Modulation of Myocardial Injury by Dietary Flaxseed

By

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Abstract

Flaxseed is one of the richest plant sources of omega-3 polyunsaturated fatty acid alpha-linolenic acid (ALA), lignan secoisolariciresinol (SDG), and dietary fiber. Dietary intake of flaxseed has been shown to suppress the progression and accelerate the regression of atherosclerotic plaques. However, the effect of flaxseed supplementation on cardiac damage induced by ischemic heart disease is not known. In the first study, myocardial infarction (MI) was induced by the surgical ligation of the left anterior descending coronary artery in rats. The addition of ground flaxseed, flax oil rich in ALA, and flax lignan rich in SDG to the diet before and after the induction of the MI decreased the incidence of arrhythmias, reduced myocardial infarct size, prevented excessive left ventricular dilatation, decreased myocardial fibrosis, and lowered the levels of inflammatory proteins and collagen compared to the untreated MI control. Dietary flax oil supplementation upregulated the expression of microRNAs 133a, 135a, and 29b which exert an antiarrhythmic, antiapoptotic and antifibrotic effect in myocardial tissue. Based on these positive results, the effect of flaxseed was further investigated in the JCR:LA-*cp* rat model of metabolic syndrome that naturally develops spontaneous myocardial ischemic lesions. A high-fat high sucrose diet was employed to exacerbate metabolic and cardiac dysfunction and any potential sex differences were also investigated. The lean genotype of JCR:LA-*cp* rats served as control animals. Dietary flaxseed supplementation reduced total cholesterol and low-density lipoprotein-cholesterol levels in obese male JCR rats. A high-fat high-sucrose diet compounded the metabolic dysfunction evident from liver injury in obese male animals. The addition of flaxseed to the diet improved liver weight and markers of hepatic injury. A high-fat high-sucrose diet increased the frequency of myocardial ischemic lesions in male and female obese JCR rats and abolished the protective effect of female sex on cardiac structure and function. Dietary flaxseed supplementation reduced myocardial

ischemic lesions and improved cardiac systolic and diastolic function in JCR:LA-*cp* rats. Collectively, these findings in myocardial ischemia and in MIs further extend the beneficial cardiovascular effects of flaxseed. It can also be concluded that dietary flaxseed has cardioprotective potential as a therapeutic option in conditions of ischemic heart disease or cardiac complications associated with metabolic syndrome.

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Contribution of authors

The manuscripts included in this thesis are multi-authored. Therefore, an outline of contribution of authors is included below in the order of appearance in the thesis.

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- Parikh: literature collection and analysis, data interpretation, writing of the manuscript, creation of figures
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- Pierce: writing and editing of the manuscript

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- Parikh: creation of figures, writing of the manuscript
- Maddaford: data collection and analysis
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- Netticadan: intellectual input and editing of the manuscript
- Pierce: writing and editing of the manuscript

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- Kura: animal feeding, data collection and analysis

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- Austria: animal feeding, performed fatty analysis, data collection
- Yu: echocardiography
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- Proctor: intellectual inputs on study design
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List of abbreviations

ACE	Angiotensin converting enzyme
ACS	Acute coronary syndrome
ALA	Alpha-linolenic acid
ALT	Alanine aminotransferase
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
APAF-1	Apoptotic protease activating factor-1
$ApoE^{-/-}$	Apolipoprotein E knock-out
ARBs	Angiotensin II receptor blockers
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BP	Blood pressure
CABG	Coronary artery bypass grafting
CAL	Coronary artery ligation
CFlax	Control diet supplemented with 10% ground flaxseed
CHD	Coronary heart disease
CKMB	Creatine kinase myocardial band
CMR	Cardiac magnetic resonance
CO	Cardiac output
CT	Computed tomography
CTGF	Connective tissue growth factor
CTn	Cardiac troponin

CVD	Cardiovascular diseases
DAPK2	Death associated protein kinase 2
DBP	Diastolic blood pressure
DHA	Docosahexaenoic acid
DiHETrE	Dihydroxy-eicosatrienoic acid
ECG	Electrocardiogram
EDV	End-diastolic volume
EF	Ejection fraction
END	Enterodiol
ENL	Enterolactone
e-NOS	Endothelial nitric oxide synthase
EPA	Eicosapentaenoic acid
EpETrE	Epoxy-eicosatrienoic acid
ESV	End-systolic volume
FS	Fractional shortening
GJA1	Gap junction protein alpha 1
GP	Glycoprotein
HCN	Hydrogen cyanide
HDL	High-density lipoprotein
HF	Heart failure
HFHS	High-fat high-sucrose
HFlax	HFHS diet supplemented with 10% ground flaxseed
HMGB1	High mobility group box 1

HR	Heart rate
hs-cTnT	high-sensitivity cardiac troponin T
HSP	Heat shock protein
IHD	Ischemic heart disease
IL	Interleukin
Irx5	Iroquois homeobox domain 5
IVRT	Isovolumetric relaxation time
IVS	Interventricular septum
KCND2	Shal-related family member 2
LA	Linoleic acid
LAD	Left anterior descending coronary artery
LDL	Low-density lipoprotein
$LDLr^{-/-}$	LDL receptor knock-out
LGE-CMR	Late gadolinium enhancement of CMR
LV	Left ventricle
LVID	Left ventricular internal diameter
LVPW	Left ventricular posterior wall thickness
MABP	Mean arterial blood pressure
MCP-1	Monocyte chemoattractant protein-1
MI	Myocardial infarction
miRs	MicroRNAs
mPTP	Mitochondrial permeability transition pore
mRNA	Messenger RNA

MUFA	Monounsaturated fatty acid
MV	Mitral valve
n-3	Omega-3
NCX1	Sodium-calcium exchanger type 1
NF-kB	Nuclear factor-kB
NLRP3	NOD-like receptor containing pyrin 3
NSTE-ACS	Non-ST-segment elevation acute coronary syndrome
NSTEMI	Non-ST-segment elevation MI
PAD	Peripheral artery disease
PCI	Percutaneous coronary intervention
PGPC	1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine
POVPC	1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine
PPARs	Peroxisome proliferator-activated receptors
PTP1B	Protein tyrosine phosphatase 1B
PUFAs	Polyunsaturated fatty acids
PVC	Premature ventricular contraction
RAGE	Receptors for advanced glycation end products
RISC	RNA-induced silencing complex
ROS	Reactive oxygen species
RV	Right ventricle
SBP	Systolic blood pressure
SCD	Sudden cardiac death
SCFAs	Small chain fatty acids

SDG	Secoisolariciresinol diglucoside
sEH	Soluble epoxide hydrolase
SFA	Saturated fatty acid
SHR	Spontaneously hypertensive rats
SPARC	Secreted protein acidic and rich in cysteine
SPECT	Single-photon emission computed tomography
SREBP-1c	Sterol regulatory element-binding protein-1c
STEMI	ST-segment elevation MI
SV	Stroke volume
TAC	Transverse aortic constriction
TC	Total cholesterol
TF	Tissue factor
TG	Triglycerides
TGF- β	Transforming growth factor-β
TIMI	Thrombolysis in Myocardial Infarction
TNF-α	Tumor necrosis factor-α
TRPM7	Transient receptor potential melastatin 7
TSP	Thrombospondin
TXA_2	Thromboxane A_2
TXNIP	Thioredoxin-interacting protein
VEGF	Vascular endothelial growth factor
VF	Ventricular fibrillation
VT	Ventricular tachycardia

CHAPTER 1: LITERATURE REVIEW

1.1. Introduction

Cardiovascular diseases (CVD) are a leading cause of death globally according to the World Health Organization. According to the Heart Disease and Stroke Statistics – 2020 Update, ischemic heart disease (IHD) is the most common type of CVD, accounting for 42.6% of all the CVD related deaths in the US (1). Although recent advances have resulted in a substantial improvement in the outcomes of IHD, it remains the major cause of morbidity and mortality (2). Therefore, new scientific discoveries are needed for improving the management of IHD. Dietary intake of omega-3 polyunsaturated fatty acids (n-3 PUFAs) is an attractive therapeutic option. While marine-derived n-3 PUFAs are widely studied and have been shown to reduce the risk of CVD, plant-based n-3 PUFAs are less explored. A plant grown on the Canadian prairies, flax, is one of the richest plant sources of n-3 PUFAs in the form of alpha-linolenic acid (ALA). The n-3 ALA is contained within the seed of flax. In addition, flaxseed contains the lignan secoisolariciresinol and dietary fiber. Dietary flaxseed supplementation in animals has suppressed atherosclerotic plaque formation in growing (3-5) and established plaques (6), whereas in human studies, flaxseed has lowered cholesterol levels (7) and blood pressure in peripheral artery disease patients with elevated blood pressure (8, 9). The beneficial vascular effects of flaxseed provide the rationale for further examinations of the effect of dietary flaxseed on cardiac damage caused by myocardial ischemia. The objectives of this thesis are fourfold: i) to investigate the effects of dietary flaxseed on arrhythmias and left ventricular remodeling after an MI induced surgically by ligation of the left anterior descending coronary artery; ii) to explore the molecular mechanisms responsible for the potential cardioprotective effect of flaxseed in an MI state; iii) to study the effect of dietary flaxseed in a model of spontaneous myocardial ischemia compounded by

metabolic syndrome; and iv) to examine whether a high-fat and high sugar diet will induce aspects of CVD and determine if dietary flaxseed can protect against this.

1.2. Cardiovascular diseases

1.2.1. Incidence

Cardiovascular diseases are the leading cause of death, as they claim more lives every year than all forms of cancer and respiratory diseases combined (10). According to the latest estimates by the Heart Disease and Stroke Statistics – 2020 update, approximately 1 of every 3 deaths occur due to CVD (1). Ischemic heart disease is the major cause of mortality as 43.2% of cardiovascular deaths are attributed to it alone, followed by stroke (16.9%) (10). Physical inactivity, genetics, smoking, elevated levels of blood pressure and cholesterol, diabetes, hormonal changes, and a diet rich in salt and fat are important risk factors for CVD (11).

1.2.2. Ischemic heart disease

Ischemic heart disease (IHD), also termed as coronary heart disease (CHD) is a condition that arises when there is an imbalance between myocardial oxygen supply and demand which results in myocardial hypoxia and accumulation of waste metabolites (12). IHD is most often caused by coronary atherosclerosis and thus it is also called coronary artery disease (CAD) (12). In addition to CAD, other conditions that may result in an imbalance between myocardial oxygen supply and demand can also cause myocardial ischemia. Decreased oxygen supply due to hypotension, anemia, or excessive bleeding and increased oxygen demand in conditions such as extreme exercise, tachycardia, acute hypertension, or aortic stenosis may cause myocardial ischemia in the absence of coronary atherosclerosis (12).

1.2.3. Consequences of ischemia

A reduction in coronary blood flow reduces the amount of oxygen being delivered to the myocardium thus resulting in the cessation of aerobic metabolism, a shift to anaerobic glycolysis, depletion of creatine phosphate, and a fall in tissue ATP levels. When ischemia continues, tissue acidosis is produced which results in potassium efflux, and reduced ATP levels fail to maintain the membrane function leading to myocyte death (11). Brief periods of ischemia that cause a reversible injury are tolerated by myocytes as no cell death occurs. However, extended periods of more than 20 minutes results in an irreversible injury that extends as a wavefront of necrosis from the subendocardial to the subepicardial layer of the heart (13). The cardiomyocyte death due to this irreversible injury results in the development of a myocardial infarction.

