway. The cyclooxygenase pathway gives rise to prostanoids and thromboxanes while the lipooxygenase pathway gives rise to leukotrienes. Thromboxanes and prostanoids modulate thrombosis, inflammation and chemotaxic responses whereas leukotrienes modulate vascular permeability and vascular and bronchial constriction [30]. The two families of PUFAs (n-3 or n-6) yield eicosanoids with different biological activity. In general, eicosanoids formed from long-chain n-6 PUFA stimulate platelet aggregation, are pro-inflammatory, strongly promote chemotaxis of white blood cells and promote vasoconstriction of blood vessels.

Eicosanoids derived from the n-3 PUFAs are less biologically active than those derived for n-6 PUFA [30]. These eicosanoids have anti-thrombotic and vasodilatory effects, and are relatively weak promoters of white blood cell chemotaxis and inflammation [10].

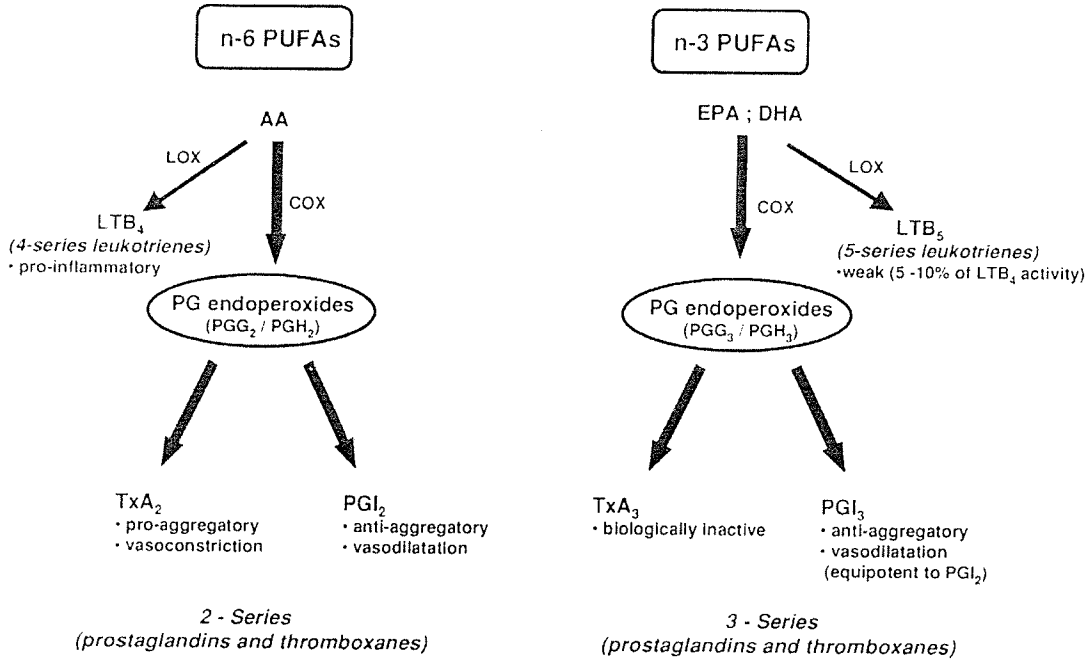


**Figure 8. Eicosanoids derived from n-3 and n-6 polyunsaturated fatty acids.** The n-3 and n-6 polyunsaturated fatty acid families produce different groups of eicosanoids with varied biological potencies. AA, arachidonic acid; EPA, eicosapentaenoia acid; DHA, decosahexaenoic acid; COX, cyclooxygenase; LOX, lipoxegenase; LT, leukotriene; PG prostaglandin; Tx, thromboxane.
*Abeywardena, M.Y. and R.J. Head, Longchain n-3 polyunsaturated fatty acids and blood vessel function. Cardiovasc Res, 2001. 52(3): p. 361-71.*

Once produced, the eicosanoids are released from the cell and act in an autocrine or paracrine fashion on cell surface receptors linked to G-proteins [30]. Changes in the ratio of n-6 and n-3 fatty acids in the cell membrane (which is influenced by diet) will affect eicosanoid production and, therefore, modify eicosanoid mediated functions such as platelet aggregation, inflammation, vascular tone and chemotaxis.

## F. Cardioprotective Effects of Omega-3 Fatty Acids

Saturated fatty acids have been known to have negative effects on the development of cardiovascular disease. These fats increase low density lipoprotein, decrease high density lipoprotein, and increase circulating blood lipids. These effects will promote atherogenesis and hypertension, two primary determinants of coronary artery disease. Omega-3 fatty acids, however, have distinctly different effects on the evolution of cardiovascular disease. They are thought to be cardioprotective. The vast majority of the information in this regard has been produced through extensive study of marine fish oils. Table 1 summarizes the cardioprotective benefits of EPA and DHA.

### 1. Fish Oils: EPA and DHA

It is now widely accepted that omega-3 fatty acids from marine fish, namely EPA and DHA are cardioprotective. Consumption of omega-3 EPA and DHA by the Greenland Eskimos showed an inverse relationship of n-3 fatty acid consumption and the incidence of coronary artery disease [2, 4, 34-36]. These fatty acids have proven to be hypolipidemic, anti-inflammatory, anti-thrombotic, anti-arrhythmic, reduce blood

**Table 1. Cardioprotective Mechanisms of EPA and DHA**

- Reduced ventricular arrhythmias
- Antithrombotic effects (ie reduced blood platelet reactivity, moderately longer bleeding times, reduced plasma viscosity)
- Lipid lowering (reduced fasting TAG and VLDL levels with moderate rises in HDL cholesterol, attenuation of postprandial TAG response)
- Improved endothelial relaxation (via enhancement of nitric oxide-dependant and nitric oxide-independent vasodilation)
- Inhibition of atherosclerosis and inflammation (via inhibition of smooth muscle proliferation, altered eicosanoid synthesis, reduced expression of cell adhesion molecules)
- Suppressed production of inflammatory cytokines (interleukins, tumor necrosis factor) and mitogens

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

pressure from a hypertensive state and can be anti-atherogenic [4, 35, 36]. Most of the vascular benefits of n-3 PUFAs are mediated at the vessel wall where they affect cellular function by influencing cell signaling events, ion channels, eicosanoid production, NO release, and lipid metabolism.

The anti-atherogenic effects of fish oils observed in numerous studies are due in part to their effect on plasma lipids and lipoproteins. N-3 fatty acid consumption reduced plasma total cholesterol, VLDL cholesterol, HDL cholesterol, total triacylglycerol and VLDL triacylglycerol concentrations [37]. This is achieved through a number of mechanisms. For example, consumption of fish oil inhibits the synthesis of triacylglycerol and VLDL in the liver. In additon, fish oils greatly reduce the

postprandial lipemia associated with high fat intake. Postprandial lipemia and the associated lipoproteins are known to be atherogenic and thrombotic [37].

The mechanism whereby n-3 fatty acids are cardioprotective extends beyond effects on circulating lipids. First, a diet low in SFA and supplemented with n-3 fatty acids resulted in the most favorable outcome on platelet function and platelet vascular interactions [37]. The increased accumulation of n-3 fatty acids in platelets reduced platelet adhesiveness and aggregation [4]. Secondly, because PUFA are more susceptible to oxidation, they may act as a sink to scavenge free radicals [37]. Thirdly, the pathogenesis of atherosclerosis is strongly influenced by the action of growth factors and cytokines. DHA has been shown to reduce the expression of adhesion molecules and pro-inflammatory cytokines [38]. N-3 fatty acids also act to reduce the growth of the atherosclerotic plaque. They do so by; inhibiting vascular smooth muscle cell proliferation, affecting signaling pathways thereby inhibiting monocyte migration, and by reducing the production and release of platelet derived growth factor [37]. EPA and DHA may also bring about their cardio-protective effects through their action on eicosanoid production. EPA inhibits the synthesis of AA derived eicosanoids such as thromboxane $A_2$, which promotes platelet aggregation, vasoconstriction, inflammation and thrombosis [4, 39]. Conversely EPA enhances the production of prostacyclin, a potent vasodilator [39].

Another significant action of n-3 fatty acids is their ability to attenuate endothelial dysfunction, a predetermining event in the pathology of vascular disease. Studies have shown that increasing the number of double bonds in the cell membrane can inhibit the activation of the endothelium that occurs in the early stages of atherogenesis [25]. As

well, n-3 fatty acids enhance the action of nitric oxide, an important regulator of vascular function [4, 37]. The reduced production of NO that is associated with endothelial dysfunction is limited by pre-treatment with DHA [25]. In addition, n-3 PUFA have been shown to antagonize n-6 derived eicosanoid specific receptors and to inhibit the release of vasoconstricting agents such as endothelins from the enodothelium [25].

**2. Flaxseed: ALA**

ALA is found in flaxseed, canola and soybeans. Flaxseed is a grain that contains the richest known plant source of ALA (Flax Council of Canada). The whole seed contains 41% fat, 28% fibre (both soluble and insoluble) and 21% protein as well as vitamins, minerals, some carbohydrates and lignans (Flax Council of Canada). Flaxseed oil is 73% PUFA, 18% MUFA and 9% SFA. 56% of the PUFA found in flaxseed has been shown to have beneficial effects on the secondary prevention of heart disease, in reducing the incidence of sudden death from cardiac arrhythmias and in the treatment of certain forms of cancer and inflammatory disorders (Flax Council of Canada). ALA can be converted to EPA and DHA and so flaxseed may have its effects via the same mechanisms as these fatty acids [39].

Prasad et al conducted three studies comparing the effect of three sources of flaxseed on hypercholesterolemic rabbits [11, 40, 41]. They tested the effects of type-1 flaxseed (containing a high ALA content and lignans), type-2 CDC-flaxseed (which has the same lignan and oil content but with a lower ALA concentration), and secoisolaricitesinol (SDG) (a lignan isolated form of flaxseed) on the development of hypercholesteremic atherosclerosis and on serum lipid and cholesterol levels in rabbits.

Their results showed that type-1 flaxseed reduced plaque formation by 46%, while the type-2 CDC-flaxseed reduced atherosclerosis by 69% and, SDG reduced it by 73%[11, 40, 41]. The findings on serum lipid levels showed type-1 flaxseed to have no significant effect on total serum cholesterol. The type-2 CDC flaxseed reduced total cholesterol and LDL-C and the SDG flaxseed reduced total and LDL cholesterol as well as increased HDL-C. The conclusion from these studies was that the beneficial effects of flaxseed on hypercholesterolemic atherosclerosis are due to the lignans in the flaxseed.

However, the mechanism responsible for the beneficial effects of flaxseed may be more complex those originally put forth by Prasad [40,41]. A study conducted by de Lorgerill et al showed that ALA was more effective than LA at reducing the incidence of death in patients who have had a myocardial infarction[42]. The mechanism for this was not elucidated but it was thought to be due to a reduction in thrombogenesis.

It appears that flaxseed may have many physiological effects. Flaxseed may influence the progression of atherosclerosis by: i) its antioxidant effect; ii) by positively affecting endothelial derived NO synthesis and thereby improving endothelial-dependent relaxation; iii) by inhibiting the production of pro-inflammatory cytokines and eicosanoids derived from arachidonic acid, and/or, iv) by lowering plasma lipid levels.

We hypothesize a dietary supplementation of ground flaxseed would exhibit beneficial effects on the contractile characteristics and atherosclerotic plaque formation in the aortic vessel of rabbits fed a high cholesterol diet. We would expect an improved endothelial dependant relaxation in response to acetylcholine and reduced plaque formation in the vessels of animals fed a diet high in cholesterol and supplemented with

ground flaxseed high in ALA in comparison to those rabbits fed cholesterol and not supplemented with flaxseed.

# III. MATERIALS AND METHODS

## 1. MATERIALS

| Product | Source |
|---|---|
| New Zealand White Male Rabbits | Southern Rose Rabbitry Farm (St. Claude, MB) |
| Rabbit Chow | Federal Co-operatives Ltd. (Saskatoon SK) |
| Flaxseed | Polar Foods Inc. (Fisher Branch, MB) |
| Cholesterol | Federal Co-operatives Ltd. (Saskatoon SK) |
| Coffee Grinder | Braun |
| Chloroform Methanol | Fisher Scientific (Nepean, ON) |
| Methanol Benzene | Fisher Scientific (Nepean, ON) |
| Sodium Sulfate | Sigma-Aldrich Canada Ltd. (Oakville, ON) |
| Acetyl Chloride | Fluka |
| EDTA Vacutainer Tubes | Becton Dickinson (Oakville, ON) |
| Heparin Vacutainer Tubes | Becton Dickinson (Oakville, ON) |
| Vet Test 8008 Blood Chemistry Analyzer | (IDEXX Laboratories Inc., ME, USA) |
| Isolated Tissue Bath System | Experimentria Ltd. (Budapest, Hungary) |
| S.P.E.L. Software | Experimentria Ltd. (Budapest, Hungary) |
| Norepinephrine | Sigma-Aldrich Canada Ltd. (Oakville, ON) |
| Acetylcholine | Sigma-Aldrich Canada Ltd. (Oakville, ON) |
| Sodium Chloride | Sigma-Aldrich Canada Ltd. (Oakville, ON) |

| Product | Source |
|---|---|
| Potassium Chloride | Sigma-Aldrich Canada Ltd. (Oakville, ON) |
| Sodium Bicarbonate | Sigma-Aldrich Canada Ltd. (Oakville, ON) |
| Magnesium Sulphate | 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 | Sigma-Aldrich Canada Ltd. (Oakville, ON) |
| Paraformaldehyde | Fisher Scientific (Nepean, ON) |
| PBS | Sigma-Aldrich Canada Ltd. (Oakville, ON) |
| HCL | Sigma-Aldrich Canada Ltd. (Oakville, ON) |
| Ethanol | Fisher Scientific (Nepean, ON) |
| Heparin | Sigma-Aldrich Canada Ltd. (Oakville, ON) |

## 2. METHODS

### 1. Animal Groups and Dietary Interventions

Male albino New Zealand white rabbits were used in all experiments. Each rabbit weighed between 2.5-3 kg upon arrival to the Central Animal Care Facility at St. Boniface Hospital Research Centre. The animals were left for one week after delivery for observation before the nutritional intervention began. Two durations were chosen for the nutritional intervention work. In the first, animals were fed for 6 weeks and in the subsequent study, they were fed for 8 weeks. These durations for the dietary interventions were chosen because they induce modest and more severe atherosclerotic plaque formation, respectively, in the New Zealand White rabbits when the rabbits are placed on a high cholesterol diet. Each study contained 32 male New Zealand white rabbits. The animals were randomly divided into 4 groups of 8 animals and assigned to a control or experimental diet. The four different diets included a control diet (RG) of regular rabbit chow (CO-OP Complete Rabbit Ration, Federal Co-operatives Limited Saskatoon SK, Canada), the same diet supplemented with 10% ground flaxseed (Braun coffee grinder) (FX) by weight, (wt/wt, ALA compromises 70% of the total fatty acids, Promega Flax from Polar foods Inc., Fisher Branch, MB, Canada), or supplemented with 0.5% cholesterol (CH) by weight (CO-OP Complete Rabbit Ration, Federal Co-operatives Limited Saskatoon SK, Canada), or a diet supplemented with 0.5% cholesterol and 10% ground flaxseed (CF) by weight. The rabbits were fed 125 grams of their diet per day. This amount was determined based on the recommended rations to meet nutritional requirements. Food intake was limited because it was determined in a pilot

study that rabbits ate excessive amounts of the flaxseed supplemented diet. The daily 125 g ration of RG, FX, CH and CF supplemented diets did not significantly differ in their caloric content, containing 423, 445, 421, 450 calories, respectively.

The diets were made by grinding the required amounts of all components, regular chow, cholesterol and flax, mixing them, adding water and re-pelleting the diet by running it through a meat grinder. The diets were fan-dried and stored in a 4° C refrigerator in containers to protect them from light.