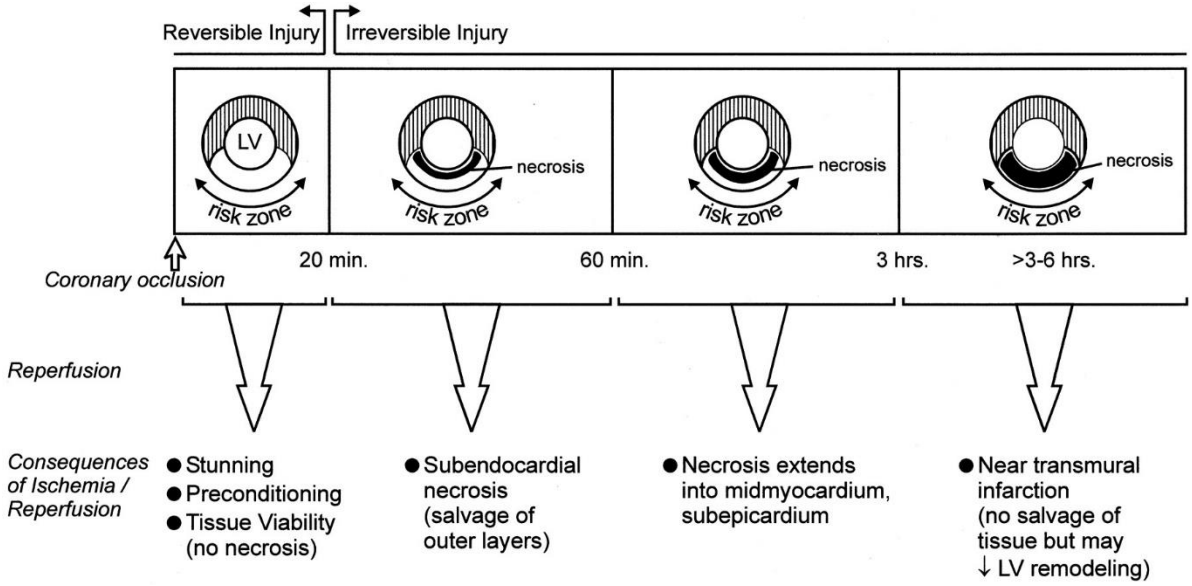


Figure 1. Effect of ischemia and reperfusion on heart-based studies in an anesthetized canine model of proximal coronary artery occlusion. Brief periods of ischemia of <20 minutes followed by reperfusion are not associated with the development of necrosis (reversible injury). Brief ischemia/reperfusion results in the phenomenon of stunning and preconditioning. If the duration

of coronary occlusion is extended beyond 20 minutes, a wavefront of necrosis marches from subendocardium to subepicardium over time. Reperfusion before 3 hours of ischemia salvages ischemic but viable tissue. (This salvaged tissue may demonstrate stunning.) Reperfusion beyond 3 to 6 hours in this model does not reduce myocardial infarct size. Late reperfusion may still have a beneficial effect on reducing or preventing myocardial infarct expansion and left ventricular (LV) remodeling (13). Kloner RA, Jennings RB. Consequences of Brief Ischemia: Stunning, Preconditioning, and Their Clinical Implications. Circulation. 2001;104(24):2981-9. *Reprinted with permission.*

1.2.4. Acute coronary syndrome

Acute coronary syndrome (ACS) is a term that is used to describe a group of clinical characteristics compatible with myocardial ischemia (14). ACS includes unstable angina (UA), non-ST-segment elevation myocardial infarction (NSTEMI), and ST-segment elevation myocardial infarction (STEMI) (15-17). UA can serve as an imminent precursor to myocardial infarction and has a similar pathophysiology to NSTEMI. UA and NSTEMI are collectively termed as non-ST-segment elevation ACS (NSTE-ACS) (18).

ACS results from a sudden total (STEMI) or partial (NSTEMI) coronary artery occlusion caused frequently by the rupture or erosion of atherosclerotic plaques that form an intraluminal thrombus interrupting blood flow, and thus reducing the oxygen supply to the myocardium (15-17). The end result of ACS is myocyte necrosis that may be detected by the elevation of cardiac biomarker levels depending upon the severity of ischemia which is determined by the degree and duration of vessel occlusion, the amount of myocardium supplied by the occluded vessel, and the presence of collateral circulation (18).

1.3. Myocardial infarction (MI)

The fourth universal definition by the joint European Society of Cardiology (ESC)/American College of Cardiology (ACC)/American Heart Association (AHA)/World Heart Federation (WHF) of a clinical myocardial infarction is the presence of acute myocardial injury detected by abnormal cardiac biomarkers and the evidence of acute myocardial ischemia (19).

1.3.1. Clinical presentations of MI

The STEMI and NSTEMI have similar signs and symptoms that are not differentiated until the ECG examination has been completed. The common presentation in both cases of an MI is chest pain which often lasts longer than 20 mins and radiates to the left arm, left shoulder, neck, or jaw. The patients may also experience diaphoresis, vomiting, palpitations, dyspnea, or syncope (11). Approximately 20% of acute myocardial infarctions are silent (painless) or atypical (all other symptoms except significant pain), and are most common in women, elderly patients, and in diabetics (20).

1.3.2. Diagnosis of MI

The diagnosis of MI usually relies on the triad of chest discomfort, the detectable quantities of markers for myocardial necrosis and the changes in the electrocardiogram (ECG) suggestive of ischemia or infarction (18, 20).

Cardiac troponin (CTn) isoforms CTnI and CTnT are components of the cardiac contractile machinery and are the preferred biomarkers for cardiac injury (19). The ischemia from myocardial injury in a STEMI and NSTEMI, but not UA, is severe enough to produce detectable quantities of CTn in the blood from the necrotic myocardium (21). CTn levels are detectable within 2-3 hours, and peak within 24-48 hours of MI (22). The detection of cardiac troponin (CTn) levels above the 99^{th} percentile upper reference limit (URL) indicates myocardial injury (19). Since the

development of a high-sensitivity cardiac troponin T (hs-cTnT) assay, there has been an improvement in differential diagnosis with a 20% increase in the diagnosis of NSTEMI and a concomitant decrease in the diagnosis of UA (17). CTn can remain in the circulation for 7-10 days or even longer in cases of renal failure. So, unless its levels are falling or subsequently rising again, serial CTn detection is limited to detecting early ischemic events or recurrent MI (18). Other biomarkers such as creatine kinase myocardial band (CKMB) are less specific and sensitive. Both the US and European guidelines emphasize CTn as the preferred biomarker and CKMB is less sensitive in detecting MI (18). The injury is acute if there are dynamic rise and fall of the CTn values above the 99th percentile URL, and chronic if values are persistently elevated (19).

A 12-lead electrocardiogram (ECG) is obtained and examined for ischemic changes within 10 mins of patients arriving at the emergency department (20). Electrocardiographic (ECG) changes in NSTEMI and UA include ST-segment depression and T wave inversion. According to the fourth universal definition of MI, ST-segment depression should be new horizontal or downward sloping in at least 2 contiguous leads with ≥0.5 mm and T wave inversion ≥1 mm with prominent R waves (19). For STEMI, new ST-segment elevation at the J-point should be ≥1 mm in at least two contiguous leads. The J-point which is a junction between QRS complex termination and the onset of ST-segment serves as a reference point for determining the magnitude of the ST-segment shift (19). A STEMI typically results in transmural MI as the injury transects the thickness of the myocardial wall. As a consequence, pathologic Q waves are seen on the ECG in a STEMI, however, as NSTEMI is limited to the subendocardium layer only, such changes are not observed (15).

Other laboratory examinations in an MI patient may include a complete blood count, lipid and metabolic profile. Patients may have a slightly elevated white blood cell count because of the

pro-inflammatory conditions that commonly accompany a MI. Moreover, it is imperative to assess the presence of anemia or perform a platelet count if a patient is going to receive a thrombolytic agent. Potassium and magnesium ion levels are closely monitored as alterations can result in fatal arrhythmias (23). The initial risk assessment of MI patients needs to address the risk of an early adverse outcome. Two validated models that have been developed to assess this risk include the Thrombolysis in Myocardial Infarction (TIMI) and the Global Registry of Acute Coronary Events (GRACE) models (20).

Non-invasive imaging techniques commonly used for diagnosing and characterizing myocardial injury and an MI include echocardiography, a myocardial perfusion scan (MPS) using single-photon emission computed tomography (SPECT) or positron emission tomography (PET), computed tomography (CT), and cardiac magnetic resonance (CMR) (15). The important imaging parameters are myocardial perfusion, viability, thickening, and motion. Each of the techniques provides information about myocardial perfusion and function to a certain extent. While the radionuclide-based technique gives a direct assessment of myocardial viability because of the tracers used, other techniques provide an indirect assessment (10).

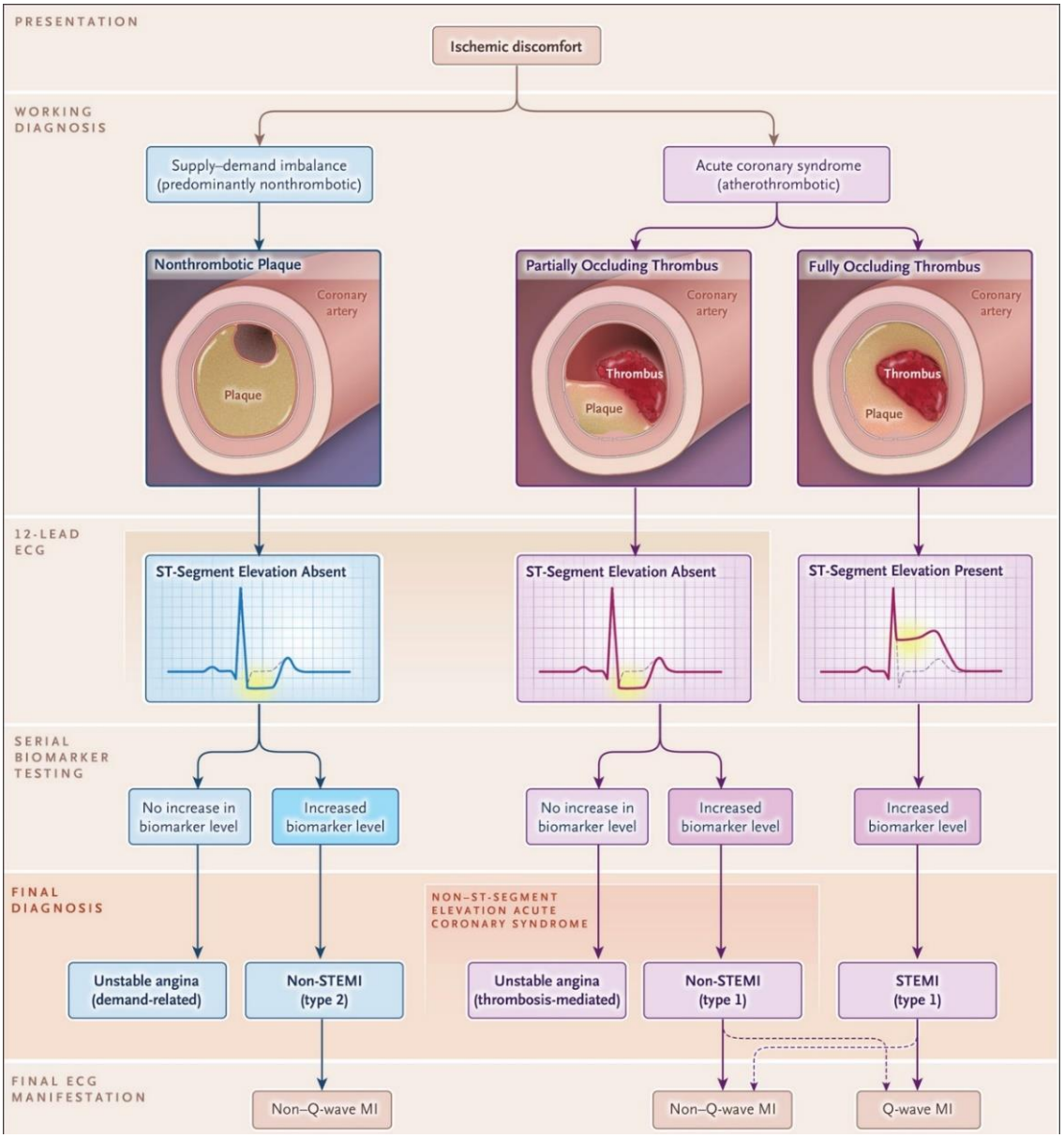


Figure 2. The spectrum of Pathologic and Clinical ST-Segment Elevation Acute Myocardial Infarction (STEMI) and Non-STEMI Acute Coronary Syndromes. Reproduced with permission from Anderson JL, Morrow DA. Acute Myocardial Infarction. New England Journal of Medicine. 2017;376(21):2053-64, Copyright Massachusetts Medical Society.

1.3.3. Types of MI

Type 1

A Type 1 MI is caused by atherothrombotic coronary artery disease and is usually aggravated by atherosclerotic plaque rupture or erosion. It is defined by the following criteria: the rise and fall of CTn values above the 99th percentile URL with at least one of the following a) symptoms of acute myocardial ischemia, b) ECG changes suggestive of new ischemia, c) pathological q wave formation, d) regional wall motion abnormality or new loss of viable myocardium evident on imaging studies, or e) postmortem or intracoronary imaging demonstration of a coronary thrombus (19). The intracoronary thrombus rupture may lead to distal coronary embolism that results in myocyte necrosis (24). The ECG findings are integral in classifying a Type 1 MI into ST-elevation myocardial infarction (STEMI) or non-ST elevation myocardial infarction (NSTEMI), which then can be treated by appropriate guidelines (19).

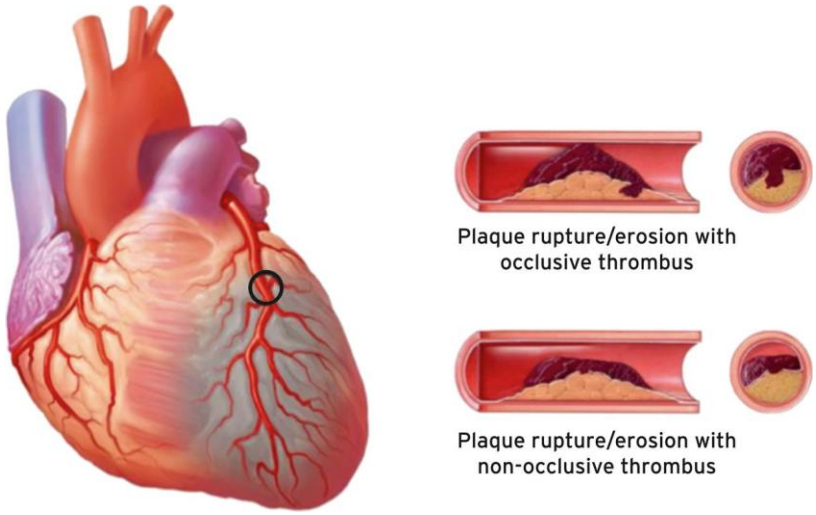


Figure 3. Type 1 myocardial infarction. Thygesen K, Alpert JS, Jaffe AS, Chaitman BR, Bax JJ, Morrow DA, et al. Fourth Universal Definition of Myocardial Infarction (2018). Journal of the American College of Cardiology. 2018;72(18):2231-64. *Reprinted with permission.*

Type 2

An MI caused by ischemic injury due to an imbalance of myocardial oxygen supply and demand is designated as Type 2 MI. The criteria for defining a Type 2 MI is similar to the ones for Type 1, except for the identification of a coronary thrombus. Atherothrombotic plaque disruption is not a characteristic of a Type 2 MI. It is more common in women and mortality rates for patients with Type 2 MI have been reported to be higher than for Type 1 MI patients (25). The imbalance produced by the insufficient blood flow to the myocardium to meet the increased demand for oxygen is multifactorial. Several stressors such as coronary atherosclerosis, coronary embolism, microvascular dysfunction, coronary artery spasm, coronary artery dissection, arrhythmias, hypotension/hypertension, severe anemia, and respiratory failure have been identified as causative factors (19). The treatment strategy includes the correction of the underlying ischemic imbalance by blood pressure management, volume adjustment, heart rate control, and respiratory support (26).

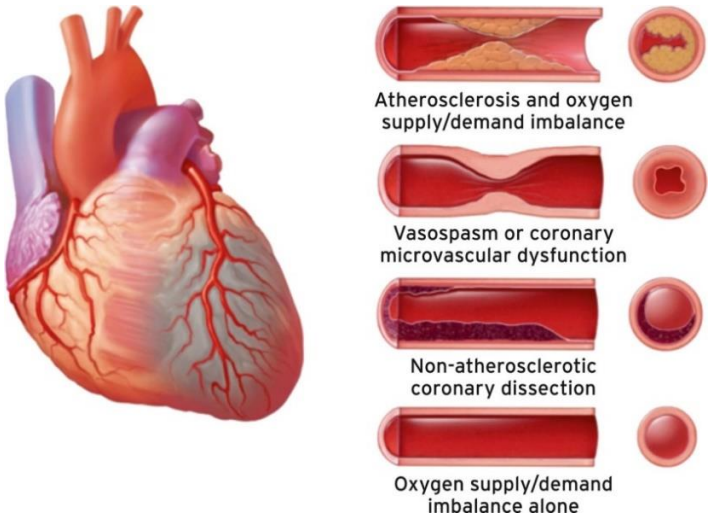


Figure 4. Type 2 myocardial infarction. Thygesen K, Alpert JS, Jaffe AS, Chaitman BR, Bax JJ, Morrow DA, et al. Fourth Universal Definition of Myocardial Infarction (2018). Journal of the American College of Cardiology. 2018;72(18):2231-64. *Reprinted with permission.*

Type 3

An MI with suspected acute myocardial ischemic injury but lacking the cardiac biomarker evidence is designated as a Type 3 MI (19, 27). Usually, these patients have typical clinical manifestations of myocardial ischemia/infarction including ischemic ECG changes as well as ventricular fibrillation. However, mortality rates escalate soon after the onset of symptoms and before the elevation of cardiac biomarkers can occur or before the blood can be collected for the detection of cardiac biomarkers (19, 27). This category facilitates differentiation of fatal MI from sudden death that may be non-ischemic or non-cardiac in origin (19). Once a Type 3 MI diagnosis is made and a post-modern examination reveals the presence of a fresh or recent thrombus in the infarct-related artery, Type 3 is redesignated as Type 1 MI (19). A Type 3 MI is a rare clinical observation with an annual incidence of 10/100,000 person-years and a frequency of only 3-4% among all MI types (28).

Type 4a

The myocardial injury may occur during or after the cardiac revascularization procedures such as percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG). In the former case, it reflects a periprocedural issue, while the latter case could be because of complications of a device used in the procedure such as early or late stent thrombosis or in-stent restenosis for PCI, or graft occlusion or stenosis with CABG (19). The increased values of CTn in the patients following a PCI or CABG reflect the procedural myocardial injury which can be assessed by late gadolinium enhancement on cardiac magnetic resonance (LGE-CMR) (29, 30).

The development of a PCI-related MI in patients within 48 hours of the index procedure is defined as Type 4a MI. It is characterized by a post-PCI elevation of CTn >5 times the 99th

percentile URL in the MI patients with normal baseline values of CTn. In the patients with preprocedural elevated CTn values that are stable (<20%) or falling, but the post-PCI rise is >20% to an absolute value of >5 times the 99th percentile URL are also classified as having a Type 4a MI (19). In addition to the elevated CTn values, one of the following criteria is also required to define a Type 4a MI; a) ECG changes suggestive of new ischemia, b) pathological q wave formation, c) regional wall motion abnormality or new loss of viable myocardium evident on imaging studies, or d) angiographic or postmortem examinations revealing a procedure-related complication such as coronary artery dissection, epicardial artery or a side branch occlusion/thrombosis, collateral flow disruption, or distal embolization (19).

Type 4b

A subcategory of PCI-related MI is stent/scaffold thrombosis or Type 4b MI which needs to be confirmed by angiography or autopsy examination along with the criteria used for a Type 1 MI (19). The timing of the occurrence of the stent/scaffold thrombosis associated with the timing of the PCI procedure is essential. The following temporal categories are advised: 0-24 hours, acute; >24 hours to 30 days, subacute; >30 days to 1 year, late; and >1 year, very late after the stent/scaffold implantation (31).

Type 4c

An MI can occur occasionally by in-stent restenosis or restenosis following balloon angioplasty in the infarct artery. This PCI-related MI is classified as a Type 4c MI and defined as focal or diffuse restenosis, or a complex lesion using the same criteria for a Type 1 MI (19).

Type 5

Several factors can result in procedural myocardial injury during a CABG. When estimated by LGE-CMR, 32-44% of CABG patients suffer from procedural myocardial injury (19). A CABG-related MI that can occur within 48 hours of the index procedure is defined as an elevation of CTn values >10 times the 99th percentile URL in patients with normal baseline values or with elevated preprocedural CTn values that are stable or falling. However, a post-CABG elevation must be >10 times the 99th percentile URL. One of the following criteria is also required for designating a Type 5 MI: a) pathological q wave formation, b) regional wall motion abnormality or new loss of viable myocardium evident on imaging studies, or c) angiographic evidence of a graft occlusion or a new native coronary artery occlusion (19).

1.3.4. Etiopathogenesis of MI

A MI is primarily caused by the reduction in blood flow to the myocardium which is mainly due to atherosclerotic plaque formation complicated by coronary thrombosis. The inciting event is the plaque rupture that exposes the necrotic core of the plaque to the blood that activates platelets and clotting factors and thus results in thrombosis (24, 32). As mentioned earlier, there can be other causes as well for the decrease in myocardial oxygen supply and the demand imbalance causing a MI.