**2.Diet Analysis**

The diets were analyzed for percent fatty acid content with a Vrian GC (3800)/MS (2000) CP-sil 88 gas chromatograph using methods developed by Folch et al and Lepage et al [43,44]. All glassware used during the process was rinsed with 2:1 $CHCl_3$:MeOH prior to use (i.e., graduated cylinder, small glass beaker(s), separatory funnel(s), round-bottom flasks, Erlenmeyer flasks, filter funnels, graduated cylinders and scoopula). The first step was to prepare a 1g sample of each diet mixed with 40mL of 2:1 $CHCl_3$:MeOH. A sample of each diet was taken and ground using a clean food grinder (Braun food grinder). A Kim Wipe dampened with a small volume of 2:1 $CHCl_3$:MeOH was used to wipe out the metal part of the grinder prior to use. This was allowed to dry before the food was added. A 1g sample from each ground diet was taken for analysis. The 1g sample was carefully poured into a separatory funnel. 40 mL of 2:1 $CHCl_3$:MeOH was measured into a 50 mL graduated cylinder. Using a Pasteur pipette, 10 mL of this was transferred to the beaker containing the initial 1g sample of food to rinse down the sides of the beaker transferring any adhered food to the bottom of the

beaker. This solution was then swirled and then in one motion, its contents were transferred to the separatory funnel. This was repeated until all the solvent was used up. Therefore, the total volume of 2:1 $CHCl_3$:MeOH in the separatory funnel is 40 mL. The next step is the extraction of lipids from each diet sample. 8.4 mL of NaCl solution (0.73% or 0.2V) was added to the separatory funnel using a serological pipette. It is important to prepare a 500 mL stock solution of 0.73% NaCl solution (w/v; g/ml) using 3.65 g of NaCl in 500 mL of $H_2O$. The separatory funnel was then shaken vigorously and allowed to vent. The solution was then left for 12 hours or overnight in order to separate the 2 layers. The MeOH generates a new phase that accepts hydrophilic molecules only letting hydrophobic molecules into the $CHCl_3$ layer. The $CHCl_3$ fraction was collected into an Erlenmeyer flask. The food was then re-extracted in the separatory funnel using 40 mL of 2:1 mixture. The mixture was left and allowed to separate out overnight. Using a scoopula, some $Na_2SO_4$ (sodium sulfate) was added to the $CHCl_3$ solution obtained in the Erlenmeyer flask. This will remove any water that may have been transferred into the flask during separation. The solution was then swirled and if necessary more $Na_2SO_4$ was added until all of the water was gone. The solution was then filtered into a pre-weighed 250 mL round bottomed flask. Caution was used to ensure the glassware was not touched with bare hands as finger-grease will alter the true weight of the round bottomed flask. The original flask was rinsed using 10 mL of $CHCl_3$ and transferred to the filter funnel. This was then combined with the original filtrate and the process was repeated two more times. The solvent was removed using rotary evaporation (the bath temperature was set to 40 °C). Once the solvent was removed, the round bottomed flask was purged with $N_2$ (g) to evaporate any remaining solvent. It is

important to use $N_2$ (g) not $O_2$ (g) as this will oxidize the lipids. Once the round bottomed flask returned to room temperature, it was reweighed. This provided the mass of the round bottomed flask with the total lipids. This value was recorded. Next, the lipids were solubilized using 2.0 mL of benzene measured out via a Hamilton syringe. The lipids were transferred using a Pasteur pipette into a glass vial. This was repeat to complete the transfer of all the lipids. Therefore, the total volume of benzene is 4.0 mL. The vial was capped with a Teflon lid, which is solvent resistant and sealed with Parafilm. The samples were then stored at –20 °C for up to 6 months. The following calculation was used to determine the percent total lipid content. First we subtracted the empty round bottomed flask weight from the full weight in order to get the total lipid amount (g). Next we compared the mass of lipids to that of the original sample mass in order to get the percent of total lipids. All diets were extracted and analyzed in triplicate.

In addition, a sample of each diet was sent to the Northwest Labs (Lethbridge, AB.) for analysis of crude protein, carbohydrate, fat, fiber, ash and digestible energy content.

**3. Blood Sampling and Analysis**

After 0, 6 or 8 weeks of dietary intervention, a blood sample was taken from the left or right marginal ear vein prior to daily feeding. The sample was collected in vacutainer tubes containing EDTA (Becton Dickinson, Oakville, ON, Canada). The samples were centrifuged at 4500 x *g* for 10 minutes at room temperature. The plasma was pipetted and stored in Eppendorf tubes at –80 ° C. For total blood cholesterol and triglyceride measurements, the plasma samples were removed from the –80° C freezer,

allowed to thaw and centrifuged at 6800 x *g*. Plasma levels of cholesterol and triglycerides were analyzed using the VetTest 8008 blood chemistry analyzer (IDEXX Laboratories Inc., Westbrook, ME, USA). The chemistry test slides that were used for the analysis were brought to room temperature before use.

The fatty acids were extracted from the plasma sample and derivatized using the Lepage and Roy method (43). 100µl of plasma was added to 2ml of methanol-benzene (4:1) in a test tube. This solution was vortexed before adding 200 µl of acetyl chloride. The tubes were then sealed and heated to 90° C for one hour. To neutralize the solution, 5ml of 6% $K_2CO_2$ was then added. 0.5 µl of the upper benzene layer was removed and analyzed by gas chromatography. A Varian CP-3800 gas chromatograph, equipped with a flame ionization detector and Varian CP-Sil 88 capillary column (50m x 0.25mm x 0.20um) was used. The benzene layer was injected with the CP-8400 auto sampler at a split ratio of 1:100. The flow rate of the helium carrier gas was 1 ml/min. The initial oven temperature was at 80° C. The temperature was held here for 1 minute then raised 30° C/ min to 140° C and then raised again at 5° C/min to 225° C and held for 10 minutes. The total time to run each sample was 30 min. The fatty acid contents of the sample were identified by comparison with authentic standards (NuCheck Prep, Elysian, MN, USA).

**4. Fatty Acid Analysis of Aortic and Carotid tissue**

Aortic and carotid tissue was flash frozen in liquid nitrogen immediately after being cleaned from extraneous tissue (43,44). The tissue was wrapped in plastic wrap and stored at –80° C. The first phase in the processing of the tissue for fatty analysis by gas chromatography was the homogenization of the tissue to obtain the total lipid content.

Each tissue was weighed and then minced into small pieces using scissors (aorta's ranged from 0.25 to 0.65g; carotid's from 0.055 to 0.18g). After transferring the tissue to a homogenization tube, the sample, which was placed on ice, was homogenized in a 5 mL aliquot of 2:1 chloroform:methanol ($CHCl_3$:MeOH). Prior to usage, all glassware was pre-rinsed with 2:1 $CHCl_3$:MeOH. The homogenate was then transferred to a graduated cylinder, and the previous step was repeated with an additional 5 ml of organic solvent. This procedure was repeated once again such that a total of 15 mL of organic solvent was present in the graduated cylinder. The contents of the graduated cylinder were then transferred to a separatory funnel. Using 5 mL of organic solvent, the graduated cylinder was rinsed out and poured into the same separatory funnel. Now a total of 20 mL of organic solvent was present plus the volume of water present from the tissue sample. Assuming that tissue has the specific gravity of water, enough dd $H_2O$ was added to the separatory funnel such that the total volume of tissue was 1 mL. Therefore, 21 ml of solvent was present in the separatory funnel. Next, 4.2 mL of 0.73% NaCl solution was added. The separatory funnel was shaken vigorously and the aqueous and organic layers were allowed to separate out overnight. Following separation, the $CHCl_3$ fraction was collected and dried over sodium sulfate, filtered and then evaporated to dryness using a rotary evaporator. The lipid was then further dried under a steady stream of $N_2$ (g). Once dry, the sample was dissolved in chloroform and then stored at -80°C until required.

The second phase involved the esterification of the extracted lipid to form the fatty acid methyl ester (FAME) derivative. Using a Drummond micropipette, 100 μL of sample extract was aliquoted into a glass derivatization tube. The solvent was then removed *via* purging with $N_2$(g). Next, 1 mL of 3:2 (v/v) MeOH:benzene solution was

added (which contained 0.1 mg/mL of C13:0 and C17:0, which are the internal standards) to the tube containing the dried lipid. The sample was put to a vortex and stored at -20°C until the next day. This procedure was repeated for each sample. Once all of the samples had been subjected to the above procedure, the samples were refunneled to room temperature. Next, 1 mL of 5:100 (v/v) acetyl chloride:MeOH solution was added to each tube and put to a vortex. Prior to the methanolysis step, the tube weights were recorded. Next, all sample tubes were placed on a 100°C pre-heated heating block for 1 hr, with vortexing every 15 minutes. After 1 hour, the tubes were allowed to return to room temperature and then reweighed. Any changes in sample loss were noted and samples with greater than 3% difference were rejected and rederivatized. Next, the reaction mixture was neutralized by the addition of 5 mL of 6% potassium carbonate. The tubes were vortexed and then centrifuged at room temperature for 5 minutes at 4500 g. Following centrifugation, the upper benzene layer was collected an analyzed by gas chromatography (GC).

GC analysis using FID detection was used to quantitate the FAME's within our tissues. The instrument was calibrated using the FAME standard GLC 462 purchased from Nu-Chek Prep, Inc. at a range of 0.003-0.700 mg/mL. The methyl esters were chromatographed on a CP-Sil 88 column from Varian, which is a 60m fused silica column with an internal diameter of 0.25mm. Analysis was performed using a Varian GC/MS/MS instrument equipped with a CP-3800 GC, CP-8400 autosampler and an FID detection system. Helium was used as the carrier gas and nitrogen as the make-up gas. Column flow was set to 1.5 mL/min. The following split ratio was used with the initial state being 5, then from 0.01 to 1 min. a split of 50 was employed, returning back to 5 at

1.00 min. The injection port temperature was 250°C and the FID was 270°C. The oven temperature began at 80°C with a 1 min. hold followed by a 30°C/min ramp to 140°C over a 3 minute period. This was followed by a steady temperature increase at 5°C/min to 200°C over a 15 minute time interval ending with a final temperature of 225°C obtained by the same rate increase as the previous step and held for 10 minutes. Sample sets included two blanks, several verification standards and sample spikes. All samples were analyzed in duplicate.

**5. Experimental Protocol for Assessing Vascular Contractile Function**

After feeding, the animals were anesthetized with $CO_2$ and ventilated. The animals were given heparin to inhibit their blood from clotting at the time of termination. The rabbit's chest was shaved and the chest was opened and the heart removed. The lungs were removed and the diaphragm was cut away to expose the thoracic and abdominal aorta. The distal end of the abdominal aorta was clamped with forceps and the aorta was grossly dissected from just proximal to the diaphragm to the proximal cut from the removal of the heart. Cuts were made along either side of the vessel and along the ventral surface of the vertebrae in a superior direction. The aorta was removed with caution not to stretch the fibers and smooth muscle cells, and immediately placed in a cold Krebs Henseleit solution (115 mM NaCl, 25mM $NaHCO_3$, 1.38 mM $KH_2PO_4$, 2.5 mM KCl, 2.46 mM $MgSO_4$, 1.9 mM $CaCl_2$, 11.2 mM dextrose, pH 7.4). The aorta was then cleaned to the level of the adventitia and prepared for vascular function testing, gas chromatography (GC), sectioning and en face staining.

To evaluate vessel contractile function, the vessel was cut just distal to the aortic arch in a 3 mm width ring. The ring was then mounted in a tissue bath system (Experimentria Ltd., Budapest, Hungary). The force transducers of the system were calibrated to 0.2V/1g. This calibration was confirmed with 1g, 10g and 20g weights. The aortic ring was fastened with surgical wire to the bath and the other end to a force transducer. The bath was perfused with Krebs Henseleit solution as described above at a temperature of 37°C and aerated with 95% $O_2$ and 5% $CO_2$, maintaining a pH of 7.4. 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. Once hung in the bath, the rings were stretched to a baseline tension of 5.5 to 6.5 grams. The level of basal tension and ring width was determined from preliminary experiments to deliver optimal contractile response of the aorta. The tissue weight was determined after the experiment was over. Each ring was dabbed on a paper towel to remove excess fluid and weighed to the $10^{-4}$ gram. Contractile response of the vascular rings was expressed as gram of tension/gram of tissue.

The response of the tissue to a variety of agonists was studied. The tissue was first equilibrated with the administration of 47 mM KCl to the organ bath. The vessel was allowed to contract until it reached its plateau and then the KCl was washed out with Krebs solution and allowed to return to basal tension. This equilibration was conducted two more times to ensure the vessel was at maximal contraction. At the end of the third

administration of 47 mM KCl, the tissue was washed out with Krebs and allowed to return to baseline tension. This equilibrium period lasted approximately 60 minutes.

Following this equilibrium period, a dose response curve to norepinephrine (NE) was performed. NE was introduced to the Krebs solution in the organ bath. The NE concentration was gradually increased from $10^{-9}$ M to $10^{-4}$ M. After the addition of each dose, the tissue was allowed to reach a steady state before the next dose was added. Each dose took approximately 5 minutes. After the final dose ($10^{-4}$M) of NE, the tissue was washed out with Krebs solution and allowed to return to baseline tension.

We next tested the ability of the tissue to relax after pre-contraction with NE. A second dose of $10^{-6}$ M NE was administered to the bath and the tissue was once again allowed to reach a steady state of contraction. Acetylcholine (ACh) was then added to the bath at gradually increasing concentrations from $10^{-8}$ M - $10^{-5}$ M with 5-minute intervals between each dose to create a relaxation dose response curve to ACh. After the final dose of ACh ($10^{-5}$ M), the tissue was rinsed with Krebs solution and weighed as described above.

**6. Tissue Processing for the Evaluation of Atherosclerotic Lesions**

A section of the distal aorta was opened longitudinally to measure the extent of plaque formation in the intima of the aorta by en face analysis. The open strips of aorta were pinned to a culture dish and a digital picture was taken. A Nikon Cool Pics 990 Digital Camera was used to take the digital image. The camera settings were as follows. The camera was set to automatic, fine and close up. The shot was taken with the timer on and the camera mounted with the specimen in place. Once a digital image was made, the

image was adjusted using Corel Photo-Paint. Using the software, the area of plaque was selected and saved as a separate file. The images (total and plaque) were then converted to grayscale. The lesion area of the aorta was quantified as the percentage of total area using the Silicon Graphics Imaging (SGI) computer software system. Carotid artery analyses were carried out in an identical manner.

**7. Sectioning and Staining of Tissue**

The rings were prepared for sectioning after being tested for their contractile response. These cross sections were then used for lipid analysis and histology. Specifically, the tissue was immersed in a 4% paraformaldehyde (PFA) solution at 4°C overnight. The PFA was poured off and the tissue was rinsed three times with a 1X PBS solution. The tissue was then immersed in a 30% sucrose solution and left on a slow shaker overnight at 4°C. The rings were removed from the sucrose, rinsed in Optima Cutting Temperature (OCT) compound, then submerged, mounted and frozen in OCT and stored at –80°C until sectioned. The rings were sectioned on a Shandon Cryotome to 7um-9um width sections and mounted on Fisher superfrost plus slides. The demonstration of lipids in the tissue was determined with an Oil Red O staining technique. The solution of Oil Red O, made up of 60% isopropanol imparts an orange-red color to fat droplets in the tissue (45).

A Nikon Cool Pics 990 Digital Camera was used to take the digital image of each stained section through a microscope. The camera settings were as follows. The camera was set to automatic, fine and close up. The aperture was set to a minimum and the white balance was focused on a white area on the slide. Once a digital image was made, the

image was adjusted using Corel Photo-Paint to brighten the plaquened area. Using the magic wand function, two separate files were made 1) the total section 2) the area of plaque was selected and saved as a separate file. The images (total and plaque) were then converted to grayscale. The lesion area of the aorta was quantified as the percentage of total area covered in plaque using the Silicon Graphics Imaging (SGI) computer software system. Using the threshold function on the SGI, the total area and area covered in plaque were determined. Calculating the plaque area as a percentage of the total area (100%), the percent area covered in plaque was determined.

**8. Statistical Analysis**

The data is expressed as the mean ± standard error (S.E.). Statistical comparisons were made using the one-way analysis of variance, followed by the Student-Neuman-Keuls test for multiple comparison using Sigma Stat software. Differences between means were considered significant when $p < 0.05$.

# IV. RESULTS

## A. Diet Analysis

The diets were analyzed for crude composition of fat, carbohydrate, protein, fiber, ash and energy. Each diet was sampled once. Table 2 shows these findings. The crude fat content was increased approximately 60% in the flax and cholesterol-flax diets as compared to control and cholesterol diets. Table 3 shows the total percent lipid extracted from a 1g sample of food. As expected from the results of the crude data the flaxseed and cholesterol-flax diets have a 60% greater total lipid content as compared to the control and cholesterol fed groups. Table 4 contains the common names, abbreviations and codes of some of the fatty acids present in the animals' diets and tissues. The fatty acid analysis of the diets is shown in Table 5. The values represented are the means ± SEM for the percent of lipids extracted from a 1g sample of ground food, for control, flaxseed, cholesterol, and cholesterol plus flaxseed (Chol-Flax) groups.

**Table 2. Nutritional Composition of the diets (g/kg dry diet)**

| | Control | Flax | Cholesterol | Cholesterol-flax |
|---|---|---|---|---|
| Crude Protein (%) | 21.3 | 20.5 | 20.4 | 20.5 |
| Crude Fat (%) | 5.4 | 8.1 | 5.2 | 8.9 |
| CHO (%) | 51.4 | 51.7 | 52.5 | 50.6 |
| Crude Fiber (%) | 13.5 | 11.7 | 13.6 | 12.4 |
| Ash (%) | 8.43 | 8.08 | 8.12 | 7.65 |
| Digestible E (cal/g) | 3.38 | 3.56 | 3.37 | 3.60 |

**Table 3. Percent of lipid extracted from a 1g sample of food from each diet**

| | Control | Flaxseed | Cholesterol | Chol/Flax |
|---|---|---|---|---|
| **wt. of food (g)** | 1.0003 g | 1.0000 g | 1.0000 g | 1.0003 g |
| **g extr'd lipids** | 0.060 g | 0.094 g | 0.057 g | 0.098 g |
| **% lipids** | 6.00% | 9.40% | 5.65% | 9.75% |

## Table 4. Nomenclature of Fatty Acids

| Fatty Acid Code | Common Name | Abbreviation |
|---|---|---|
| C14:0 | Myristic Acid | |
| C14:1 | Myristoleic Acid | |
| C16:0 | Palmitic Acid | |
| C16:1 | Palmitoleic Acid | |
| C18:0 | Stearic Acid | |
| C18:1 (n-9) | Oleic Acid | Ol |
| C18:1 | Elaidic Acid | |
| C18:2 (n-6) | Linoleic Acid | LA |
| C18:3 (n-6) | Gamma Linolenic Acid | GLA |
| C18:3 (n-3) | Alpha Linolenic Acid | ALA |
| C20:0 | Arachidic Acid | |
| C20:1 (n-9) | Eicosenoic Acid | |
| C20:2 (n-6) | Eicosadienoic Acid | |
| C22:0 | Behenic Acid | |
| C20:3 (n-6) | di-Homo-Gamma Linolenic Acid | |
| C20:3 (n-3) | Eicosatrienoic Acid | |
| C20:4 (n-6) | Arachidonic Acid | AA |
| C20:5 (n-3) | Eicosapentaenoic Acid | EPA |
| C22:4 | Docosatetraenoic Acid | |
| C24:1 (n-9) | Tetracosenoic Acid | |
| C22:6 (n-3) | Decosahexenoic Acid | DHA |

| Table 5. Fatty acid composition of the 4 animal diets | | | | |
|---|---|---|---|---|
| **FAME** | **Control** | **Flaxseed** | **Cholesterol** | **Chol-Flax** |
| | **(mg/g)** | **(mg/g)** | **(mg/g)** | **(mg/g)** |
| **C14:0** | 0.29 ± 0.01 | 0.37 ± 0.01 | 0.27 ± 0.00 | 0.34 ± 0.02 |
| **C14:1** | 0.13 ± 0.00 | 0.00 ± 0.00 | 0.03 ± 0.03 | 0.00 ± 0.00 |
| **C16:0** | 6.50 ± 0.25 | 9.57 ± 0.35 | 6.34 ± 0.04 | 9.36 ± 0.23 |
| **C16:1** | 0.40 ± 0.02 | 0.54 ± 0.03 | 0.36 ± 0.00 | 0.44 ± 0.00 |
| **C18:0** | 2.20 ± 0.15 | 3.94 ± 0.16 | 2.05 ± 0.01 | 3.64 ± 0.19 |
| **C18:1 Ol** | 10.69 ± 0.33 | 17.06 ± 0.86 | 10.29 ± 0.01 | 16.36 ± 0.84 |
| **C18:1 Vac** | 1.72 ± 0.15 | 2.78 ± 0.00 | 1.64 ± 0.06 | 2.61 ± 0.07 |
| **C18:2 LA** | 11.19 ± 0.34 | 11.78 ± 0.51 | 12.33 ± 0.17 | 13.71 ± 0.49 |
| **C20:0** | 0.11 ± 0.01 | 0.17 ± 0.01 | 0.12 ± 0.00 | 0.16 ± 0.03 |
| **C18:3n-6 GLA** | 0.00 ± 0.00 | 0.12 ± 0.00 | 0.00 ± 0.00 | 0.13 ± 0.00 |
| **C20:1** | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| **C18:3n-3 ALA** | 1.99 ± 0.12 | 20.08 ± 0.84 | 2.12 ± 0.04 | 22.54 ± 0.68 |
| **C20:2** | 0.09 ± 0.01 | 0.13 ± 0.01 | 0.09 ± 0.00 | 0.11 ± 0.01 |
| **C22:0** | 0.16 ± 0.01 | 0.20 ± 0.00 | 0.17 ± 0.01 | 0.20 ± 0.04 |
| **C22:1** | 0.11 ± 0.03 | 0.29 ± 0.01 | 0.10 ± 0.01 | 0.09 ± 0.04 |
| **C20:3** | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.19 ± 0.00 |
| **C22:6 DHA** | 0.00 ± 0.00 | 0.22 ± 0.00 | 0.11 ± 0.11 | 0.12 ± 0.12 |

Mean ± SE are shown.

**B. Body weights**

Animals were weighed upon arrival to animal holding prior to feeding and at termination. Table 6 shows the mean body weights of the animals for both trials. Whole animal weights were measured prior to feeding (0-weeks) and at termination (6 or 8-weeks) for the four groups. No statistically significant differences were observed between the groups in either trial.

**Table 6: Animal Body Weight (Kg)**

| Group | | 6 Week Duration | | 8 Week Duration | |
|---|---|---|---|---|---|
| | | 0 weeks | 6 weeks | 0 weeks | 8 weeks |
| Control | X±SEM | 2.37±0.09 | 3.51±0.06 | 2.83±0.09 | 3.72±0.09 |
| | n | 8 | 8 | 8 | 6 |
| | | | | | |
| Flax | X±SEM | 2.25±0.09 | 3.51±0.11 | 2.88±0.06 | 3.75±0.11 |
| | n | 7 | 7 | 8 | 7 |
| | | | | | |
| Cholesterol | X±SEM | 2.34±0.11 | 3.50±0.05 | 2.84±0.04 | 3.62±0.07 |
| | n | 8 | 8 | 8 | 7 |
| | | | | | |
| Chol-Flax | X±SEM | 2.44±0.06 | 3.73±0.05 | 2.84±0.07 | 3.75±0.11 |
| | n | 8 | 8 | 8 | 7 |

Abbreviations: cholesterol and flax fed, (chol-flax). Values represent means ± standard error (X±SEM). No statistical significance exists amongst the groups ($p>0.05$).

### C.) Plasma Analysis

Blood was collected from the rabbits at 0, 6 and 8 weeks of dietary intervention. The plasma was separated and analyzed for triglyceride, cholesterol and long chain fatty acid composition. Figure 9 shows the changes in plasma triglyceride levels from 0 to 8 weeks in all four groups. No statistically significant change in plasma triglyceride levels was measured in any dietary intervention. Figure 10 shows the changes in plasma cholesterol levels from 0 to 8 weeks in all four groups. No significant change was measured in control and flax fed groups over the course of the study. Both the cholesterol and the cholesterol-flax supplemented groups exhibited statistically significant increases in comparison to control and flax groups. A 10-fold increase in plasma cholesterol was measured in the cholesterol-supplemented group and a 13-fold increase in the cholesterol-flax fed group over the 8 weeks of dietary intervention. No significant difference occurred between the cholesterol and the cholesterol-flax groups.

The levels of plasma long chain fatty acids after 6 and 8 weeks of feeding are shown in Table 7. The 0 week data is not shown but the following significant changes occurred. Significant differences occurred between the control and flax groups at 0 weeks in C:14:0, C:18:0, C:18:3 and in total plasma fatty acid content. The control group measured the high end of the mean and the flax group represented the lower end of the mean in all measured plasma fatty acids. The control plasma fatty acid at 0 week was

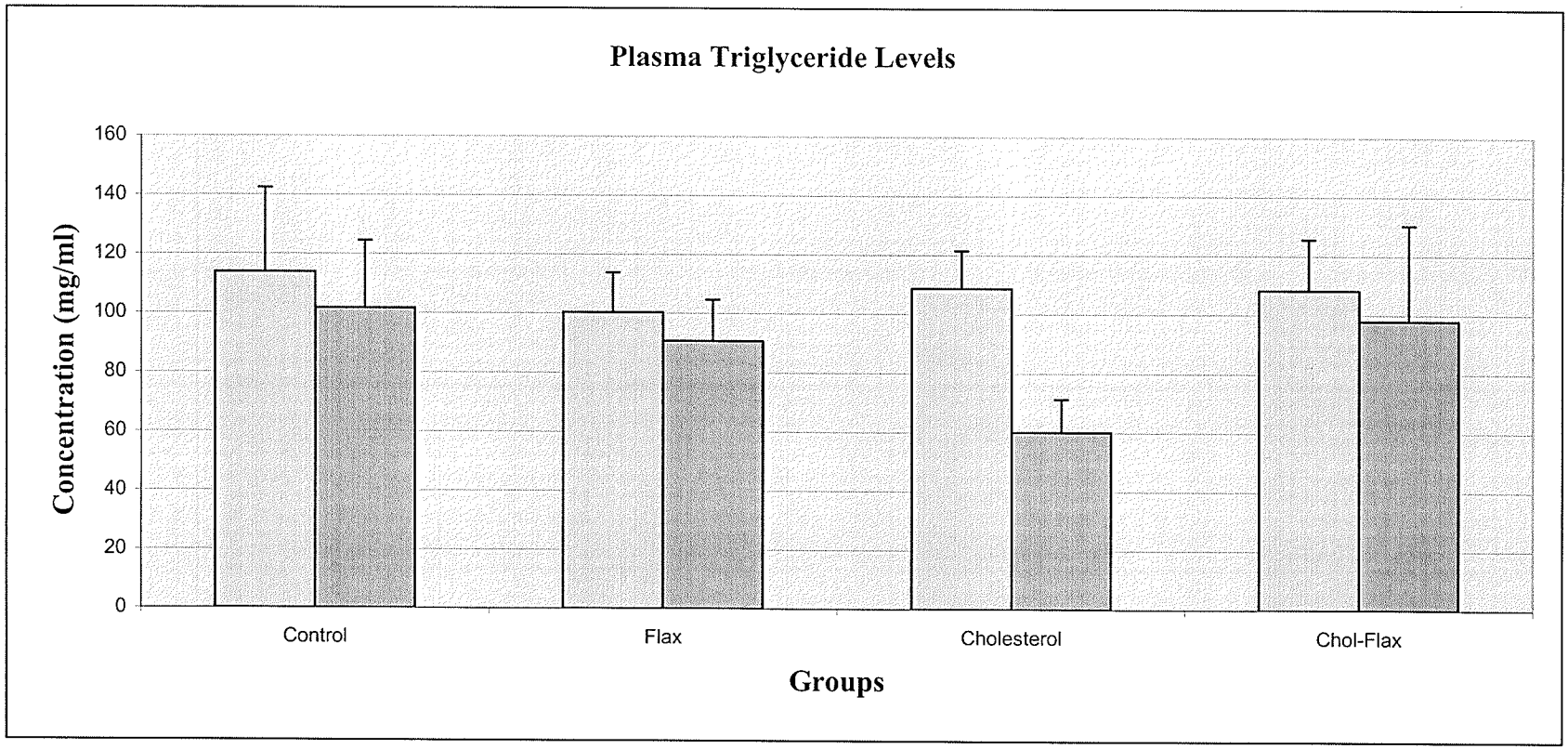


**Figure 9. Plasma triglyceride levels.** Mean values ± standard error are shown for each of the groups at 0 and 8 weeks of dietary intervention. The light bars represent plasma triglyceride levels at 0 weeks. The dark bars represent plasma triglyceride levels at 8 weeks ($p>0.05$). n = 8 in all four groups at 0 weeks. n = 7 for control and cholesterol and n = 8 for flax and cholesterol-flax at 8 weeks

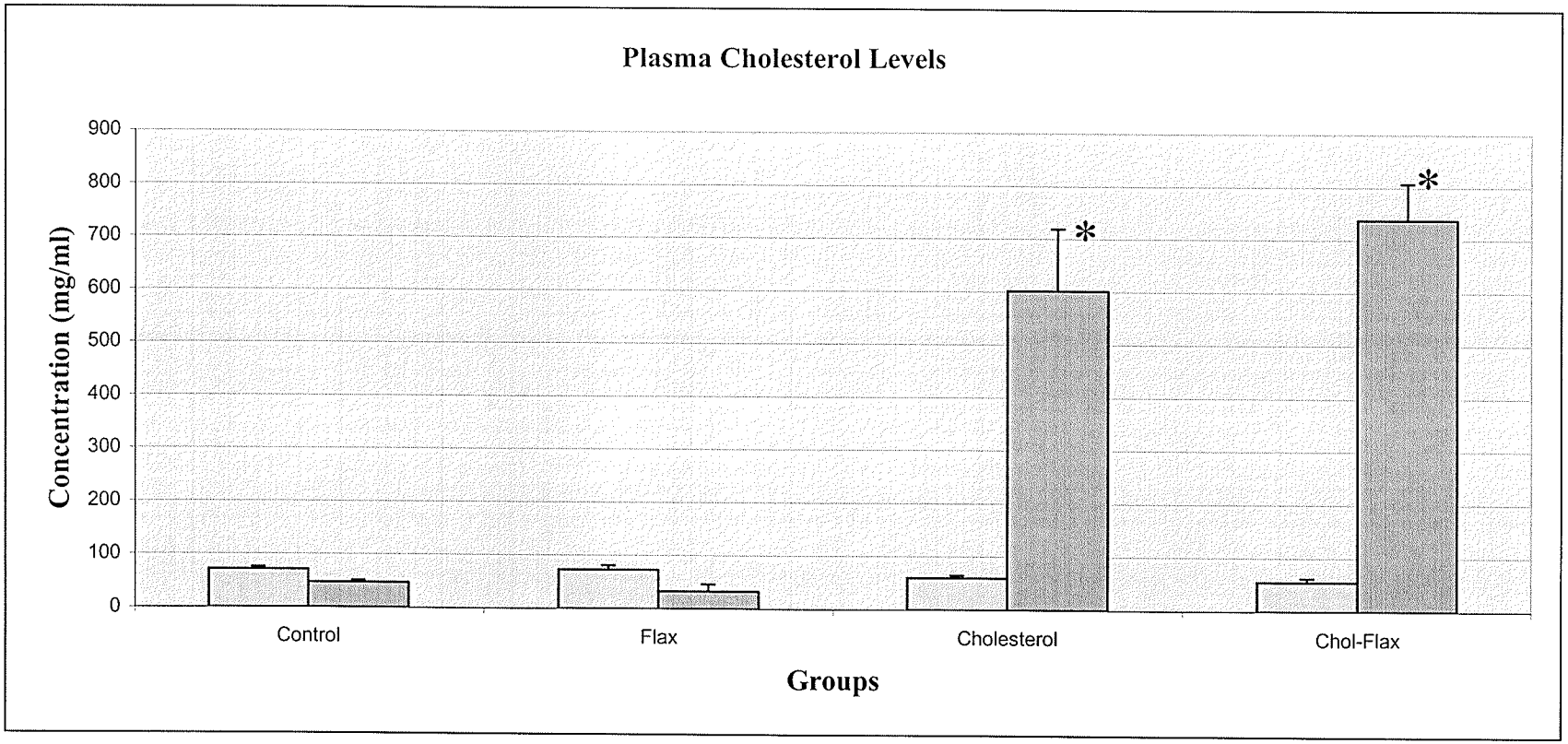


**Figure 10. Plasma cholesterol levels.** Mean values ± standard error are shown for each of the groups at 0 and 8 weeks of dietary intervention. The light bars represent plasma triglyceride levels at 0 weeks. The dark bars represent plasma triglyceride levels at 8 weeks. * Represents statistical significance ($p<0.05$) in cholesterol and cholesterol-flax fed groups compared with control. n = 8 in all four groups at 0 weeks. n = 7 for control and cholesterol and n = 8 for flax and cholesterol-flax at 8 weeks

significantly higher than that measured in controls after 6 and 8 weeks of feeding in C:14:0, C:16:0, C:18:2, C:20:4, and in total plasma fatty acid levels.

Significant changes in plasma fatty acid levels occurring between the four groups after 6 weeks and between the four groups after 8weeks took place as follows (Table 7). A significant differences in the plasma fatty acid level of arachidonic acid (AA) occurred between the control and flax fed animals after 6 weeks and after 8 weeks of feeding. There was no significant difference measured in any of the other plasma fatty acids between the control and flax fed animals. AA levels at 0 weeks averaged 2.1mg/dl. After 6 weeks of feeding, controls plasma AA levels dropped to 0.1, and then increased again to 1.9 after 8 weeks of feeding. Flax fed animals exhibit a slow decline from 2 mg/dl at 0 weeks to 1.6 mg/dl after 6 weeks and then to 1.2 mg/dl after 8 weeks of feeding. After 6 weeks C:22:0 and C:22:6 were the only plasma fatty acids having no significant changes measured. After 8 weeks of feeding a significant increase from below detectable limits (bdl) to 0.3mg/dl occurred in plasma C:22:0 occurred in the cholesterol-flax fed animals, C:22:6 remained unchanged. All other plasma fatty acids measured had significant changes between control and cholesterol and cholesterol/flax and between flax and cholesterol and cholesterol/flax groups. Similar changes were seen with the four groups after 6 weeks or 8 weeks of feeding in C:14:0, C:16:0, C:16:1, C:18:0, C:18:1, C:18:2, C:20:0, C:20:1, C:20:2, C:20:3, C:22:0, C:22:6. A significant increase in plasma C:20:1 from bdl to 0.1 mg/dl after 6 weeks and 1.2 mg/dl after 8 weeks was measured in the cholesterol fed animals as compared to the other 3 groups.