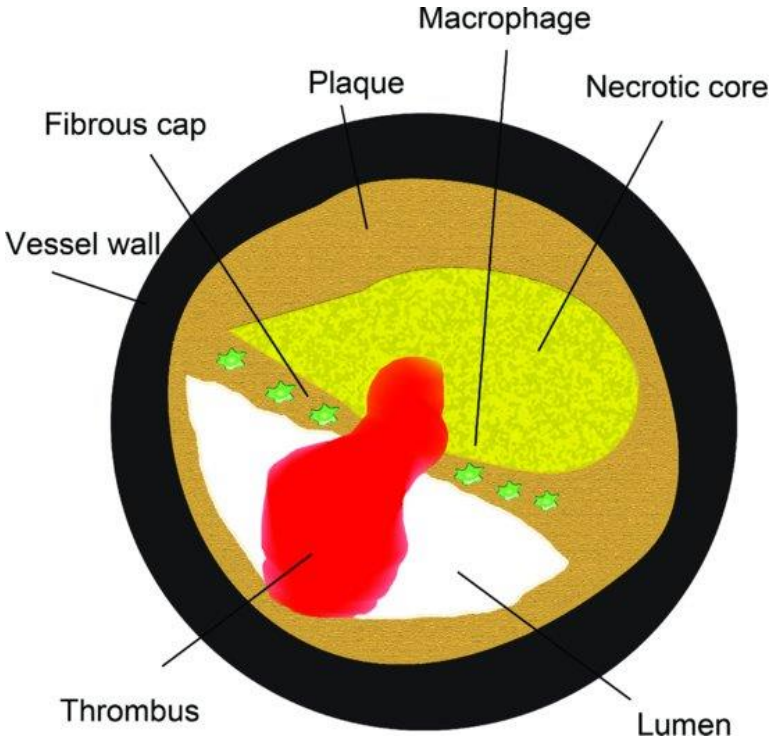


Figure 5. Myocardial infarction is usually caused by rupture of a vulnerable plaque leading to formation of an occlusive thrombus in a coronary artery. A "vulnerable plaque" is often associated with inflammation, activation of macrophages in the fibrous cap, and proteolytic degradation of the matrix. Frangogiannis NG. Pathophysiology of Myocardial Infarction. Comprehensive Physiology. 2015;5(4):1841-75. *Reproduced with permission.*

Platelets play an essential role in the initiation and progression of the thrombotic event (33). Endothelial dysfunction caused by several risk factors including hypertension, smoking, and hyperlipidemia, can result in immediate platelet adhesion to the exposed collagen via von Willebrand factor (34). Various mediators such as thromboxane A2, adenosine diphosphate, and thrombin, facilitates the activation of platelets. After the activation process, the content of the α-granules is released from platelets which further contributes to platelet activation and vasoconstriction. Platelet activation also results in a conformation change and thus exposure of the

glycoprotein (GP) receptors IIb/IIIa on the platelet surface. Fibrinogen binds to 2 GP IIb/IIIa receptors on the adjacent platelets forming platelet aggregation and platelet-rich thrombus (33). Atherosclerotic plaques are rich in tissue factor (TF) which is responsible for the initiation of the coagulation cascade (35). This TF comes into contact with blood and combines with clotting factor VIIa when an atherosclerotic plaque is disrupted. This complex of TF-VIIa activates factor X which results in the conversion of prothrombin to thrombin and fibrinogen to fibrin. The crosslinking of the fibrin polymers and entrapment of the red blood cells complete the coronary thrombus formation occluding the vessel (33).

The flow restriction due to severely narrowing plaques results in the development of ischemia (36). The cellular effects of ischemia begin within seconds of hypoxia and results in the sudden cessation of aerobic metabolism. Cardiomyocytes under ischemic conditions use pathways other than oxidative phosphorylation to generate adenosine triphosphate (ATP). The supplies of creatine phosphate, which is the source of high energy phosphate, are rapidly depleted and anaerobic glycolysis becomes the main metabolic pathway to generate ATP (36, 37). However, in the absence of perfusion, glucose cannot be supplied to the cardiomyocytes and thus intracellular glycogen stores serve as the main substrate for glycolysis. Moreover, anaerobic glycolysis is not as efficient as oxidative phosphorylation in producing ATP at a rate commensurate to its consumption. The end-product lactate accumulates creating intracellular acidosis which inhibits the activity of many enzymes involved in glycolysis. As a result of this, within 15-20 min of ischemia, the rate of anaerobic glycolysis decreases and the process is halted. With a marked reduction in the ATP levels, ischemic cardiomyocytes cannot maintain homeostasis and thus undergo irreversible changes (36). The metabolic changes during ischemia result in profound electrophysiologic changes with disturbances of ionic balance (38). An efflux of potassium ions

(K^+) is associated with ischemia and accumulation of extracellular K^+ causes a reduction in the cardiac action potential. As more cardiomyocytes die during irreversible injury, more K^+ is released into the extracellular spaces resulting in conduction block and inexcitability. Acidosis due to ischemia also affects the function of various ion channels causing a reduction in resting membrane potential, prolongation of the action potential duration and development of early-after depolarizations. With the reduction in outward pumping, increases in the intracellular sodium (Na^+) and calcium (Ca^{2+}) levels occur. These alterations can create an arrhythmogenic environment (36).

Both apoptosis and necrosis have been implicated in the death of cardiomyocytes following an MI (36). Recent evidence indicates that both types of cell death are intertwined and occur in a continuum (39). Apoptosis, a programmed cell death process, results in the removal of dead cardiomyocytes without eliciting an inflammatory reaction, whereas necrosis, which is an unregulated process, acts as a danger signal and thus triggers intense inflammation (36). The relative contribution of apoptosis and necrosis in cardiomyocyte death following an infarct remains unclear. However, studies have suggested that during ischemia the death of cardiomyocytes may be due to necrosis, while reperfusion may stimulate proapoptotic pathways resulting in apoptotic cell death (40). In the infarcted myocardium, apoptosis appears to be mediated through the activation of an intrinsic pathway involving mitochondrial signaling as well as an extrinsic pathway facilitated by the activation of membrane death receptors (36). Although apoptosis begins with the onset of ischemia, its major impact is on ventricular remodeling after an MI (11). The critical event activating apoptosis in cardiomyocytes involves the permeabilization of the outer mitochondrial membrane (OMM) which results in the release of apoptogens such as cytochrome C and apoptosis-inducing factor into the cytoplasm. Cytochrome C binds to the apoptotic protease

activating factor 1 (Apaf-1) and activates procaspase-9. Subsequently, caspase-9 is activated which ultimately cleaves several cellular proteins causing apoptosis (36, 41). In contrast, necrosis is mediated through the opening of an inner mitochondrial pore termed the mitochondrial transition pore (mPTP). Cytosolic calcium in the ischemic cardiomyocytes enter mitochondria triggering the opening of mPTP and resulting in a) a sudden loss of the mitochondrial membrane potential and subsequent cessation of ATP production, and b) mitochondrial swelling due to the influx of water. These alterations can rupture the OMM and cause a release of apoptogens with cell death as a consequence (42).

The role of autophagy is implicated in the survival of cardiomyocytes following an MI (43). During a stress condition, autophagy can promote the survival of cardiomyocytes by releasing energy substrates through the degradation of unnecessary cellular components and by removal of damaged or dysfunctional constituents (44). However, an uncontrolled autophagic process can deplete cells of essential constituents and organelles resulting in autophagic cell death (45). In ischemic cardiomyocytes, autophagy is mediated through the activation of the AMP-activated protein kinase (AMPK)-dependent pathway (46). Increasing evidence suggests a positive impact of autophagy on cardiomyocytes during an MI (47), however, the effect of autophagy at this stage is controversial as deleterious effects have also been observed (43).

Due to a negligible regenerative capacity of the heart, the death of a billion cardiomyocytes following an MI results in their replacement with a non-contractile collagen-based scar that maintains cardiac structural integrity and prevents catastrophic complications such as cardiac rupture (36). The cardiac repair following an MI occurs in three distinct infarct healing phases: a) the inflammatory phase, b) the proliferative phase, and c) the maturation phase (36).

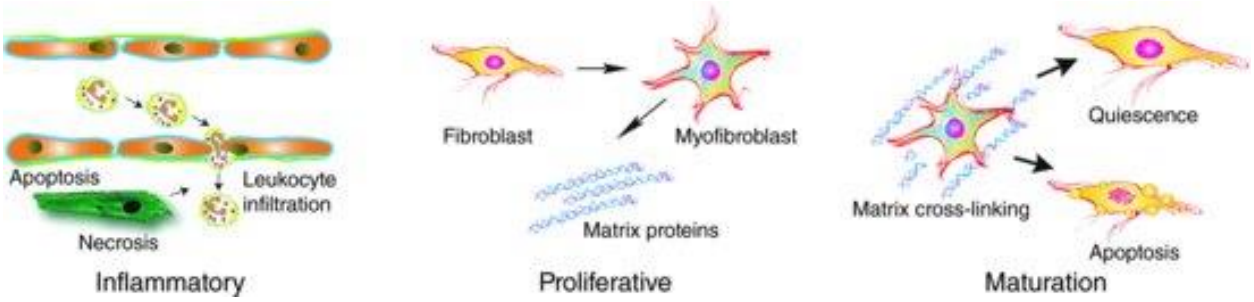


Figure 6. The phases of repair following myocardial infarction. Alarmins released by necrotic cardiomyocytes trigger an intense inflammatory reaction that serves to clear the infarct from dead cells and matrix debris. Removal of dead cells induces suppression of proinflammatory signaling, leading to the transition to the proliferative phase. Fibroblasts acquire a synthetic myofibroblast phenotype and deposit extracellular matrix proteins and a rich neovascular network is formed. Finally, during the maturation phase, the extracellular matrix is cross-linked, while infarct fibroblasts become quiescent and may undergo apoptosis. Frangogiannis NG. Pathophysiology of Myocardial Infarction. Comprehensive Physiology. 2015;5(4):1841-75. *Reproduced with permission.*

The inflammatory phase is characterized by the activation of the innate immune system to remove the dead cells. Necrotic cell death releases a range of endogenous molecules called 'alarmins', that triggers an immune response by activating toll-like receptors (TLR), receptors for advanced glycation end products (RAGE), and interleukin (IL) receptors (48). Alarmins such as high mobility group box 1 (HMGB1) are critical regulators of immune pathways following an MI (49). Other intracellular contents such as members of the S100 family (S100A8/A9), interleukin (IL-1α), heat shock proteins, as well as matrix proteins including fibronectin and hyaluronan released during the degradation of extracellular matrix by the activation of latent collagenases, are

also implicated in activating immune responses (36). The normal heart has a substantial antioxidant defense system that inhibits the generation of reactive oxygen species (ROS) through the activity of glutathione peroxidase, superoxide dismutase, and catalase enzymes. However, excessive ROS production following an MI overwhelms the defense system. Generation of ROS is essential for cardiac healing following an MI, however, excessive ROS-mediated signaling promotes cell apoptosis and induces degradation of the extracellular matrix (36). Overactive ROS then promotes leukocyte chemotaxis, activates the complement system, and upregulates the expression of cytokines and chemokines through the activation of the nuclear factor (NF)-kB system (50). In the infarcted myocardium, the NF-kB system induces the expression of several pro-inflammatory cytokine genes (36). Chemokines such as monocyte chemoattractant protein (MCP-1) play an important role as a chemoattractant in the infarcted myocardium (51).