**Table 7: Plasma fatty acid concentration in control, flax-supplemented, cholesterol-supplemented and cholesterol with flax supplemented animals (mg/ml) after 6 and 8 weeks of feeding.**

| Fatty Acid | 6 weeks | | | | 8 weeks | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | Flax | Chol | Chol-Flax | Control | Flax | Chol | Chol-Flax |
| C 14:0 | 0.2 [2,3] | 0.3 [4,5] | 1.5 [2,4] | 1.4 [3,5] | 0.5 [2,3] | 0.1 [4,5] | 1.7 [2,4,6] | 1.1 [3,5,6] |
| ±*SEM* | *0.1* | *0* | *0.2* | *0.2* | *0.1* | *0.1* | *0.2* | *0.2* |
| C:16:0 | 12.7 [2,3] | 9.2 [4,5] | 71.3 [2,4] | 76.1 [3,5] | 14.8 [2,3] | 9.4 [4,5] | 81.7 [2,4] | 80.1 [3,5] |
| ±*SEM* | *1.7* | *1.1* | *8.1* | *7.1* | *1.6* | *1* | *7.4* | *5.9* |
| C:16:1 | 1.2 [2,3] | 0.5 [4,5] | 17.8 [2,4] | 13.6 [3,5] | 1.9 [2,3] | 0.8 [4,5] | 19.9 [2,4,6] | 13.9 [3,5,6] |
| ±*SEM* | *0.2* | *0.2* | *2.2* | *1.8* | *0.3* | *0.1* | *1.9* | *1.3* |
| C:18:0 | 13.6 [2,3] | 11.3 [4,5] | 26.2 [2,4] | 29.2 [3,5] | 14.5 [2,3] | 10.7 [4,5] | 28.3 [2,4] | 33.7 [3,5] |
| ±*SEM* | *1.2* | *0.8* | *3.1* | *4.6* | *1.1* | *0.8* | *2.4* | *2.8* |
| C:18:1 | 15.6 [2,3] | 9.2 [4,5] | 122.1 [2,4] | 135.8 [3,5] | 17.4 [2,3] | 10.2 [4,5] | 142.8 [2,4] | 142.8 [3,5] |
| ±*SEM* | *3.5* | *1* | *13.3* | *14.7* | *2.5* | *1* | *10.4* | *12.3* |
| C:18:2 ω-6 | 13.5 [2,3] | 9.1 [4,5] | 61.9 [2,4] | 74.9 [3,5] | 12.5 [2,3] | 8.6 [4,5] | 76.8 [2,4] | 81.2 [3,5] |
| ±*SEM* | *2.7* | *1.1* | *9.4* | *7.1* | *2.3* | *1.2* | *8.6* | *7.1* |
| C:18:3 ω-3 | 0.6 [3] | 4.3 [5] | 12 [6] | 96.6 [3,5,6] | 0.5 [3] | 5.9 [5] | 16.6 [6] | 108.0 [3,5,6] |
| ±*SEM* | *0.5* | *0.4* | *1.7* | *11.8* | *0.3* | *1* | *1.9* | *13.9* |
| C:20:0 | bdl | bdl | 0.6 [2,4] | 0.8 [3,5] | bdl | bdl | 0.8 [2,4] | 1.0 [3,5] |
| ±*SEM* | | | *0.1* | *0.1* | | | *0.1* | *0.1* |
| C:20:1 | bdl | bdl | 0.1 [4,6] | bdl | bdl | bdl | 1.2 [2,4,6] | bdl |
| ±*SEM* | | | *0* | | | | *0.1* | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| C:20:2 | bdl | bdl | 1 [2,4] | 0.8 [3,5] | bdl | bdl | 1.2 [2,4] | 1.1 [3,5] |
| ±*SEM* | | | *0.1* | *0.2* | | | *0.1* | *0.3* |
| C:20:3 ω-6 | bdl | bdl | 0.7 [2,4] | 0.6 [3,5] | bdl | bdl | 1.0 [2,4] | 0.8 [3,5] |
| ±*SEM* | | | *0.2* | *0.1* | | | *0.1* | *0.1* |
| C:20:4 ω-6 | 0.1 [1,4,5] | 1.6 [1,4,5] | 3.3 [2,4] | 3.2 [3,5] | 1.9 [1,2,3] | 1.2 [1,4,5] | 3.7 [2,4] | 3.3 [3,5] |
| ±*SEM* | *0* | *0.2* | *0.3* | *0.1* | *0.1* | *0.3* | *0.2* | *0.1* |
| C:20:5 ω-3 | bdl | bdl | 0.4 [2,4] | 0.5 [3,5] | bdl | bdl | 2.7 [2,4,6] | 0.9 [3,5,6] |
| ±*SEM* | | | *0.1* | *0.1* | | | *0.1* | *0.1* |
| C:22:0 | bdl | bdl | bdl | bdl | bdl | bdl | 0.2 | 0.3 [3,5] |
| ±*SEM* | | | | | | | *0.1* | *0.1* |
| C:22:6 ω-3 | 0.3 | bdl | bdl | 0.4 | bdl | bdl | bdl | bdl |
| | *0.3* | | | *0.4* | | | | |
| **Total Fatty Acids** | | | | | | | | |
| | 65.8 [2,3] | 49 [4,5] | 329.8 [2,4,6] | 453.2 [3,5,6] | 68.8 [2,3] | 50.1 [4,5] | 393.3 [2,4,6] | 484 [3,5,6] |
| | *10* | *4.2* | *39.8* | *46.3* | *7.8* | *5.2* | *32.3* | *44* |

Mean values ± standard error are shown. Abbreviations and symbols: bdl, (below detectable limits) have trace amounts present (< 0.1 mg/dL); [1] $P < 0.05$ control versus flax within its' respective time (ie 6 weeks or 8 weeks); [2] $P < 0.05$ control versus cholesterol within its' respective time (ie 6 weeks or 8 weeks); [3] $P < 0.05$ control versus cholesterol with flax within its' respective time (ie 6 weeks or 8 weeks); [4] $P < 0.05$ flax versus cholesterol within its' respective time (ie 6 weeks or 8 weeks); [5] $P < 0.05$ flax versus cholesterol with flax within its' respective time (ie 6 weeks or 8 weeks); [6] $P < 0.05$ cholesterol versus cholesterol with flax within its' respective time (ie 6 weeks or 8 weeks). N = 7 for control and cholesterol and n = 8 for flax and cholesterol-flax at 6 and 8 weeks. The data for 0 weeks are not included. There was no significant difference in the absolute total values of serum lipids in controls between 0,6 and 8 weeks. At 0 weeks there was a significant difference [1] $P < 0.05$ control versus flax in C14:0, C18:0 and C18:3 (data not shown). N=8 in all groups at 0 weeks.

Total plasma fatty acid levels decreased remained unchanged in controls over the 6 and 8 weeks of dietary intervention from that measured at 0 weeks. Total levels in the flax supplemented group after 6 and 8 weeks decreased to a marginal significant level but statistical significance was not certain. Both the cholesterol and cholesterol-flax fed groups at 6 and 8 weeks exhibited statistically significant increases in total plasma fatty acid content in comparison to those of controls. These changes in total plasma fatty acid content were observed as changes in the levels of all types of fatty acids (SFA, MUFA, and PUFA). Cholesterol fed animals exhibited a 4.1-fold increase in total plasma fatty acids after 6 weeks of feeding and a 4.9-fold increase over the 8 weeks of feeding. Cholesterol-flax fed animals exhibited a 5.6-fold increase, after 6 weeks and a 6-fold increase over the 8 weeks of feeding. The greatest difference measured occurred in plasma ALA in the cholesterol/flax fed group as compared with control, flax and cholesterol fed animals. Animals fed flax alone had a 7.2-fold increase after 6 weeks and a 11.8-fold increase after 8 weeks in ALA over that of controls. Cholesterol fed animals increased 20-fold after 6 weeks and 33.2-fold after 8 weeks of feeding in plasma ALA levels as compared with controls values.

**D.) Tissue Analysis**

Tissue samples of the proximal aorta and carotid arteries of 8-week rabbits were flash frozen and analyzed by gas chromatography for tissue fatty acid content. Table 8 summarizes the fatty acid content of the 8-week aortic tissue. There was not enough carotid tissue for the successful completion of its fatty acid analysis. The flax fed animals exhibited significant increases in DHA content (mg/ml) as compared to the control rabbit aortic tissue. Tissues of animals fed cholesterol or cholesterol-flax exhibited similar increases in most fatty acids as compared to control and flax fed animal tissue: (palmitoleic acid, OL, oleic acid, AA, eicosanoic acid, C20:2, di-homo-GLA,C24:0, and C24:1). A significant increase was measured in ALA and EPA in the cholesterol-flax fed animals as compared to control. A significant increase was measured in DHA between the cholesterol-flax fed group and all other groups.

**Table 8: Fatty acid content of aortae of 8-week tissue (mg FAME/g of tissue).**

| FAME | Control | Flax | Cholesterol | Cholesterol-Flax |
|---|---|---|---|---|
| C14:0 | 0.14 ± 0.06 | 0.18 ± 0.03 | 0.30 ± 0.05 | 0.26 ± 0.04 |
| C14:1 | 0.00 ± 0.00 | Bdl | bdl | bdl |
| C16:0 | 2.45 ± 0.70 | 2.57 ± 0.40 | 4.76 ± 0.61 | 4.50 ± 0.64 |
| C16:1 | 0.27 ± 0.11 | $^{bd}$0.26 ± 0.06 | $^{ab}$0.65 ± 0.07 | $^{ad}$0.58 ± 0.10 |
| C18:0 | 1.14 ± 0.18 | 1.16 ± 0.08 | 1.76 ± 0.23 | 1.70 ± 0.16 |
| C18:1 (n-9) Ol | 2.49 ± 0.76 | $^{bd}$2.55 ± 0.26 | $^{ab}$8.22 ± 1.08 | $^{ad}$6.95 ± 1.01 |
| C18:1 Vac | 0.18 ± 0.05 | $^{bd}$0.17 ± 0.01 | $^{ab}$0.83 ± 0.10 | $^{ad}$0.62 ± 0.10 |
| C18:2 (n-6) LA | 1.38 ± 0.42 | $^{bd}$1.44 ± 0.11 | $^{ab}$3.83 ± 0.56 | $^{ad}$3.64 ± 0.54 |
| C20:0 | bdl | $^{bd}$0.01 ± 0.01 | $^{ab}$0.07 ± 0.01 | $^{ad}$0.06 ± 0.01 |
| C18:3 (n-6) GLA | bdl | Bdl | bdl | bdl |
| C20:1 (n-9) | 0.02 ± 0.01 | $^{bd}$0.01 ± 0.02 | $^{ab}$0.24± 0.04 | $^{ad}$0.22 ± 0.05 |
| C18:3 (n-3) ALA | 0.31 ± 0.12 | 1.27 ± 0.15 | 1.49 ± 0.52 | $^{a}$2.23 ± 0.35 |
| C20:2 (n-6) | bdl | $^{bd}$0.00 ± 0.00 | $^{ab}$0.27 ± 0.04 | $^{ad}$0.21 ± 0.04 |
| C22:0 | 0.04 ± 0.01 | 0.05 ± 0.01 | 0.07 ± 0.00 | 0.06 ± 0.00 |
| C20:3 (n-6) 8-11-14 | bdl | $^{bd}$0.00 ± 0.00 | $^{ab}$0.10 ± 0.02 | $^{ad}$0.07 ± 0.01 |
| C20:3 (n-3) 11-14-17 | bdl | $^{d}$0.00 ± 0.00 | 0.23 ± 0.12 | $^{ad}$0.28 ± 0.06 |
| C20:4 (n-6) | 0.90 ± 0.05 | $^{b}$0.75 ± 0.03 | $^{b}$0.90 ± 0.04 | 0.86 ± 0.04 |
| C24:0 | bdl | $^{bd}$0.00 ± 0.00 | $^{ab}$0.03 ± 0.01 | $^{ad}$0.03 ± 0.01 |
| C20:5 (n-3) EPA | bdl | $^{d}$0.00 ± 0.00 | 0.05 ± 0.03 | $^{ad}$0.08 ± 0.01 |
| C24:1 (n-9) | bdl | $^{bd}$0.00 ± 0.00 | $^{ab}$0.09 ± 0.02 | $^{ad}$0.06 ± 0.02 |
| C22:6 (n-3) DHA | bdl | $^{a}$0.03 ± 0.01 | $^{c}$0.02 ± 0.01 | $^{acd}$0.06 ± 0.00 |

Mean values ± standard error are shown. Abbreviations : **bdl** (below detectable limits) trace amounts present (< 0.1 mg/dL); **a** $P < 0.05$ versus control, **b** $P < 0.05$ between flax and cholesterol fed groups, **c** $P < 0.05$ between cholesterol and cholesterol-flax fed groups, **d** $P < 0.05$ between flax and cholesterol-flax fed groups.

**Table 9. Tissue wet weights of aortic rings**

| Group | 6 Week Study | 8 Week Study |
|---|---|---|
| | | |
| Control X±SEM | 0.02 (± 0.002) | 0.02 (± 0.002) |
| | N=8 | N=7 |
| | | |
| Flax X±SEM | 0.021 (± 0.002) | 0.021 (± 0.001) |
| | N=7 | N=7 |
| | | |
| Cholesterol X±SEM | 0.019 (± 0.001) | 0.023 (± 0.002) |
| | N=8 | N=7 |
| | | |
| Cholesterol-Flax X±SEM | 0.021 (± 0.002) | 0.022 (± 0.002) |
| | N=8 | N=8 |
| | | |

Mean values ± standard error (SE) are shown for each of the four groups in the 6 and 8-week interventions. N is the sample size per group.

**E.) Vascular function analysis**

Contractile and relaxation responses were measured in organ baths with aortic rings connected to a force transducer. After testing, tissues were weighed to standardize the contractile response. Table 9 shows the mean tissue weights (g) and standard error for all four groups in both the 6 and 8-week trials. No statistical significant differences were measured between the groups in either trial $P>0.05$).

The rings were first introduced to a high KCl concentration solution. This induced a depolarization of the cells resulting in contraction of the vessel ring. Figures 11 and 12 show the mean contractile response of the aortic rings to 47mM KCl in the 6 and 8-week trials respectively. No significant difference was detected amongst the groups in either trial. After 8-weeks of feeding there was a trend in all groups compared with control to have a reduced contractile response to KCl.

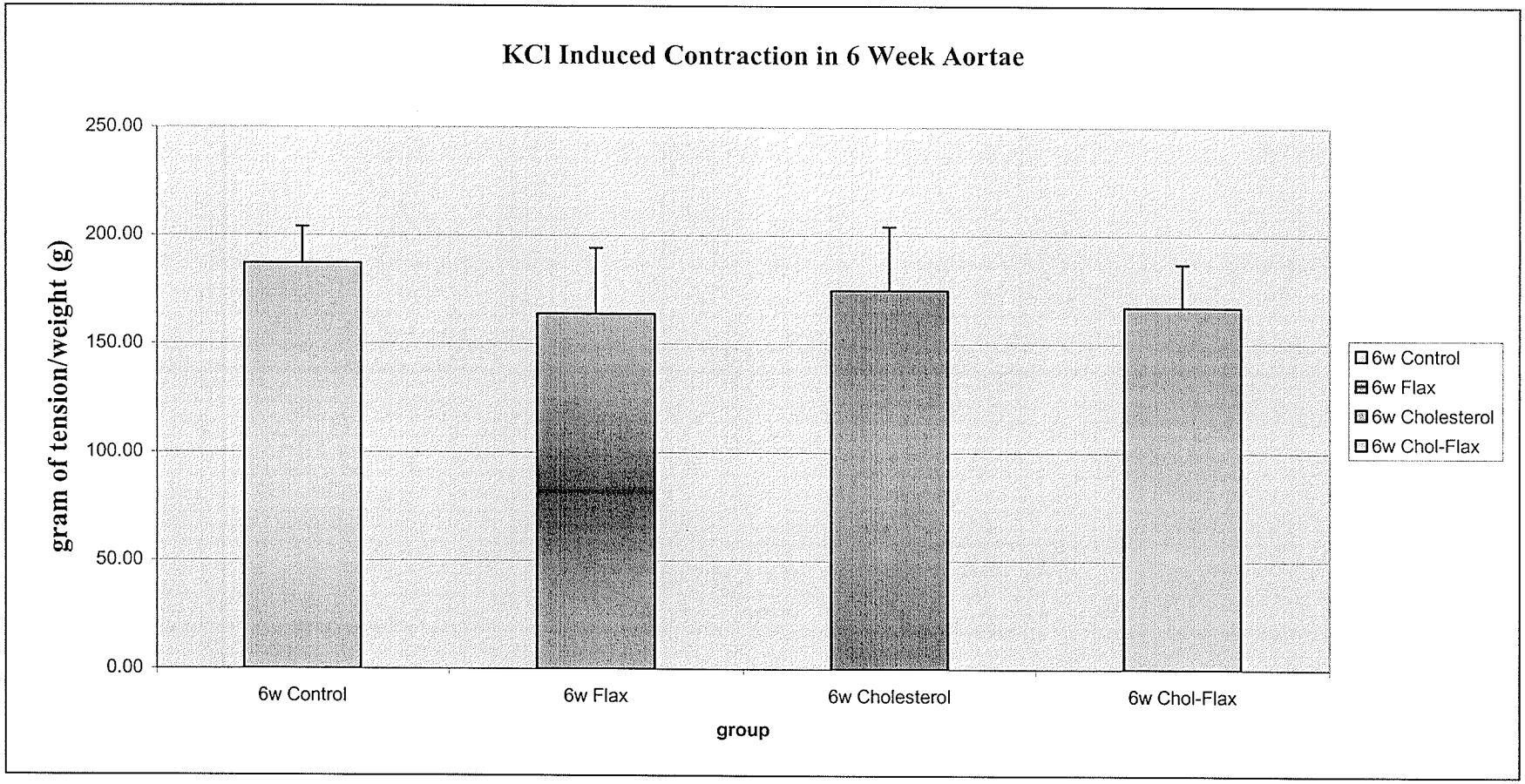


**Figure 11. KCl induced contraction in aortae after 6-weeks of nutritional intervention.** Mean values ± standard error are shown. Maximum contraction is shown as a function of tissue wet weight in grams. No statistical significance was measured. N = 8 (control, cholesterol and cholesterol-flax), n = 7(flax).