During the proliferative phase of infarct healing, anti-inflammatory pathways are triggered which suppress and resolve the inflammatory reaction. This is followed by infiltration of the infarct with activated myofibroblasts and vascular cells (36). The effective suppression or resolution of inflammation is crucial as overactive or prolonged inflammation can adversely impact cardiomyocytes and impair ventricular geometry by excessive matrix degradation and fibrotic expansion (36). Phagocytic ingestion and thus removal of dead neutrophils release anti-inflammatory molecules such as IL-10, transforming growth factor- β (TGF-β), and pro-resolving lipid mediators including lipoxins, protectins, resolvins, and maresins (52). The pro-resolving lipid mediators promote clearance of dead neutrophils by macrophages and terminate leukocyte recruitment (53). Dying neutrophils can also suppress inflammation by releasing annexin A1 and lactoferrin mediators that can abrogate further recruitment of neutrophils (54). The clearance of matrix degradation proteins is also important for the resolution of inflammation. The hyaluronan

fragments participate in the resolution of inflammation by interacting with a transmembrane adhesion molecular CD44, which is a glycoprotein that is involved in a variety of cell-cell and cell-matrix interactions (55). During this proliferative phase, cardiac fibroblasts undergo phenotypic changes and transition into a synthetic, myofibroblast-like phenotype. These cells have several unique characteristics including expression of contractile proteins, such as α-smooth muscle actin, and an increased synthesis of extracellular matrix proteins (56). Although the cellular origin of infarct myofibroblasts remains less known, however, several sources have been suggested. The resident fibroblasts are proposed as the main contributor of the activated myofibroblasts (57). Additional sources include cardiac pericytes, blood-derived fibroblast progenitor endothelial cells undergoing mesenchymal transition, epicardial cells, and vascular smooth muscle cells (36, 58). Angiotensin II, aldosterone, growth factors, matricellular proteins such as ED-A fibronectin are involved in the activation of myofibroblasts in the healing infarct (36). Growth factors such as TGF-β are required for the conversion of fibroblasts to myofibroblasts and this action is mediated through the Smad3 signaling that stimulates α-SMA expression (59). Other mediators involved in the activation of infarct myofibroblasts include fibroblast growth factor, platelet-derived growth factor, and the mast cell proteases such as tryptase and chymase (60-63). Furthermore, extracellular matricellular proteins such as thrombospondin (TSP-1, -2), osteopontin, secreted protein acidic and rich in cysteine (SPARC), tenascin-C, and periostin are upregulated in the infarcted myocardium and regulate the healing response (36).

The maturation phase is characterized by progressive changes in the composition of the infarct and the extracellular matrix. Collagen undergoes crosslinking through the upregulation of the enzyme lysyl oxidase (64). The expression of matricellular proteins decreases and the number of activated myofibroblasts is reduced. Infarct myofibroblasts may either become quiescent, or

undergo apoptosis, or exhibit suppressed synthetic capacity with reduced expression of contractile proteins (36). Defects in this process can result in excessive fibrosis and adverse ventricular remodeling (36).

On cessation of blood flow during coronary occlusion, the contractile function of the zone of myocardium supplied by that vessel is adversely impacted (11). The following four abnormal contraction patterns develop: a) dyssynchrony, which is a dissociation of contraction between adjacent segments with respect to time, b) hypokinesis, which is a reduction in the extent of shortening, c) akinesis, which is termination of shortening, and d) dyskinesis, which is expansion and systolic bulging (11). STEMI patients may also exhibit a condition termed 'ischemia at a distance', where the non-infarcted zone has impaired myocardial contractile function because of the loss of collaterals from the occluded vessel (65). The early abnormality that develops even with infarcts involving only a small portion is ventricular stiffness in diastole (11). Diastolic dysfunction impairs ventricular compliance. The ventricular relaxation is an energy-dependent process and the impaired energetics during ischemia affects ventricular filling and relaxation (66). Left ventricle contractile function is depressed when a sufficient portion of the myocardium is affected and cardiac output, stroke volume, blood pressure, and peak dp/dt decreases, whereas end-systolic volume increases. When 15% of the left ventricular segment is affected, the left ventricular ejection fraction (LVEF) drops and left ventricular pressure and volume rise. Clinical heart failure and cardiogenic shock develop when >25% and >40% of the LV myocardium is lost, respectively (11).

The consequences of a large MI are alterations in the LV geometry. The LV becomes dilated and more spherical. The infarcted zone becomes thinner while the viable myocardium undergoes hypertrophy. These changes are referred to as ventricular remodeling which affects

ventricular function and prognosis (67). Cardiac hypertrophy is an adaptive process during post-infarct remodeling that counteracts excess load, prevents progressive ventricular dilation and stabilizes cardiac contraction (67). Hypertrophy induces and regulates several transcription factors such as natriuretic peptides, insulin-like growth factor-1, and transforming growth factor-β1 (67). Infarct size, ventricular volume, loading conditions, and patency of the infarct artery determine the process of LV dilation (68). Ventricular dilation is associated with a higher risk of arrhythmias and mortality, the progression of systolic dysfunction and a worsening of prognosis (69). Elevated ventricular volume and pressure exert stress on the myocardial wall increasing the risk of infarct expansion, whereas a patent infarct artery expedites the process of scar formation and reduces the risk of infarct expansion and ventricular dilation (11). Infarct expansion usually accompanies adverse ventricular remodeling following an MI. It is described as acute thinning and dilation of the infarcted area that is not explained by additional cardiomyocyte necrosis (11, 70). Infarct expansion can be explained by several unique processes such as: a) the side-to-side slippage and thus rearrangement of cardiomyocytes, which reduces the number of layers across the ventricular wall, b) the disruption of normal cardiomyocytes; and c) the degradation of the extracellular matrix by matrix metalloproteinases that contributes to chamber dilation (11, 71). The apex is particularly vulnerable to infarct expansion as its thinnest wall is exposed to higher stress (72).

Cardiac remodeling is a complex pathological condition where many molecular components are involved. At present, many investigations are focusing on dissecting the molecular mechanisms involved in post-infarction remodeling and heart failure. Endogenous *miRs* have important roles in various cardiovascular pathologies (73, 74). Altered levels of *miR* have been reported in ischemic/reperfused hearts in animal and human studies. He *et al.* (75) observed an upregulation of 10 *miRs* and a downregulation of 6 *miRs* in Sprague-Dawley rat hearts subjected

to 1 h ischemia followed by 3 h reperfusion. In a total coronary occlusion rat model of myocardial infarction, Dong *et al.* (76) reported differential expression of 38 *miRs* in the infarcted zone and 33 *miRs* in the border zone compared with the non-infarcted zone. Bostjancic *et al.* (77) examined infarcted heart tissue from MI patients and found 77 dysregulated *miRs*, of which 47 *miRs* changed within 1-week post-MI and 30 *miRs* were altered 4 weeks post-MI.

1.4. MicroRNAs and cardiovascular disease

Several microRNAs (*miRs*) have been shown to be involved in cardiac pathological processes such as acute myocardial infarction, cardiac arrhythmias, and hypertrophy (78). The role of *miRs* is increasingly being recognized as diagnostic, prognostic markers or therapeutic targets for cardiovascular diseases. A study by Da Costa Martins *et al*. (79) reported that genetic deletion of a nuclease enzyme involved in *miR* biogenesis resulted in maladaptive cardiac remodeling and heart failure. This underlines the importance of *miR*s for cardiac function. *MiRs* can either promote or inhibit the apoptosis of cardiomyocytes, they can modulate angiogenesis, alter cardiac regeneration and reprogram cardiac fibroblasts into cardiomyocytes (80). These effects clearly support a relationship between altered *miR* expression and ischemic heart disease.

1.4.1. Biology of microRNAs

MicroRNAs are endogenous, noncoding, single-stranded RNAs of 22-26 nucleotides that inhibit messenger RNA (mRNA) expression through the Watson-Crick base pairing between sequences located in the three prime untranslated regions (3'UTR) of mRNA and *miR* (78, 80). *MiRs* are transcribed by RNA polymerase II as pri-miRs, which are ~ 2 kb in length. In the nucleus, pri-miRs are cleaved by the RNase III-type enzyme *Drosha*, *Dgcr8*, and other factors to produce a short stem-loop structure of ~ 70 nucleotides called pre-miRs (81). They are then exported out

of the nucleus through exportin-5 into the cytoplasm where they undergo further processing by the ribonuclease *Dicer* producing a double-stranded RNA molecule (80).

One strand of the *miR* duplex is selected as mature *miR* and the other one, known as the "passenger strand", is eliminated and degraded (74, 80). This selection process is determined by the strength of base pairing between the two strands of *miR* duplex (74). These then associate with the Argonaute protein to form the RNA-induced silencing complex (RISC) (80). *miRs* in RISC can bind to specific mRNAs by identifying *miR* recognition elements (seeds) located in the 3' UTR of target mRNAs. The nucleotides at the 2-8 position at the 5'-end of the *miRs* must be complementary to the 'seed' region of the target mRNAs for *miRs* to exert their functional effects of translational depression or direct degradation of the target mRNAs (78, 80). The greater degree of complementarity between *miRs* and target mRNAs is more likely to result in target mRNA degradation (82).

The *miRs* can either bind to target mRNA and undergo degradation or can be released from cells through the following export mechanisms (83, 84). The pre-miR can be incorporated into multivesicular bodies that fuse with the plasma membrane and are then released into the extracellular space and the circulation in the form of exosomes. The cytoplasmic *miRs* can also be secreted from the cells as microvesicles through membrane blebbing or shedding. They can also be released in the form of apoptotic bodies by some cells types such as endothelial cells. The circulating *miRs* can thus be found either in a "free" form complexed with proteins or in the membrane-bound bodies (83, 84). Circulating *miRs* are derived from the heart, liver, lung, kidney, and blood cells (82). As the circulating *miRs* reside either in the exosomes, microvesicles or apoptotic bodies, they are protected against degradation by the endogenous RNase activity (82).

This stability of circulating *miRs* can be being exploited to use them as diagnostic and prognostic biomarkers for diseases.

The human genome is estimated to encode approximately 1000 *miRs* that can target hundreds of distinct mRNAs (82). Based on the genomic locations of *miR* genes, *miRs* are classified into the following four types: a) the intergenic *miRs*, which are transcribed from their own genes; b) the intronic *miRs*, which are transcribed from the introns of their host protein-coding genes; c) the exonic *miRs*, which are transcribed from the exons of their host protein-coding genes; and d) the untranslated region *miRs*, which are transcribed from the 3' or 5' UTR of the protein-coding genes (85).

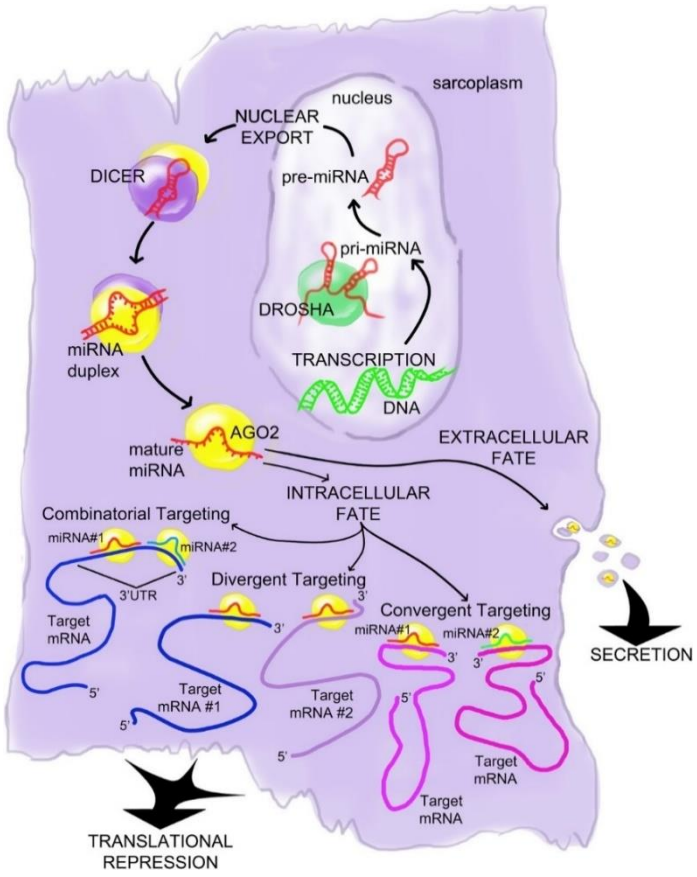


Figure 7. Schematic of miRNA Biogenesis and Function. Condorelli G, Latronico MVG, Cavarretta E. microRNAs in Cardiovascular Diseases: Current Knowledge and the Road Ahead. *Journal of the American College of Cardiology* 2014;63**:** 2177-2187. *Reprinted with permission.*

1.4.2. Myocardial infarction and microRNAs

Cardiac injury during a myocardial infarction (MI) is caused by ischemic and hypoxic conditions. An MI can cause cardiac cell death, impaired cardiac signaling which can lead to infarct expansion, amplified oxidative stress, and remodeling of surviving cardiomyocytes. Cardiac remodeling starts as an adaptive mechanism to maintain heart function. However, this process becomes maladaptive in pathological conditions and consequently, cardiac fibrosis, dilated cardiomyopathy and heart failure develop.