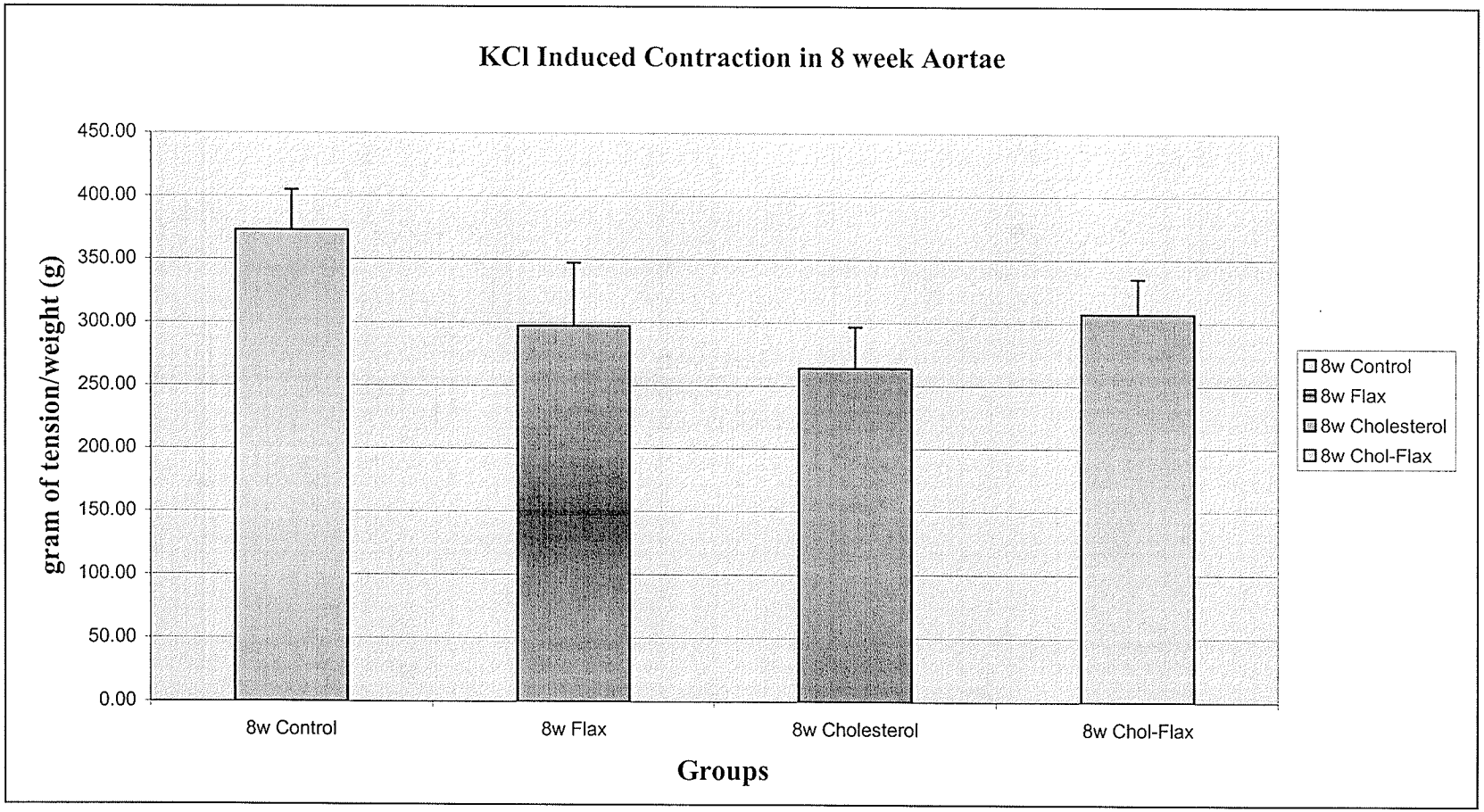


**Figure 12. KCl induced contraction in aortae after 8-weeks of nutritional intervention.** Mean values ± standard error are shown. Maximum contraction is shown as a function of tissue wet weight in grams. No statistical significance was measured. N = 7 (control, flax and cholesterol) n = 8 (cholesterol-flax).

Norepinephrine was introduced at increasing concentrations ($10^{-9}$M to $10^{-4}$ M) to the organ bath. Norepinephrine stimulates $\alpha_1$-adrenergic and angiotensin II receptors on the membrane of the smooth muscle cell causing release of $IP_3$ (inositol trisphosphate) the second messenger of these receptors, activating a G-protein response and resulting in contraction of the vessel ring. Figures 13 and 14 are contraction response curves for 6 and 8-week trials, respectively, in response to increased concentrations of norepinephrine. No statistical difference was measured in contractile response amongst the groups in either trial.

The vessel rings were pre-contracted with a dose of $10^{-6}$ M norepinephrine and then acetylcholine was administered at increasing doses ($10^{-8}$ M to $10^{-5}$ M) to determine the relaxation response of the isolated aortic rings. Acetylcholine induced an endothelial-dependant relaxation of the smooth muscle cells via the release of nitric oxide. Figures 15 and 16 show relaxation response curves for the 6 and 8-week trials, respectively. No significant difference was measured between the control group and any of the diet supplemented groups.

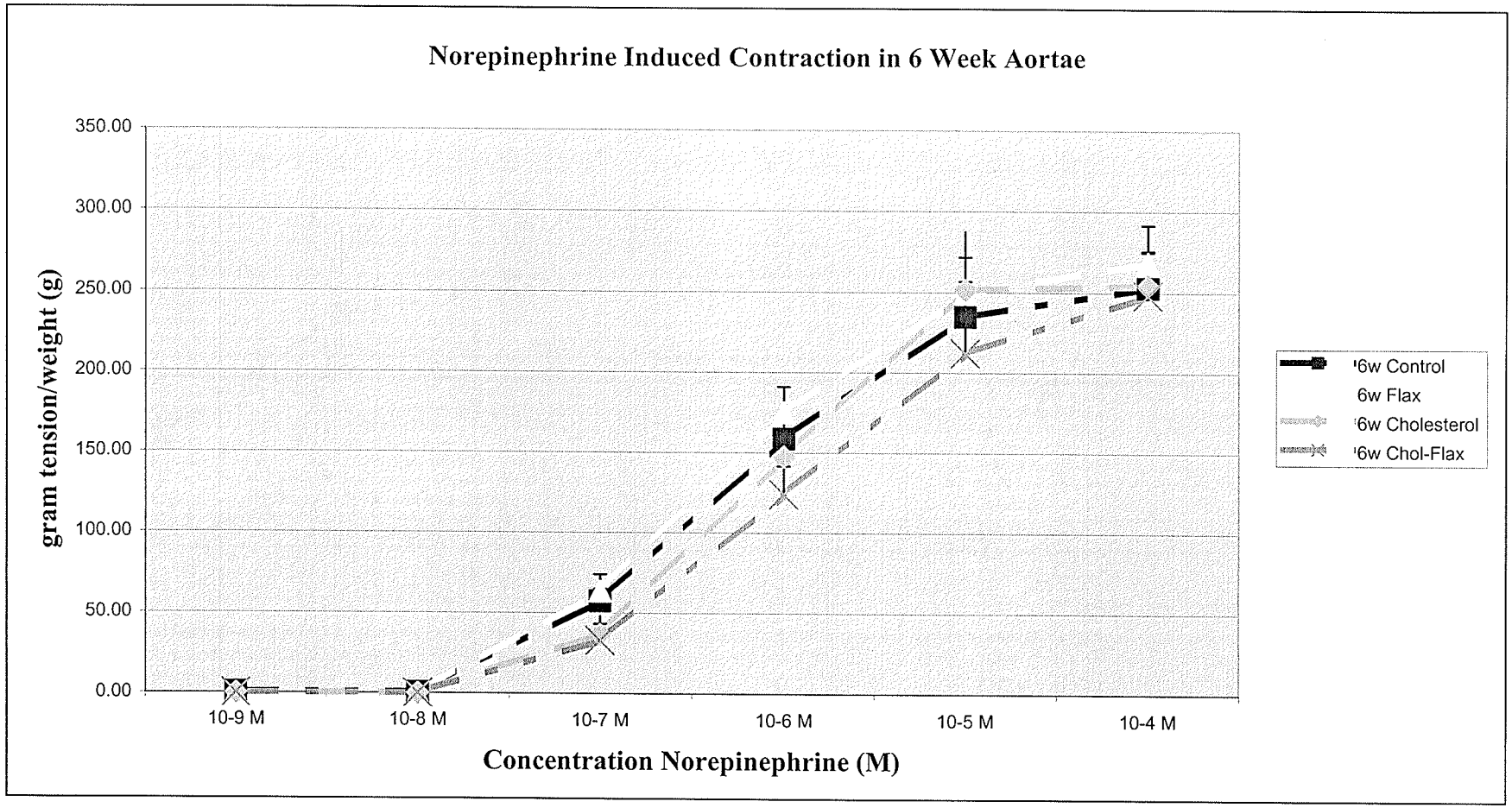


**Figure 13. Norepinephrine induced contraction response to increasing doses of norepinephrine (10-9 to 10-4 M) in aortae obtained from rabbits after 6-weeks of dietary intervention.** Maximum contraction was measured and the mean values ± standard error are shown as a function of tissue wet weight. No statistically significant difference amongst the groups was measured. N = 8 (control, cholesterol and cholesterol-flax), n = 7(flax).

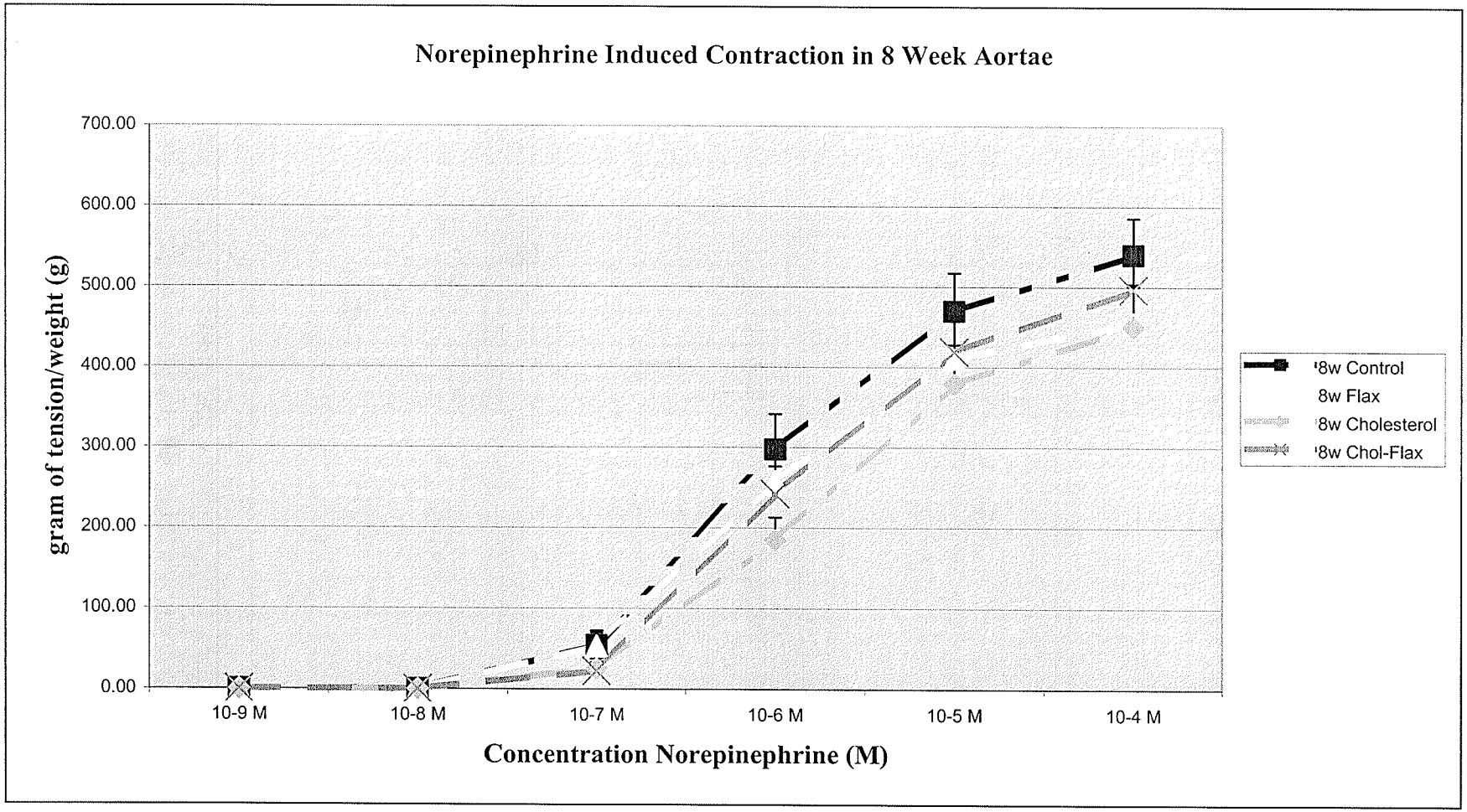


**Figure 14. Norepinephrine induced contraction response to increasing doses of norepinephrine (10-9 to 10-4 M) in aortae obtained from rabbits after 8-weeks of dietary intervention.** Maximum contraction was measured and the mean values ± standard error are shown as a function of tissue wet weight. No statistically significant difference amongst the groups was measured. N = 7 (control, flax and cholesterol) n = 8 (cholesterol-flax).

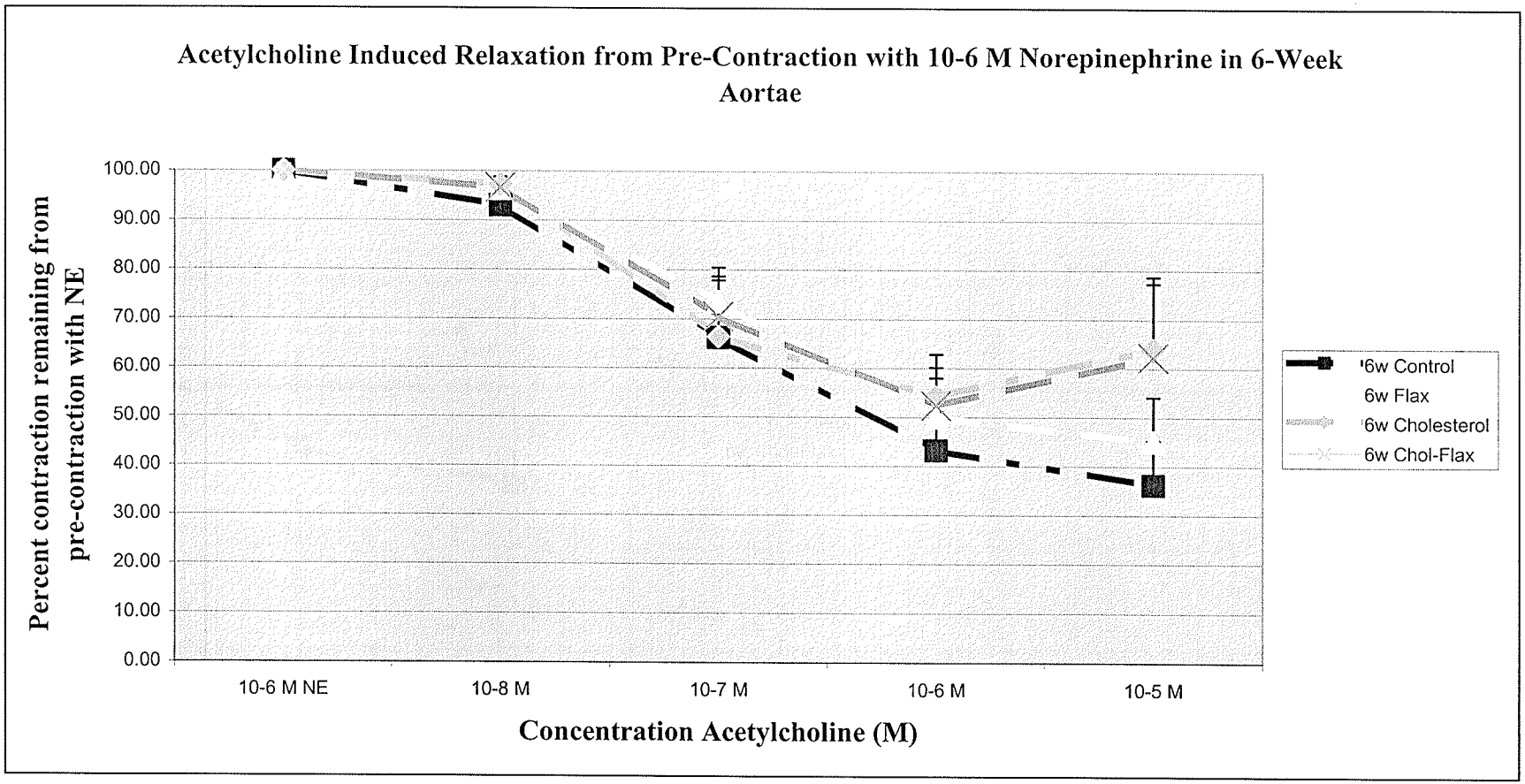


**Figure 15. Acetylcholine induced relaxation response curve after pre-contraction to 10-6 norepinephrine in 6-week aortae.** The vessels were pre-contracted and acetylcholine was introduced in increasing concentrations (10-8 to10-5 M). Maximum contraction was measured as a function of tissue wet weight. The data represented is the mean percent contraction remaining after acetylcholine administration ± standard error. No statistically significant difference amongst the groups was measured. N = 8 (control, cholesterol and cholesterol-flax), n = 7(flax).