Impairment in the function of ion channels, transporter proteins, intracellular calcium handling proteins, and other relevant proteins during an MI can create substrates which can predispose the heart to abnormalities in electrical conduction and arrhythmias. Recent studies have highlighted the importance of *miRs* in the regulation of cardiac rhythm (80). *MiRs* that play important roles in the cardiac remodeling and arrhythmias that occur during an MI are discussed in the following section.

1.4.3. MicroRNA-1

MiR-1 is one of the most abundant *miRs* expressed in the myocardium (86). Its overexpression is observed in coronary artery disease patients (87). The *miR-1* family consists of a *miR-1* subfamily containing *miR-1-1* and *miR-1-2* transcripts. However, these two subfamily members are located on two separate chromosomal regions, 20q13.33 and 18q11.2, respectively (86).

An approximately 2.6-fold increase in *miR-1* expression was accompanied by arrhythmias in a rat MI model of permanent coronary artery ligation (80). Increased expression of *miR-1* results in a widening of the QRS complex, indicating slowed cardiac conduction which explains the role of *miR-1* as an arrhythmogenic agent in ischemic hearts. *MiR-1* post-transcriptionally inhibits the potassium voltage-gated channel subfamily J member 2 (*KCNJ2*), which codes for the Kir2.1 protein. Kir2.1 is the subunit of the K+ channel which carries the inward rectifier potassium current (80). A reduction in Kir2.1 prolongs the action potential duration and QT interval, resulting in higher incidences of ventricular tachyarrhythmia (86). *MiR-1* represses the gap junction protein alpha 1 (*GJA1*), which encodes the connexin-43 gap junction channel protein, thus slowing down the cardiac conduction in the setting of an MI (87). Other targets of *miR-1* include the notch ligand delta, the Rho GTPase *Cdc42*, Iroquois homeobox domain 5 (*Irx5*), and Shal-related family member 2 (*KCND2*) (88).

These cellular mechanisms could be responsible for the arrhythmogenic potential of *miR-1*. Consistent with this hypothesis, the introduction of *miR-1* worsened arrhythmias, while suppression through antisense inhibitors reduced arrhythmias in rats undergoing an MI (88). Moreover, propranolol, a β- blocker used as an antiarrhythmic agent, can downregulate *miR-1* expression and thus improve cardiac conduction (89). Interestingly, the expression levels of *miR*-1 in the left atrial tissue from patients with atrial fibrillation were reduced. This differential regulation of *miR-1* in the left atrial and ventricular tissues suggests that the *miR*-1 levels in the myocardium should be maintained within a proper range (80). However, further studies are warranted to understand the effect of *miR-1* expression in different tissues and conditions. The induction of the *miR-1* expression in the rat model of MI lowered the expression of insulin-like growth factor-1(90), and other anti-apoptotic genes such as B-cell lymphoma-2 (Bcl-2) and heat

shock protein (HSP) -60 and -70 (73). This could be responsible for the activation of the pro-apoptotic pathways in cardiomyocytes which may ultimately impact left ventricular remodeling.

1.4.4. MicroRNA-133

miR-133 is dominantly expressed in cardiomyocytes and cardiac fibroblasts (19). Its targeted deletion, overexpression, and antisense-mediated knockdown have demonstrated its essential roles and targets in cardiac remodeling (91). The *miR-133* family has two isoforms, *-133a* and *-133b*. Both isoforms have a similar sequence except for the 3'-terminal base, where guanidine is found in *miR-133a* and adenosine in *miR-133b* (73). The *miR-133a* isoform has two further subvariants, *-133a-1* and *-133a-2*. Depending upon the species, these *miRs* are located on different chromosomes. For example, in the murine genome, *miR-133a-1* is arranged with *miR-1-2* on chromosome 18, *mir-133a-2* is clustered with *miR-1-1* on chromosome 2, and *miR-133b* is located with *miR 206* on chromosome 1. However, in the human genome, *miR-133a-1, miR-133a-2,* and *miR-133b* are located on chromosomes 18, 20, and 6, respectively (92).

MiR-133 and *miR-1* are transcribed together, however, whereas *miR-1* is pro-apoptotic, *miR-133* has an anti-apoptotic role (93). In both *in vitro* and *in vivo* models of ischemia and reperfusion injury, overexpression of exogenous *miR-133a* reduced the apoptosis of cardiomyocytes (94). The β-blocker carvedilol protected cardiomyocytes against apoptosis by upregulating *miR-133* expression (95). *MiR-133* exerts its action by inhibiting pro-apoptotic genes such as death-associated protein kinase 2 (DAPK2), apoptotic protease activating factor 1 (APAF1), caspase-9, Bcl-2 like 11, and Bcl-2 modifying factor (BMF) (73).

A downregulation of *miR-133a* levels in a rat model of MI (73), and also in cardiac tissue from MI patients has been observed (96). Conversely, circulating levels of *miR-133a* in blood have

been upregulated in acute MI patients, which could suggest cardiac damage (97). The levels of circulating *miR-133a* are sensitive to cardiac injury and are elevated before changes in cardiac troponin T (cTnT) or creatine phosphokinase (CPK) can be oberved (98). This implies that *miR-133a* could provide strong diagnostic and prognostic information compared to the traditional markers (99). In a rat model of MI, the overexpression of *miR-133* increased left ventricular ejection fraction and fractional shortening (100). Overexpression of *miR-133a* has improved cardiac function by decreasing myocardial fibrosis in the streptozotocin-induced diabetic cardiomyopathy in mice (101) and heart failure in rats (102). *miR-133a* exerted its anti-fibrotic effect by inhibiting the expression of profibrotic genes such as connective tissue growth factor (CTGF) and collagen 1A1 (103) as well as suppression of the upregulated Akt-dependent signaling pathways in heart failure (102). Inflammation plays a critical role in the adverse left ventricular remodeling after an MI (104). Overexpression of *miR-133a* reduced the inflammatory cell infiltration in the myocardium after a MI (105). *miR-133a* can also be cardiogenic by reprogramming human and murine fibroblasts via suppressing Snai1, a key modulator of epithelial to mesenchymal transition (106). It can also induce transdifferentiation of cardiac fibroblasts by inhibiting the transforming growth factor-β (TGF-β) signaling cascade (105). *miR-133* has also been studied for its correlation with the incidences of ventricular fibrillation (VF). The downregulation of *miR-133a/b* was associated with the development of VF in MI patients by increasing the expression of hyperpolarization-activated cyclic nucleotide-gated ion channel 2 (HCN2), which determines cardiac automaticity. This indicates that increased expression of *miR-133a/b* could be used therapeutically for reducing the incidence of arrhythmias in MI patients (107).

In summary, because of its anti-fibrotic, anti-apoptotic, and regenerative potential, *miR-133a* could be further explored therapeutically for its cardioprotective and regenerative effects after cardiac injury.

1.4.5. MicroRNA-29

The *miR-29* family consists of *miR-29a, miR-29b-1, miR-29b-2,* and *miR-29c* (108). Despite being transcribed from different genetic loci, both *miR-29b-1* and *miR-29b-2* have identical mature sequences (109). Thus, both are collectively termed as *miR-29b*. In the human genome, *miR-29a* and *miR-29b-1* are located on chromosome 7, whereas *miR-29b-2* and *miR-29c* are on chromosome 1 (109). A study has demonstrated that *miR-29b* is the dominant member of the *miR-29* family as its myocardial expression is 4- and 8-fold higher than *miR-29a* and *miR-29c*, respectively (110). *MiR 29* is an essential regulator of extracellular matrix proteins and pathways that are related to fibrosis (108). *miR 29* family members target 16 genes that code for extracellular matrix proteins such as collagen isoforms, fibrillin 1, elastin, matrix metalloproteinase 2, laminin γ1, and integrin β1 (111, 112).

MiR-29 is highly expressed in fibroblasts and its inhibition can induce collagen expression in both cell culture and mice after an MI (79). This demonstrates the profibrotic effect of lowering *miR-29* expression. A study by Zhang *et al* (113) demonstrated the antifibrotic effect of *miR-29b* in angiotensin II-induced cardiac fibrosis in mice. Consistent with this, carvedilol produced an antifibrotic effect in a murine model of MI by upregulating *miR-29b* (114). However, Sassi *et al* (115) demonstrated that *miR-29* promotes rather than reduces cardiac fibrosis. However, these contrasting data could be explained by the use of a mouse model for left ventricular pressure overload induced by transverse aortic constriction (TAC) by Sassi *et al* (115) unlike other studies (108, 116) that used a coronary artery ligation induced animal model of MI. There are differences

in the type of cardiac fibrosis produced in these experimental models (117). The cardiac fibrosis in TAC is mainly characterized as reactive interstitial fibrosis, which occurs as an adaptive mechanism to preserve cardiac structure and function. In contrast, in ischemic injury model, replacement fibrosis occurs to fill up the void of viable cardiomyocytes to prevent cardiac rupture. In addition, other studies have shown the beneficial effects of the upregulation of a specific variant of *miR, -29b,* while Sassi *et al* (115) assessed the global deletion of *miR* regardless of variant subtypes. Therefore, these experimental differences could be responsible for the contrasting results in these studies.

An upregulation of *miR-29b* improved cardiac function in doxorubicin-induced cardiotoxicity in rats (110). The improved cardiac function by *miR-29b* has been attributed to its inhibitory effect on the apoptosis of cardiomyocytes. Consistent with this, overexpression of *miR-29b* promoted apoptosis of cardiomyocytes *in vitro* and downregulation by of *miR-29b* by an *miR-29b* inhibitor suppressed apoptosis of cardiomyocytes *in vitro* (110). The exact mechanism behind this anti-apoptotic effect of *miR-29b* has been identified. It targeted the Bax 3'UTR to reduce Bax expression, which is a pro-apoptotic protein. It also induced the expression of Bcl-2, an anti-apoptotic protein. Furthermore, it inhibited mitochondrial membrane depolarization, cytochrome C release, and activation of caspase-3 (110). *miR-29b* has also been shown to suppress tumor necrosis factor-related receptor 5 (TRAF5) to exert its anti-apoptotic effect in hypoxia-induced cardiomyocyte injury (118). *miR-29b* may also improve cardiac function through the activation of Akt in endothelial cells that promotes endothelial cell-mediated angiogenesis (116). The induction of angiogenesis in MI is known to improve cardiac function and decrease the risk of cardiac rupture (119).

Increasing evidence now suggests that the downregulation of *miR-29* contributes to the development of cardiac fibrosis. In this setting, *miR-29* mimics that could elevate the expression levels of *miR-29* or, alternatively, pharmacological inhibitors that could prevent the downregulation of *miR-29* might represent effective therapeutic strategies (108).

1.4.6. MicroRNA-135

MiR-135 is comprised of two variants, *miR-135a* [*miR-135a-1 and miR-135a-2*], and *miR-135b* (120). *MiR-135* is less widely studied in the field of cardiovascular physiology and pathophysiology. *MiR-135a* is encoded by two genes located on chromosome 3 and 12, whereas *miR-135b* is found on chromosome 1 (121). *MiR-135b* is expressed 10-fold less in the midbrain raphe nuclei than *miR-135a* (120). Chu *et al* (122) reported a reduction in cardiac hypertrophy by the overexpression of *miR-135b* in both angiotensin II and TAC -induced models. *CACNA1C,* a gene that codes for L-type calcium channels, was the main target of *miR-135b* (122). It was also predicted to target phosphodiesterase 1A (PDE1A), and the cardiac-specific sodium-calcium exchanger (NCX1) (122). *miR-135b* improved cardiac function in a murine MI model and protected cardiomyocytes *in vitro* by downregulating the expression of caspase-1, NOD-like receptor-containing pyrin 3 (NLRP3), and interleukin-1β (IL-1β) (123).

MiR-135a expression levels are reduced in myocardial ischemia-reperfusion injury in diabetic mice (124). Upregulation of *miR-135a* was achieved by using mimics in this model. This reduced the myocardial infarct size and apoptosis by decreasing the expression of the thioredoxin-interacting protein (TXNIP) (124). TXNIP (also called the thioredoxin binding protein-2 or vitamin D3-up-regulated protein -1) regulates the cellular redox state and is associated with apoptosis (125). Wang *et al* (126) reported that upregulation of *miR-135a* decreased myocardial infarct size and apoptosis in an ischemia-reperfusion injury model. Protein tyrosine phosphatase

1B (PTP1B), which plays an important role in several cellular processes such as differentiation, proliferation, migration, and apoptosis, is a direct target of *miR-135a* (126). A negative association between PTP1B and *miR-135a* was observed (126). Wu *et al* (127) demonstrated that the antifibrotic effect of *miR-135a* is mediated through the transient receptor potential melastatin 7 (TRPM7) channel, which plays a crucial role in cellular proliferation and differentiation.

Duoung *et al* (128) reported the downregulation of *miR-135a* in the left ventricle of mice with a complete atrioventricular block induced by His-bundle ablation. The sodium-calcium exchanger type 1 (NCX1) has been identified as the main target of *miR-135a* (128). Increased expression and activity of the NCX1 that bi-directionally exchanges 3 Na^+ for 1 Ca^{++}, is an adaptive mechanism during the cardiac remodeling process to maintain cardiac function. However, this alteration can become pro-arrhythmic and produce early- and delayed-after depolarizations as a consequence (128). To support this, overexpression of *miR-135a* reduced the spontaneous beating frequency of rat neonatal cardiomyocytes by 63% (128). *miR-135a* should be explored further to exploit its anti-arrhythmic potential for modulating cardiac automaticity, spontaneous calcium release and calcium efflux.

1.4.7. Limitations of using microRNA as a therapeutic

Despite being widely studied and shown to be effective in experiments, there are some limitations to the current status of *miRs* as prospective therapeutics. *miRs* and inhibitors of *miRs* do not affect the existing proteins, but rather alter the levels of mRNA and their translation. Therefore, it would be necessary to understand the pharmacokinetics of any proposed *miR* therapy (88). Studies are also required to develop stable anti-miRs that can achieve therapeutic efficacy in the heart and to better prevent any off-target effects (88). Further, the overexpression of any *miRs* for therapeutic purposes requires viral approaches which would pose a potential challenge for any

translational studies in humans (79). Finally, in some cases, any attempts at genetic deletions have lacked a therapeutic response compared to the short-term inhibition of specific *miRs* (129). Long-term and carefully designed studies in the future will help understand the disparate outcomes and aid in the development of efficacious *miR* therapies for cardiovascular pathologies.

1.4.8. Summary

MicroRNAs are emerging as therapeutic target options for impaired heart rhythms and hemodynamic function. Unlike the standard pharmacological agents that target single molecules in a pathological pathway, *miRs* can interact with several mRNAs and regulate multiple downstream mediators affecting a number of signaling cascades together (79). In particular, *miR -1*, *-133*, *-29* and *-135* could be investigated further for their beneficial actions on regulating the function of several ion channels, inhibiting cardiac apoptosis and improving cardiac function.

1.5. Treatment and management of MI

The patients with STEMI usually have a complete arterial occlusion and therefore reperfusion is required as quickly as possible. Coronary reperfusion is achieved by means of either primary PCI or intravenous fibrinolysis (20). Approximately 80% of STEMI patients are treated with PCI, while 11% with fibrinolytic agents (130, 131). Both the US and European guidelines recommend reperfusion therapy in STEMI patients quickly and effectively (16, 132). Several studies have shown reduced infarct size and lower mortality in those who receive reperfusion therapy quickly compared to those with a delay (133). The guidelines recommend the first medical contact-to-device time, or the door-to-balloon angioplasty time, for primary PCI of 90 minutes or less (ACC-AHA class I recommendation, evidence level A). This time has been associated with improved morbidity and mortality (133). Compared to balloon angioplasty, stent placement is better and drug-eluting stents such as cobalt-chromium everolimus-eluting stents are more

effective than bare-metal stents in reducing the target vessel revascularization (20, 134, 135). Fibrinolysis is preferred in the treatment of STEMI when PCI cannot be readily performed (ACC-AHA class I recommendation, evidence level A). However, it is only 33-60% successful in restoring patency of an artery involved in an infarct (136). Fibrin derivatives such as alteplase, reteplase, tenecteplase are preferred over non-fibrin specific drugs such as streptokinase (137). Limitations of fibrinolytic therapy over PCI include a higher rate of early death, reinfarction, and intracranial hemorrhage (20). All ACS patients undergoing PCI receive an initial dose of 162-325 mg of aspirin, followed by 81 mg/day indefinitely. Aspirin as an antiplatelet agent works by inhibiting the synthesis of thromboxane A_2 (TXA_2) through an irreversible inhibition of platelet cyclooxygenase-1 (21). Concomitantly with aspirin, a $P2Y_{12}$ inhibitor such as clopidogrel, prasugrel, ticagrelor, and cangrelor, is given as early as possible (16), and preferably continued for at least 1 year (138). These $P2Y_{12}$ inhibitors block a subtype of the ADP receptor, $P2Y_{12}$, and thus prevent binding of ADP as well as expression of GP IIb/IIIa receptors on platelets, subsequently reducing platelet activation and aggregation (21). In addition to reperfusion therapy, STEMI patients on the first day of hospitalization may also receive intranasal oxygen if oxygen saturation is low, sublingual nitroglycerin to reduce preload by vasodilation, a $P2Y_{12}$ inhibitor, an anticoagulant agent such as bivalirudin, unfractionated heparin, enoxaparin, or fondaparinux; and IV β-blockers for patients with ongoing ischemia and elevated blood pressure (21). β-blockers decrease myocardial oxygen demand by reducing heart rate and contractility and improving ventricular filling and coronary perfusion. Through these effects, they reduce the risk of reinfarction and ventricular arrhythmias following an MI (21). GP IIb/IIIa receptor inhibitors (GPIs) such as abciximab, eptifibatide, and tirofiban, block the final pathway in platelet aggregation and are not preferred in the management of patients without the need of PCI because

of a significant bleeding effect (16, 21). Angiotensin-converting enzyme (ACE) inhibitors are initiated in all patients following an MI to reduce mortality and prevent the development of heart failure (HF) (16). Following an MI, statins reduce total mortality, CVD mortality and stroke (21).

In NSTEMI, the infarct artery is usually patent despite being severely stenotic and so the goal is to prevent the progression of the thrombus to complete occlusion. In cases with a higher risk of death or MI, invasive revascularization interventions with either PCI or CABG is preferred in patients with NSTEMI (21). The early pharmacotherapy in NSTEMI is similar to that of STEMI (21). However, fibrinolytics are not recommended in NSTEMI patients because of an increased risk of mortality (15). ACE inhibitors such as captopril and enalapril are effective for preventing cardiac remodeling (21). Angiotensin II receptor blockers (ARBs) such as candesartan, valsartan, and losartan improve clinical outcomes in patients with low EF and HF (139). Aldosterone antagonists such as either spironolactone or eplerenone are indicated in MI patients to attenuate myocardial and vascular fibrosis, LV hypertrophy, hypertension, endothelial dysfunction, electrolyte imbalance, and arrhythmias (140).

1.6. Primary prevention of MI

The 2019 ACC/AHA guidelines on the primary prevention of cardiovascular disease promoted the importance of a healthy lifestyle to prevent CHD by emphasizing lifestyle factors such as exercise and diet that could affect cardiovascular risk factors (141). Several studies have examined and established the independent role of physical activity in the primary prevention of CHD (142, 143). A 20-30% lower risk for CHD has been associated with physical activity and the amount of physical activity required for achieving these lower risks has been quantified (144). At least 150 minutes per week of moderate intensity aerobic exercise or 75 minutes per week of vigorous intensity aerobic exercise has been recommended to reduce the risk of atherosclerotic

CHD (141). The total accumulated amount of physical activity is focused as shorter duration exercise is equally beneficial as longer ones (145).

A healthy diet and nutrition has a potential impact on the modifiable cardiovascular risk factors such as overweight, obesity, high blood pressure, high cholesterol, and diabetes (141). The current guidelines have stressed the importance of increasing the intake of whole grains, vegetables, fruit, nuts, lean animal protein, and fish, while reducing the intake of red meat, processed red meat, trans fat, refined carbohydrates and sweetened beverages (141). Several observational studies have shown associations between dietary pattern and cardiovascular mortality. The traditional Mediterranean diet is characterized by a high intake of vegetables, fruits, nuts and olive oil with a low intake of red and processed meat (146). The PREDIMED (Prevención con Dieta Mediterránea) trial demonstrated 30% and 28% risk reduction, respectively, in cardiovascular mortality in groups that received the Mediterranean diet supplemented with either extra virgin olive oil or nuts (147). This study underlined the importance of diet and in particular unsaturated fat for cardiovascular health. A cohort study examined the association of specific dietary fats with total mortality in 2 large ongoing studies, the Nurses' Health Study with 83,349 women and the Health Professionals Follow-up Study with 42,884 men. Interestingly, the intake of saturated fat (SFA) was associated with an increased mortality, whereas a 27% reduction was observed when 5% energy from saturated fat was replaced with polyunsaturated fatty acid (PUFA) (148).