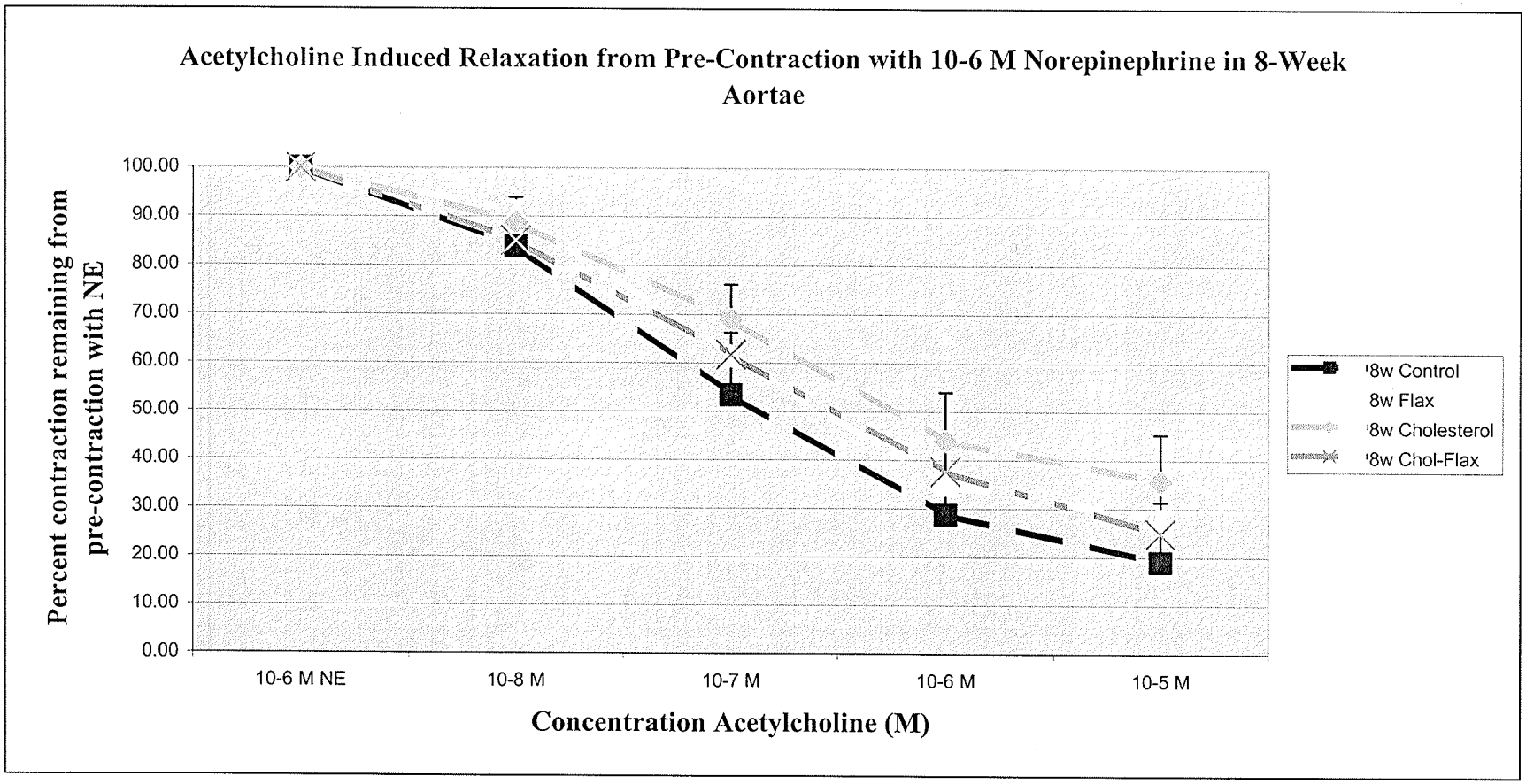


**Figure 16. Acetylcholine induced relaxation response curve after pre-contraction to 10-6 norepinephrine in 8-week aortae.** The vessels were pre-contracted and acetylcholine was introduced in increasing concentrations (10-8 to10-5 M). Maximum contraction was measured as a function of tissue wet weight. The data represented is the mean percent contraction remaining after acetylcholine administration ± standard error. No statistically significant difference amongst the groups was measured. N = 7 (control, flax and cholesterol) n = 8 (cholesterol-flax).

**F.) Mean area of plaque**

Aortae were cut open longitudinally and digitally photographed to analyze the mean area of plaque on the surface of the aorta. Figure 17 shows a representative picture of one aorta from each group after 8 weeks of dietary intervention. Representative pictures of the 6-week aortae are not shown. However, the visible representation of plaque in the 6-week aortae was similar to that found in the 8-week aortae. No plaque formation was visible in the aortae of the control and flax-fed animals. However, severe plaque formation was clearly visible in the cholesterol-fed rabbit groups. The atherosclerosis was attenuated in the cholesterol/flax-fed animals. Figure 18 shows the mean percent area of plaque formed in each of the groups after 6 weeks of feeding. Figure 19 shows the plaque formation after 8 weeks of feeding. No plaque formation occurred in the aortae of the control and flax groups. The cholesterol and cholesterol-flax fed groups had significantly greater plaque formation than the control and flax fed groups in both 6 and 8-week trials. In the 6-week trial there was a trend for the cholesterol/flax group to have less visible plaque development than the cholesterol group, no significant difference was measured between the cholesterol and cholesterol-flax groups. However, there was a statistically significant inhibition of atherosclerotic plaque formation in the cholesterol-flax group in comparison to the animals fed cholesterol alone in the 8-week trial. This method of plaque analysis visualizes and quantifies superficial plaque only.

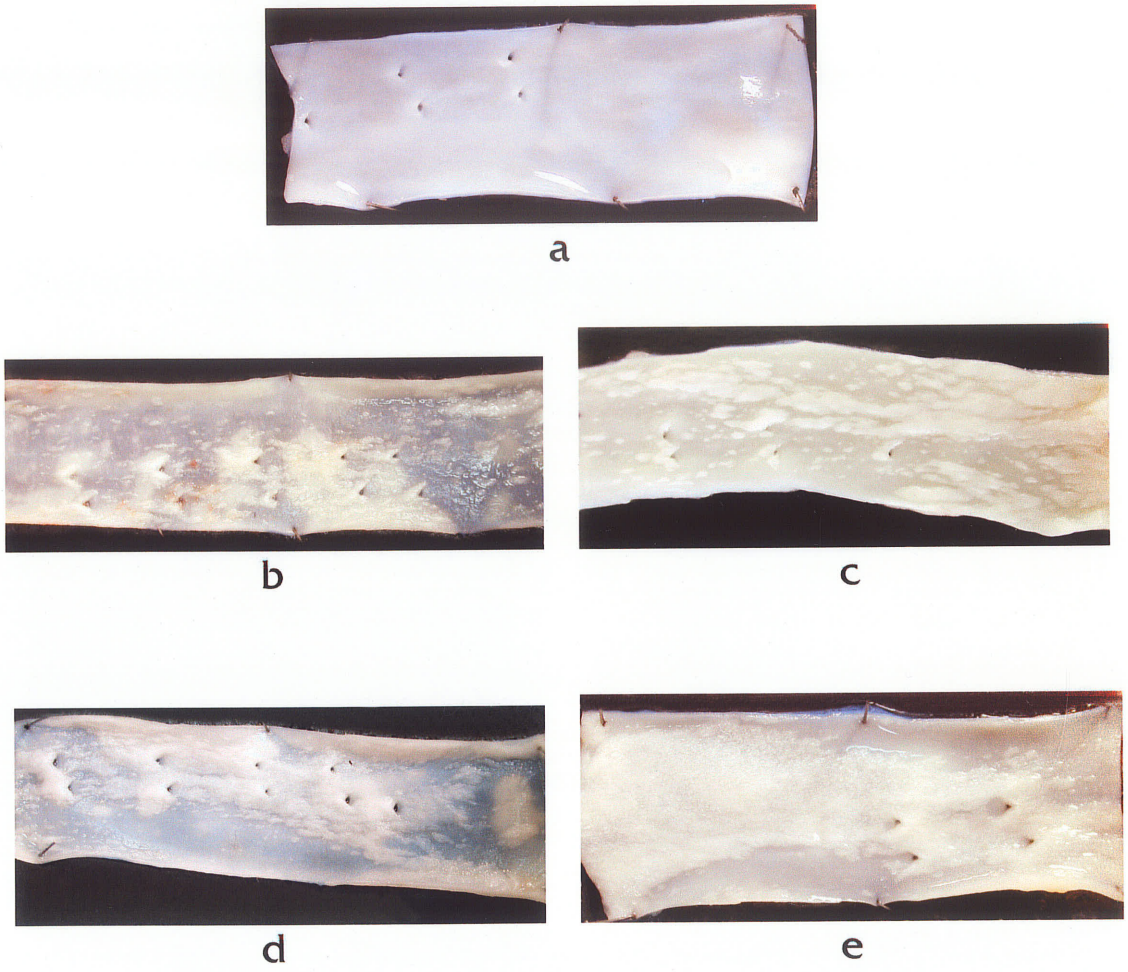


**Figure 17. Representative pictures of 6 and 8 week aortae for enface analysis of plaque formation.** The tissue was cut open longitudinally, pinned to a culture disc and digitally photographed: a) Represents the aorta of a 6-week control fed animal. No plaque was visible on the surface of these aortae. Aortae of 8-week controls and 6 and 8-week flax fed animals also had no visible plaque on their surface either. Therefore their pictures were similar to the 6-week control and are not shown. b) aorta of 6-week cholesterol-flax fed animal c) aorta of 6-week cholesterol fed animal d) aorta of 8-week cholesterol-flax fed animal e) aorta of 8-week cholesterol fed animal.

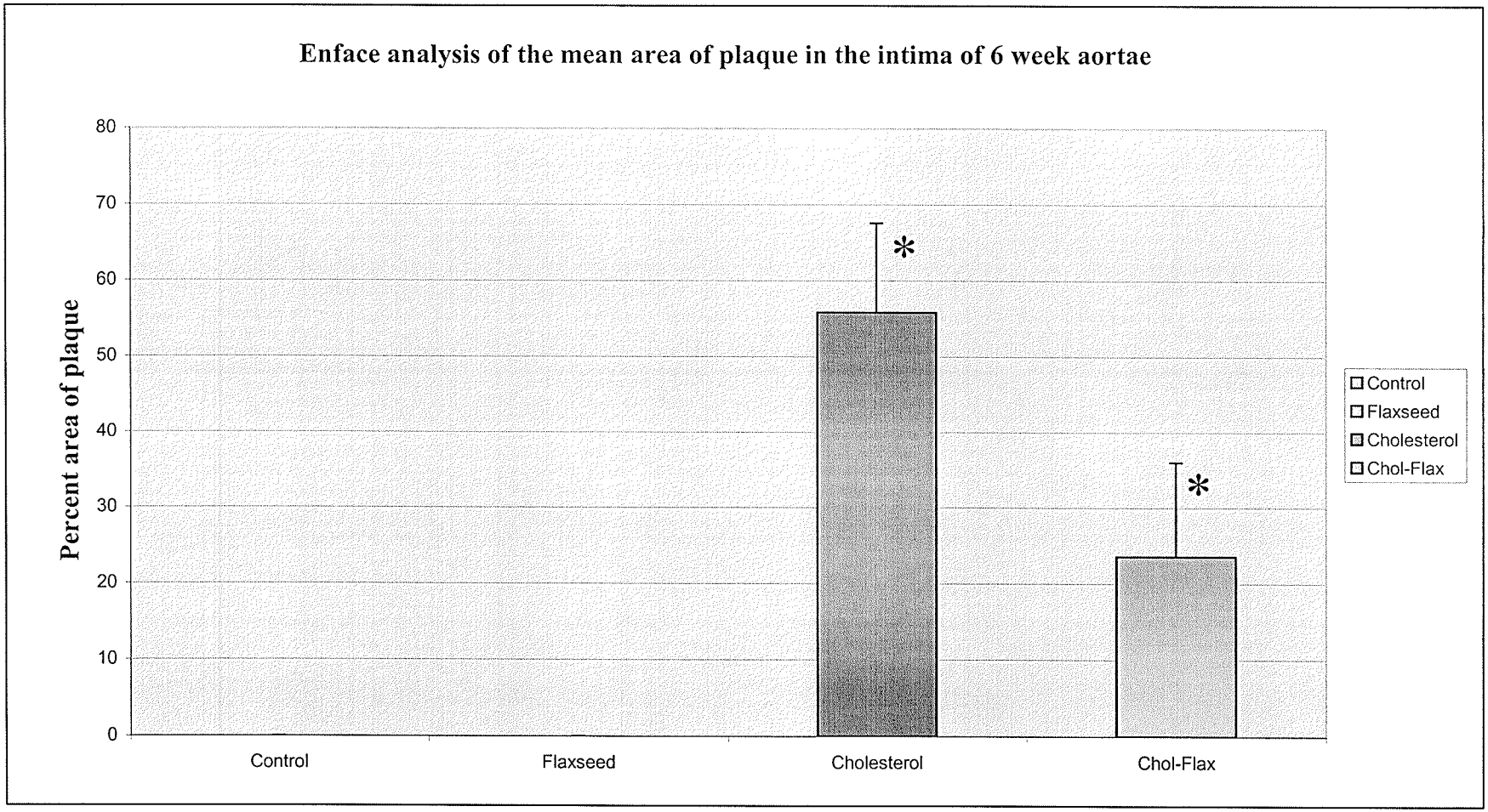


**Figure 18. Analysis of the percent plaque formation on the surface of a 6-week aortae.** Mean percents ± standard error are shown. *Represents statistical significance from control group $p<0.05$. N = 8 (control and flax), n = 7 (cholesterol) n = 6 (chol-flax).

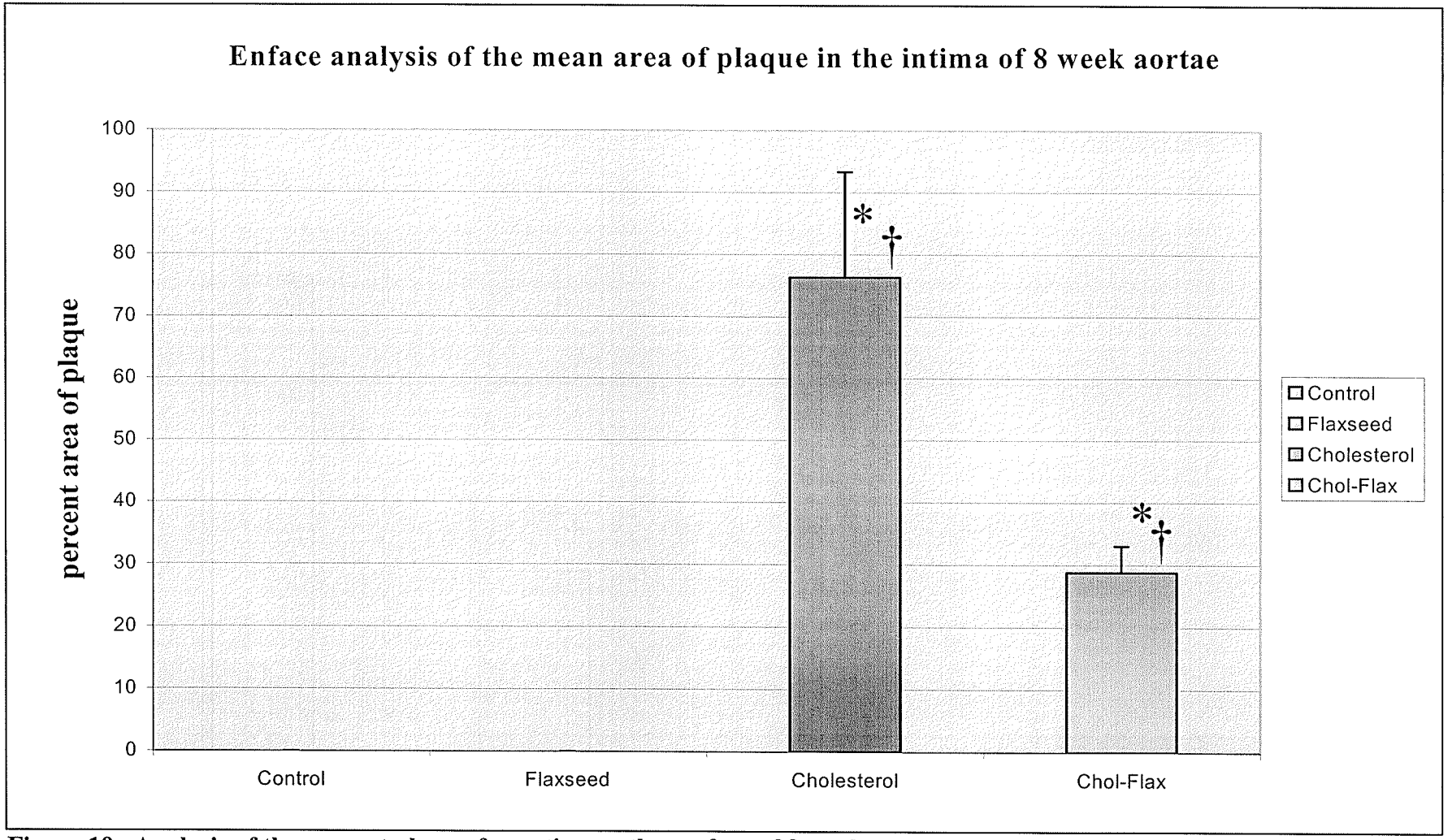


**Figure 19. Analysis of the percent plaque formation on the surface of 8-week aortae.** Mean percents ± standard error are shown. Statistical significance is represented by *or † ($p<0.05$). * Represents statistical significance in the cholesterol and cholesterol-flax fed groups compared with the control and flax fed groups. † Statistically significant difference between the cholesterol and cholesterol-flax fed group. N = 4 in all groups.

The thickness of the plaque in the intima of the aorta was measured from a cross section of the aorta stained with Oil Red O. Figure 20 shows a representative photograph of one cross section stained with Oil Red O from each of the four groups i)control ii) flax iii) cholesterol and iv) cholesterol-flax in both the a) 6- week and b) 8-week trials. Oil Red O is used to detect lipid deposition in the intima of the aorta. The stain imparts an orange-red color to fat droplets in the tissue. No plaque formation was visible in cross sections obtained from control and flax fed animals. There is significant plaque formation in the c) cholesterol and d) cholesterol-flax fed groups. Figure 21 represents the quantitative analysis of these pictures from many animals. The mean percent area of plaque for the four groups after 6 weeks of feeding is shown in Figure 21. No plaque formation was detected in any of the cross sections obtained from the control and flax fed animals. Plaque formation was significantly increased in both the cholesterol-fed and cholesterol-flax fed rabbits as compared with controls and flax fed animals. Figure 22 shows the mean area of plaque formed in the 8–week rabbits. No plaque formation was detected in any of the cross sections obtained from the control and flax fed animals. Plaque formation was significantly increased in both the cholesterol-fed and cholesterol-flax fed rabbits as compared with controls and flax fed animals. No statistical significant difference was measured between the cholesterol and cholesterol-flax groups in either trial.

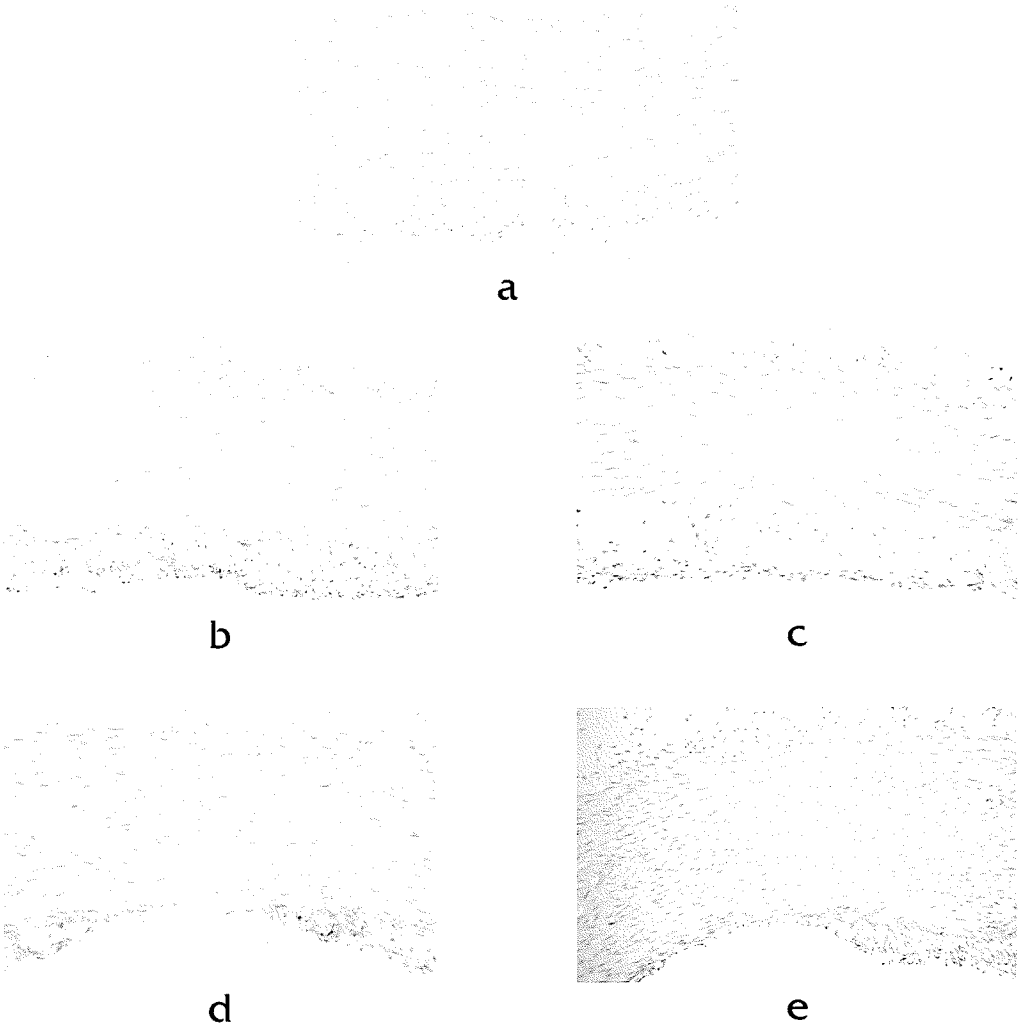


**Figure 20. Representative pictures taken of tissue cross sections stained with Oil Red O:** a) Represents the section of a 6-week control fed animal. No plaque was visible in the intima of these aortic sections as shown by no red appearance after Oil Red O staining. Cross-sections of 8-week controls and 6 and 8-week flax fed animals also had no visible plaque in the intima. Therefore their pictures were similar to the 6-week control and are not shown. b) section of aorta of a 6-week cholesterol fed animal. Note the increased deposition of lipid in the intimal layer as evident by the red staining. c) section of aorta of an 8-week cholesterol fed animal d) section of aorta of a 6-week cholesterol-flax fed animal e) section of aorta of an 8-week cholesterol-flax fed animals.

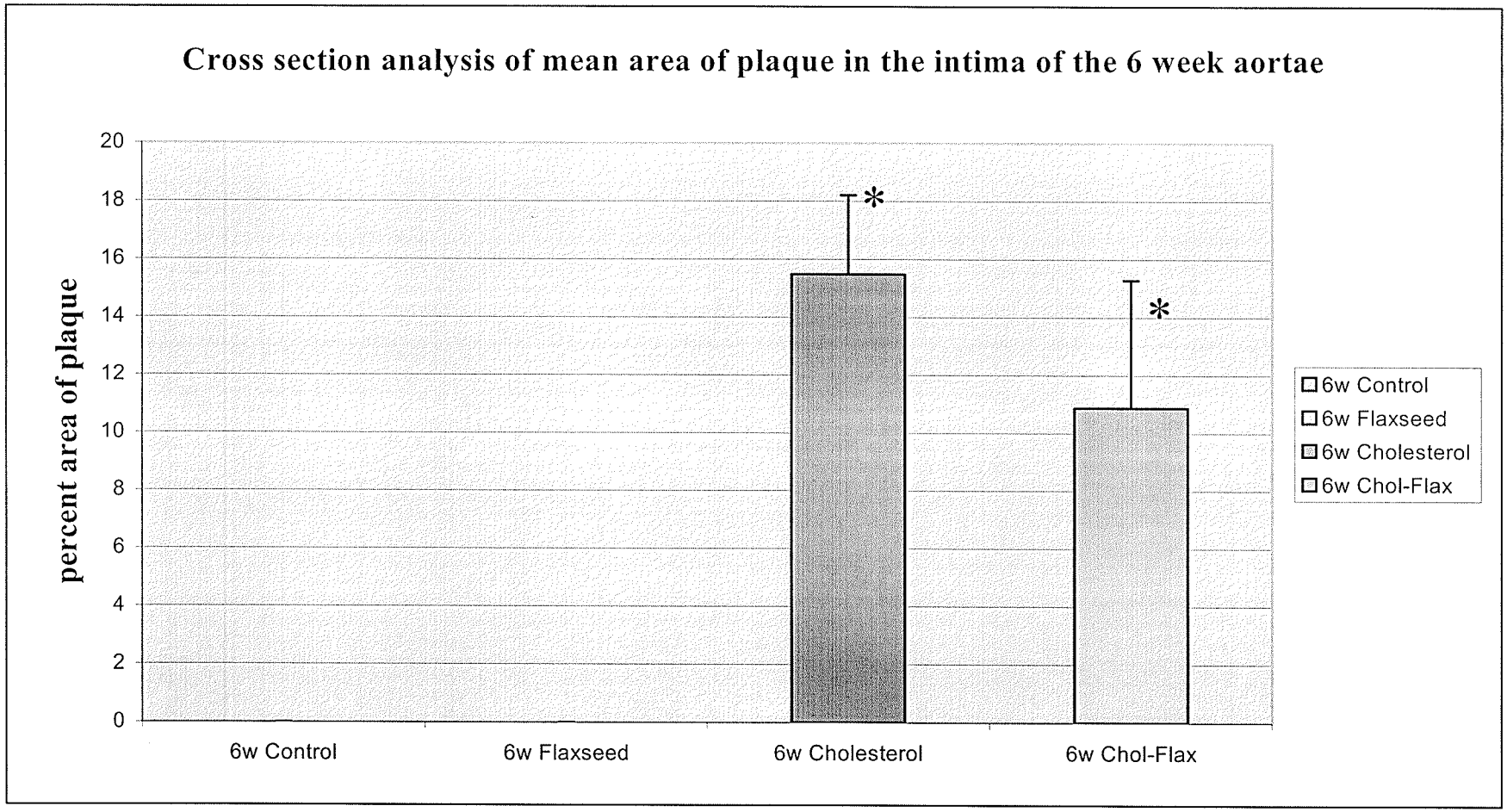


**Figure 21. Analysis of the cross sections of 6-week aortae for the mean area of plaque in the intima of the artery.** Plaque formation is detected by a red color after staining with Oil red O. Mean percents ± standard error are shown. No statistically significant difference amongst the groups was measured ($p>0.05$). * Represents statistical significance in the cholesterol and cholesterol-flax fed groups compared with the control and flax fed groups. N = 8 (control and flax), n = 5 (cholesterol and chol-flax).

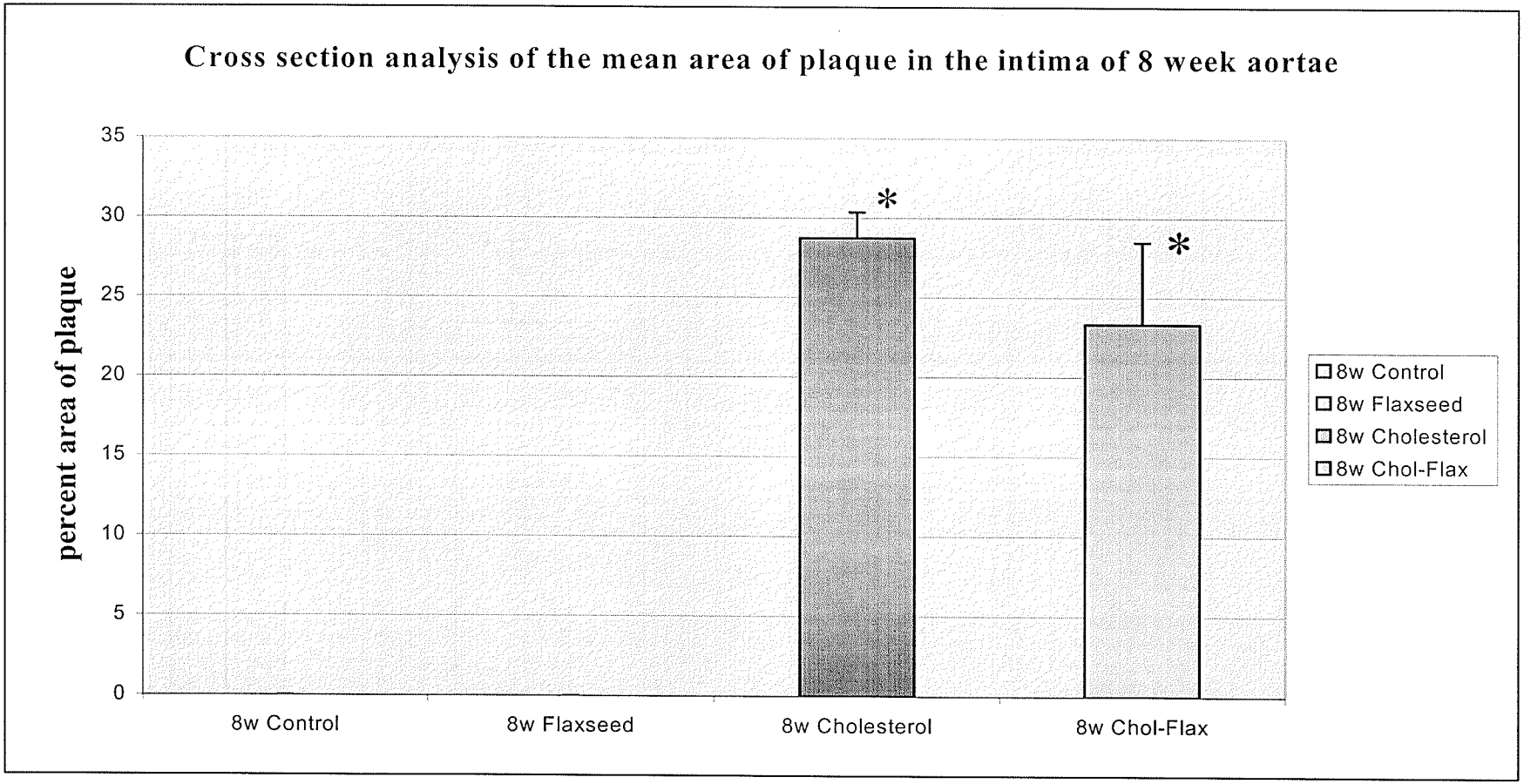


**Figure 22. Analysis of the cross sections of 8-week aortae for the percent area of plaque formation in the intima of the artery.** Plaque formation is detected by a red color after staining with Oil red O. Mean percents ± standard error are shown. No statistically significant difference amongst the groups was measured ($p>0.05$). * Represents statistical significance in the cholesterol and cholesterol-flax fed groups compared with the control and flax fed groups. N = 8 (control and flax), n = 7 (cholesterol) n = 5 (chol-flax).

The percent plaque formation on the surface of 6 and 8-week carotid arteries was measured. Figure 23 contains representative pictures of these carotid arteries. No visible plaque was present on the surface of the carotid arteries of the 6-week tissue. At eight weeks we observed a significant difference between the plaque formation that occurred in control and flax fed animals as compared to the cholesterol and cholesterol-flax fed animals. In addition the plaque formation was significantly reduced in the cholesterol-flax fed group as compared to the animals fed cholesterol alone Figure 24.

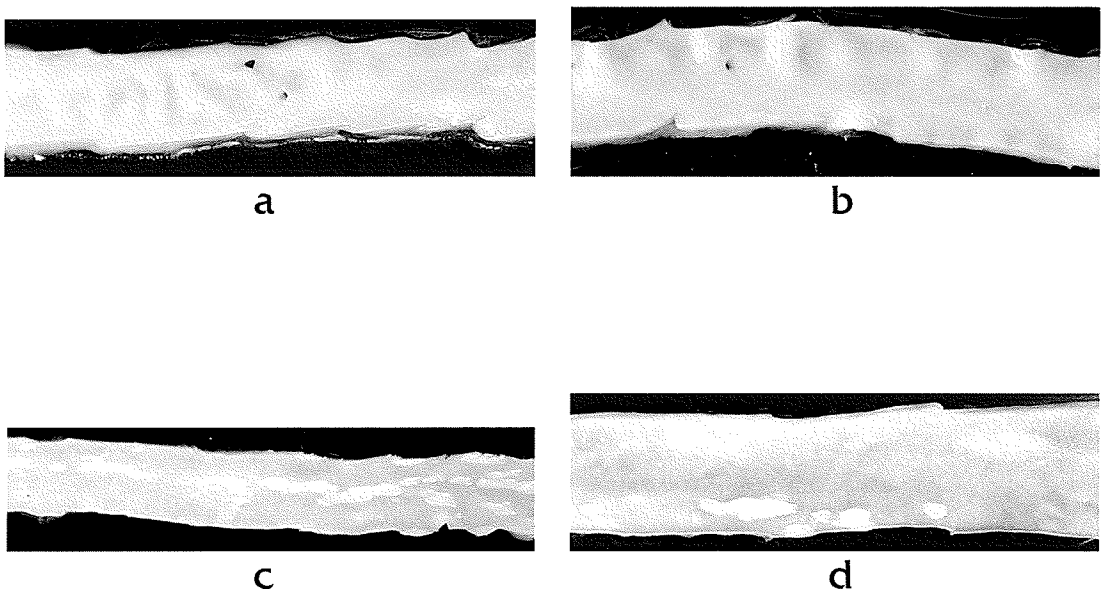


**Figure 23. Representative pictures of 6 and 8-week carotid arteries for enface analysis of plaque formation.** The tissue was cut open longitudinally, pinned to a culture disc and digitally photographed: a) carotid artery of a control fed animal b) carotid artery of a flax fed animal c) carotid artery of a cholesterol fed animal d) carotid artery of a cholesterol-flax fed animal.

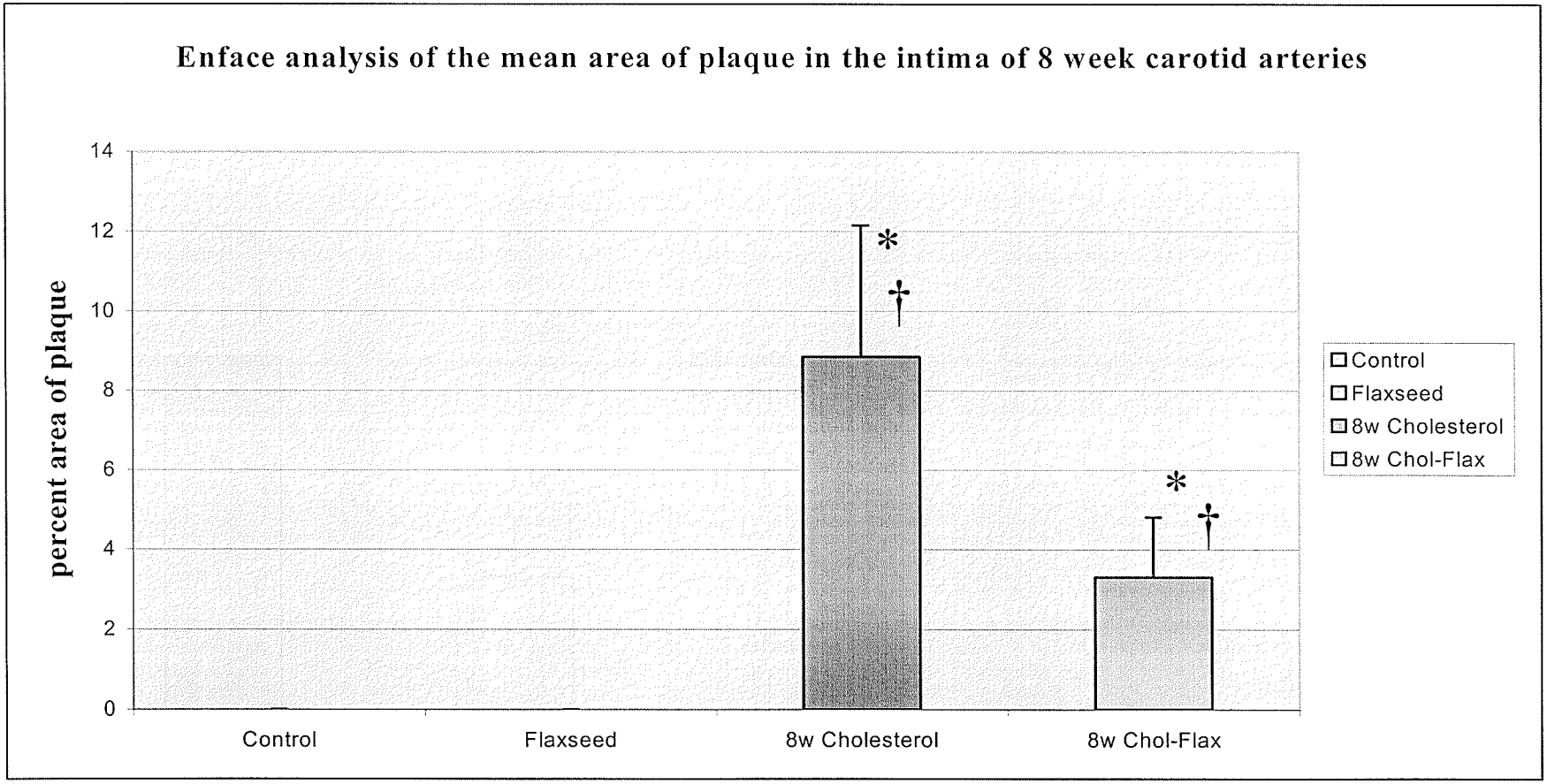


**Figure 24. Analysis of the percent plaque formation on the surface of 8-week carotid arteries.** Mean percents ± standard error are shown. Statistical significance is represented by *or † ($p<0.05$). * Represents statistical significance in the cholesterol and cholesterol-flax fed groups compared with the control and flax fed groups. † Statistically significant difference between the cholesterol and cholesterol-flax fed group. N = 4 in all groups.

# V. DISCUSSION

The hypothesis tested in this study was to whether a dietary supplementation of ground flaxseed would exhibit beneficial effects on the contractile characteristics and atherosclerotic plaque formation in the aortic vessel of rabbits fed a high cholesterol diet. Specifically, we studied the effects of a dietary supplement of flaxseed genetically bred to be high in the n-3 polyunsaturated fatty acid, ALA. We hypothesized that we would observe improved endothelial dependant relaxation in response to acetylcholine and reduced plaque formation in the vessels of animals fed a diet supplemented with flaxseed plus cholesterol in comparison to those rabbits supplemented with cholesterol alone.

This study demonstrated that increasing the dietary intake of ground flaxseed resulted in changes in plasma and tissue fatty acid levels. Significant differences were measured in plasma fatty acids in the flax, cholesterol and cholesterol-flax fed animals as compared to controls after both 6 and 8 weeks of dietary intervention. Significant changes occurred in all fatty acids, saturated, monounsaturated and polyunsaturated fatty acids in the flax, cholesterol and cholesterol-flax fed animals as compared with controls. However the main difference in plasma fatty acids levels between the flax, cholesterol and cholesterol-flax groups was in ALA. Animals fed flax alone exhibited a 7.2 fold increase and an 11.8-fold increase after 6 and 8 weeks of dietary intervention in plasma ALA levels. Cholesterol fed animals also had large plasma ALA level increases of 20-fold and 33.2 fold respectively. The cholesterol-flax supplemented groups showed the largest increases in plasma ALA increasing 161-fold and 216-fold over that of control values after the 6 and 8 week dietary intervention. It was expected that the plasma ALA

levels of both the flax and cholesterol-flax groups would have increased significantly compared to the control and cholesterol fed groups. It was surprising, however, that the cholesterol fed animals (without any flax supplementation) showed increases in plasma ALA levels greater than the flax fed animals and that the cholesterol-flax supplemented group had such immense increases in plasma ALA. This may be explained by the work of Thomson et al [46] who studied the absorption of fatty acids across the jejunum of rabbits on a high cholesterol diet. They found that cholesterol facilitated the uptake of fatty acids across the gut [46,47]. Our results may be consistent with that of Thomas et al. A cholesterol-facilitated absorption of fatty acids across the gut would increase the efficiency of ALA absorption from the flax seed as well as in the regular chow therefore resulting in elevated ALA levels in the flax, cholesterol and cholesterol-flax groups. This facilitated fatty acid uptake could also explain why the cholesterol fed and cholesterol-flax fed groups exhibited increases in all types of fatty acids, while the flax fed animals exhibited decreases in SFA and MUFA and a specific increase in plasma ALA. With regard to the other PUFA species, significant increases were also measured in AA, DHA and EPA after 6 weeks of feeding and in AA and DHA after 8 weeks of supplementation compared with controls. However, no significant difference was measured in AA between cholesterol and cholesterol-flax fed animals after 6 or 8 weeks of feeding. EPA increases were very small and DHA levels were less in cholesterol-flax fed animals than in animals fed cholesterol only. It is possible to conclude from these data, therefore, that ALA is the main polyunsaturated fatty acid being affected in the plasma by our dietary interventions.

The stimulation of ALA absorption was clearly accentuated in the cholesterol-flax fed animals. These animals exhibited a striking, significantly greater increase in plasma ALA (161-216x higher than control values). Although the fatty acid composition of the flax and cholesterol-flax diets did contain 20x the ALA content compared to the control and cholesterol diets, clearly this was not the reason for the unusually large rise in plasma ALA in the cholesterol-flax fed rabbits. It would appear that the dietary cholesterol facilitated the absorption of the ALA even more when additional ALA was present in the diet in the cholesterol-flax fed animals. This resulted in a dramatic increase in plasma ALA in the cholesterol plus flax fed animals.

These changes in plasma fatty acid composition in response to the dietary interventions were transferred to the tissue. The fatty acid composition of the aortic tissue exhibited significant changes in specific fatty acid content in response to a flaxseed enriched diet. Only ALA and DHA content in the tissue was significantly altered after inducing the flaxseed enriched diet. There was a large increase (~4 fold) in aortic ALA content after flaxseed supplementation of the diet, as expected. The content of DHA in the aortic tissue was increased but the content of DHA remained relatively low. Both the cholesterol and cholesterol-flax fed groups exhibited increases in many fatty acids as compared to control. Only the C14:0, C14:1, C18:0, C22:0 and C20:4 fatty acid species were unaltered after these dietary interventions. It is concluded once again that the cholesterol-induced stimulation of fatty acid absorption that led to increases in plasma ALA was transferred through to the tissue.

It was hypothesized that a diet supplemented with cholesterol would have a negative effect on endothelial dependant relaxation (as measured by the relaxation

response to acetylcholine). Additionally, it was hypothesized that flaxseed fed in combination with cholesterol may attenuate these negative effects. Our results from vascular function testing did not support these hypotheses. The deleterious effects of cholesterol supplementation were mild and did not achieve statistical significance. Several limiting factors may have influenced our results. First, it is possible that our in vitro methods of measuring contractility limited the effects of the dietary interventions. The tissue was removed from the body and tested without the influence of the high circulating levels of fatty acids in the blood. The vascular tissue had to rely entirely on its own endogenous stores of fatty acids to induce any changes in contractile performance. Therefore, our study eliminated any potential effects by the fatty acids on blood/vessel wall interaction (such as scavenging free radicals, influencing cell signaling events, ion channels, eicosanoid production, NO release, reducing platelet adhesiveness and altering lipid metabolism).

A significant beneficial effect of flaxseed-supplementation was observed on atherosclerotic plaque formation on the surface of the aortic and carotid vessels. As expected, supplementation of the diets of rabbits with cholesterol resulted in accelerated plaque formation. After 6 weeks of dietary supplementation with cholesterol, significant plaque formation was visible on the surface of aortic vessels of the animals. After 8 weeks of feeding, animals ingesting diets containing cholesterol exhibited more extensive plaque formation on the surface of both the carotid and aortic vessels. At 6-weeks, a reduction of 30% in the formation of visible plaque was measured in the cholesterol-flax fed group as compared to the cholesterol alone fed animals ($P > 0.05$). After 8 weeks, the reduction in the cholesterol-flax group increased to 46% compared to the cholesterol

alone fed group ($P < 0.05$). The intrinsic variability in plaque development within the animals was more evident at 6 weeks than after 8 weeks. This may have contributed to the difference not achieving statistical significance at this earlier period of dietary intervention. Regardless supplementation of flax in combination with cholesterol did result in significantly reduced plaque formation in the vessels of cholesterol-flax fed animals as compared to those animals fed cholesterol alone.

The anti-atherogenic effects of flaxseed supplementation may have been induced by a number of components within the flaxseed. For example, the beneficial effect of the cholesterol-flax diet on plaque formation could be due to changes in the fatty acid composition of the aortae. Increased n-3 fatty acid concentration has been shown to inhibit vascular smooth muscle proliferation, monocyte migration and release of platelet-derived growth factor [4,5] all occurrences that would have beneficial effects on reducing plaque formation since the presence of the above moieties are implicated in atherogenesis. DHA administered at high doses resulting in high levels of plasma DHA has been shown to reduce the expression of growth factors, adhesion molecules and pro-inflammatory cytokines [38]. There is potential that the beneficial effects of the flaxseed may be due to its ALA content which as part of the n-3 fatty acid family may induce similar beneficial cardioprotective effects to that of DHA and EPA. The marine fish oils EPA and DHA have been shown to be cardioprotective by reducing ventricular arrhythmias, exhibiting antithrombotic effects (ie reduced blood platelet reactivity, moderately longer bleeding times and reduced plasma viscosity), lowering plasma lipid levels (ie reduced fasting TAG and VLDL levels with moderate rises in HDL cholesterol and the attenuation of postprandial TAG response), improving endothelial relaxation (via

enhancement of nitric oxide-dependant and nitric oxide-independent vasodilation), inhibition of atherosclerosis and inflammation (via inhibition of smooth muscle proliferation, altered eicosanoid synthesis and the reduced expression of cell adhesion molecules), suppressing the production of inflammatory cytokines such as interleukins and tumor necrosis factor and suppressing the production of mitogens.

Plasma cholesterol levels and saturated fatty acids have been known to have negative effects on the development of Atherosclerosis [4]. These lipids increase low density lipoprotein, decrease high density lipoprotein, and increase circulating blood lipids. These effects will promote atherogenesis. However, the plasma cholesterol levels of our animals were not significantly reduced in the cholesterol-flax group as compared to the cholesterol fed animals. Therefore, it is unlikely that the anti-atherogenic effects of flaxseed were achieved through a reduction in plasma cholesterol levels. This point brings forth the question, how much a key player is plasma cholesterol in affecting atherogenesis. Another possibility to explain the anti-atherogenic effects of flaxseed is the presence of lignans in the flaxseed. Prasad et al (11,40,41) conducted three studies comparing the effects of three sources of flaxseed on hypercholesterolemic rabbits: one high in ALA with lignans, one lower in ALA with lignans, and one enriched in a purified form of lignan. They showed that the lignan isolated from flaxseed resulted in reduced cholesterol levels and had greater inhibitory effects on atherosclerosis than the high ALA form of flaxseed. They concluded that the lignan content of the flaxseed was primarily responsible for the anti-atherosclerotic effects. Finally, the antioxidant capacity of lignans may be responsible for its anti-atherogenic actions [11,40,41]. Although we did not examine this mechanism, it is a reasonable hypothesis and would be consistent with our

data. Antioxidants are known to have potent anti-atherogenic effects in animal models of atherosclerosis [48,49].

The results of our study have relevance for humans to consider with regard to the supplementation of flaxseed in the diet. Flaxseed supplementation may be beneficial for people suffering from or at risk of developing atherosclerosis. Further studies should be done to determine the clinical influence of dietary flaxseed. It is important therefore, to discuss the relevance of our dietary intervention to humans. A 10% dietary supplementation with flax provided approximately 13% of the total energetic intake to the rabbits in the present investigation. One clinical trial [50] provided 50 grams of flaxseed per day to subjects. This resulted in a mild but significant improvement in the circulating lipid levels in these subjects. This daily flaxseed supplementation represented approximately 13% of the total energetic intake in these subjects. Thus, our choice of a 10% flaxseed supplementation is equivalent in energetic intake to the 50 gram per day dosage that was used successfully previously.

Potential problems associated with flaxseed supplementation should be addressed such as altered platelet function and optimal daily intake. Dietary intervention with flaxseed could influence platelet aggregation. Fish oil and DHA and EPA are known to increase bleeding times by interfering with platelet aggregation [1,2,4]. Changes in these plasma fatty acid levels may also affect eicosanoid production and other aspects of platelet function. The ALA content of flaxseed may be detrimental to patients undergoing surgical procedures or in patients who already exhibit difficulties in forming blood clots. The dosage of flaxseed is another concern when attempting to relate the present data to the human condition. The optimal daily intake of these fatty acids is

unclear. Our data also indicate the potentially dangerous effect of combining a diet high in cholesterol with flaxseed supplementation. Such a combination may increase the absorption of the n-3 fatty acids to extremely high levels. These effects of ALA need to be studies in controlled clinical studies.

## VII. REFERENCES


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