1.7. Omega-3 PUFAs

Omega-3 fatty acids (n-3) are a family of PUFAs that play an essential role in various cellular process such as maintaining membrane structure and function, cellular signaling and gene expression (149). Marine animals such as fish are an important source of n-3 PUFAs such as

eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (150). Studies have shown an inverse association between fish intake and CVD risk (151). A recent meta-analysis of 13 randomized controlled trials with a total of 127,477 participants demonstrated a significant reduction in the risk of MI, total CAD, and total CVD through supplementation of the diet with marine n-3 PUFAs (152). However, fish consumption is lower than advised because of various concerns such as the smell and taste of fish, as well as the content of potential toxins (153). Moreover, it is not a dietary source for vegetarians and vegans. Alpha linolenic acid (ALA) is a form of n-3 obtained from plants, found mainly in some seeds and nuts (151). Flaxseed, *linum usitatissium*, is the richest plant source of ALA (153).

References

1. Virani SS, Alonso A, Benjamin EJ, Bittencourt MS, Callaway CW, Carson AP, et al. Heart disease and stroke statistics - 2020 update: A report from the american heart association. Circulation. 2020;141(9):e139-e596.

2. Arnett DK, Blumenthal RS, Albert MA, Buroker AB, Goldberger ZD, Hahn EJ, et al. 2019 acc/aha guideline on the primary prevention of cardiovascular disease: A report of the american college of cardiology/american heart association task force on clinical practice guidelines. Circulation. 2019;140(11):e596-e646.

3. Dupasquier CM, Weber AM, Ander BP, Rampersad PP, Steigerwald S, Wigle JT, et al. Effects of dietary flaxseed on vascular contractile function and atherosclerosis during prolonged hypercholesterolemia in rabbits. American journal of physiology Heart and circulatory physiology. 2006;291(6):H2987-96.

4. Dupasquier CM, Dibrov E, Kneesh AL, Cheung PK, Lee KG, Alexander HK, et al. Dietary flaxseed inhibits atherosclerosis in the ldl receptor-deficient mouse in part through antiproliferative and anti-inflammatory actions. American journal of physiology Heart and circulatory physiology. 2007;293(4):H2394-402.

5. Bassett CM, McCullough RS, Edel AL, Patenaude A, LaVallee RK, Pierce GN. The alpha-linolenic acid content of flaxseed can prevent the atherogenic effects of dietary trans fat. American journal of physiology Heart and circulatory physiology. 2011;301(6):H2220-6.

6. Francis AA, Deniset JF, Austria JA, LaVallee RK, Maddaford GG, Hedley TE, et al. Effects of dietary flaxseed on atherosclerotic plaque regression. American journal of physiology Heart and circulatory physiology. 2013;304(12):H1743-51.

7. Edel AL, Rodriguez-Leyva D, Maddaford TG, Caligiuri SP, Austria JA, Weighell W, et al. Dietary flaxseed independently lowers circulating cholesterol and lowers it beyond the effects of cholesterol-lowering medications alone in patients with peripheral artery disease. The Journal of nutrition. 2015;145(4):749-57.

8. Rodriguez-Leyva D, Weighell W, Edel AL, LaVallee R, Dibrov E, Pinneker R, et al. Potent antihypertensive action of dietary flaxseed in hypertensive patients. Hypertension (Dallas, Tex : 1979). 2013;62(6):1081-9.

9. Caligiuri SPB, Rodriguez-Leyva D, Aukema HM, Ravandi A, Weighell W, Guzman R, et al. Dietary flaxseed reduces central aortic blood pressure without cardiac involvement but through changes in plasma oxylipins. Hypertension (Dallas, Tex : 1979). 2016;68(4):1031-8.

10. Benjamin EJ, Muntner P, Alonso A, Bittencourt MS, Callaway CW, Carson AP, et al. Heart disease and stroke statistics-2019 update: A report from the american heart association. Circulation. 2019;139(10):e56-e528.

11. Braunwald's heart disease : A textbook of cardiovascular medicine. Eleventh edition. ed. Zipes DP, Libby P, Bonow RO, Mann DL, Tomaselli GF, Braunwald E, editors. Philadelphia, PA: Elsevier/Saunders; 2018.

12. Pathophysiology of heart disease : A collaborative project of medical students and faculty. Lilly LS, editor. Philadelphia: Wolters Kluwer; 2016.

13. Kloner RA, Jennings RB. Consequences of brief ischemia: Stunning, preconditioning, and their clinical implications. Circulation. 2001;104(24):2981-9.

14. Kumar A, Cannon CP. Acute coronary syndromes: Diagnosis and management, part i. Mayo Clin Proc. 2009;84(10):917-38.

15. Amsterdam EA, Wenger NK, Brindis RG, Casey DE, Ganiats TG, Holmes DR, et al. 2014 aha/acc guideline for the management of patients with non–st-elevation acute coronary syndromes. A Report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. 2014;64(24):e139-e228.

16. O'Gara PT, Kushner FG, Ascheim DD, Casey DE, Jr., Chung MK, de Lemos JA, et al. 2013 accf/aha guideline for the management of st-elevation myocardial infarction: A report of the american college of cardiology foundation/american heart association task force on practice guidelines. Circulation. 2013;127(4):e362-425.

17. Roffi M, Patrono C, Collet J-P, Mueller C, Valgimigli M, Andreotti F, et al. 2015 esc guidelines for the management of acute coronary syndromes in patients presenting without persistent st-segment elevation: Task force for the management of acute coronary syndromes in patients presenting without persistent st-segment elevation of the european society of cardiology (esc). European heart journal. 2016;37(3):267-315.

18. Reed GW, Rossi JE, Cannon CP. Acute myocardial infarction. Lancet (London, England). 2017;389(10065):197-210.

19. Thygesen K, Alpert JS, Jaffe AS, Chaitman BR, Bax JJ, Morrow DA, et al. Fourth universal definition of myocardial infarction (2018). Circulation. 2018;138(20):e618-e51.

20. Anderson JL, Morrow DA. Acute myocardial infarction. New England Journal of Medicine. 2017;376(21):2053-64.

21. Rogers KC, Denus Sd, Finks SW, Spinler SA. Acute coronary syndromes. In: DiPiro JT, Talbert RL, Yee GC, Matzke GR, Wells BG, Posey LM, editors. Pharmacotherapy: A pathophysiologic approach, 10e. New York, NY: McGraw-Hill Education; 2017.

22. Morrow DA, Cannon CP, Jesse RL, Newby LK, Ravkilde J, Storrow AB, et al. National academy of clinical biochemistry laboratory medicine practice guidelines: Clinical characteristics and utilization of biochemical markers in acute coronary syndromes. Circulation. 2007;115(13):e356-75.

23. Harrington DH, Stueben F, Lenahan CM. St-elevation myocardial infarction and non-st-elevation myocardial infarction: Medical and surgical interventions. Critical care nursing clinics of North America. 2019;31(1):49-64.

24. Bentzon JF, Otsuka F, Virmani R, Falk E. Mechanisms of plaque formation and rupture. Circ Res. 2014;114(12):1852-66.

25. Cediel G, Gonzalez-del-Hoyo M, Carrasquer A, Sanchez R, Boqué C, Bardají A. Outcomes with type 2 myocardial infarction compared with non-ischaemic myocardial injury. Heart (British Cardiac Society). 2017;103(8):616-22.

26. Saaby L, Poulsen TS, Hosbond S, Larsen TB, Pyndt Diederichsen AC, Hallas J, et al. Classification of myocardial infarction: Frequency and features of type 2 myocardial infarction. The American journal of medicine. 2013;126(9):789-97.

27. Thygesen K, Alpert JS, Jaffe AS, Simoons ML, Chaitman BR, White HD, et al. Third universal definition of myocardial infarction. European heart journal. 2012;33(20):2551-67.

28. Jangaard N, Sarkisian L, Saaby L, Mikkelsen S, Lassen AM, Marcussen N, et al. Incidence, frequency, and clinical characteristics of type 3 myocardial infarction in clinical practice. The American journal of medicine. 2017;130(7):862.e9-.e14.

29. Rahimi K, Banning AP, Cheng AS, Pegg TJ, Karamitsos TD, Channon KM, et al. Prognostic value of coronary revascularisation-related myocardial injury: A cardiac magnetic resonance imaging study. Heart (British Cardiac Society). 2009;95(23):1937-43.

30. Selvanayagam JB, Porto I, Channon K, Petersen SE, Francis JM, Neubauer S, et al. Troponin elevation after percutaneous coronary intervention directly represents the extent of irreversible myocardial injury: Insights from cardiovascular magnetic resonance imaging. Circulation. 2005;111(8):1027-32.

31. Garcia-Garcia HM, McFadden EP, Farb A, Mehran R, Stone GW, Spertus J, et al. Standardized end point definitions for coronary intervention trials: The academic research consortium-2 consensus document. Circulation. 2018;137(24):2635-50.

32. Boateng S, Sanborn T. Acute myocardial infarction. Disease-a-Month. 2013;59(3):83-96.

33. Brener SJ. Insights into the pathophysiology of st-elevation myocardial infarction. American heart journal. 2006;151(6 Suppl):S4-10.

34. Kroll MH, Harris TS, Moake JL, Handin RI, Schafer AI. Von willebrand factor binding to platelet gpib initiates signals for platelet activation. The Journal of clinical investigation. 1991;88(5):1568-73.

35. Wilcox JN, Smith KM, Schwartz SM, Gordon D. Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. Proceedings of the National Academy of Sciences of the United States of America. 1989;86(8):2839-43.

36. Frangogiannis NG. Pathophysiology of myocardial infarction. Comprehensive Physiology. 2015;5(4):1841-75.

37. Braasch W, Gudbjarnason S, Puri PS, Ravens KG, Bing RJ. Early changes in energy metabolism in the myocardium following acute coronary artery occlusion in anesthetized dogs. Circulation Research. 1968;23(3):429-38.

38. Carmeliet E. Cardiac ionic currents and acute ischemia: From channels to arrhythmias. Physiological reviews. 1999;79(3):917-1017.

39. Konstantinidis K, Whelan RS, Kitsis RN. Mechanisms of cell death in heart disease. Arteriosclerosis, thrombosis, and vascular biology. 2012;32(7):1552-62.

40. Gottlieb RA. Cell death pathways in acute ischemia/reperfusion injury. Journal of cardiovascular pharmacology and therapeutics. 2011;16(3-4):233-8.

41. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, et al. Cytochrome c and datp-dependent formation of apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell. 1997;91(4):479-89.

42. Baines CP, Kaiser RA, Purcell NH, Blair NS, Osinska H, Hambleton MA, et al. Loss of cyclophilin d reveals a critical role for mitochondrial permeability transition in cell death. Nature. 2005;434(7033):658-62.

43. Lavandero S, Troncoso R, Rothermel BA, Martinet W, Sadoshima J, Hill JA. Cardiovascular autophagy: Concepts, controversies, and perspectives. Autophagy. 2013;9(10):1455-66.

44. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature. 2008;451(7182):1069-75.

45. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: Crosstalk between autophagy and apoptosis. Nature reviews Molecular cell biology. 2007;8(9):741-52.

46. Matsui Y, Takagi H, Qu X, Abdellatif M, Sakoda H, Asano T, et al. Distinct roles of autophagy in the heart during ischemia and reperfusion: Roles of amp-activated protein kinase and beclin 1 in mediating autophagy. Circ Res. 2007;100(6):914-22.

47. Kanamori H, Takemura G, Goto K, Maruyama R, Ono K, Nagao K, et al. Autophagy limits acute myocardial infarction induced by permanent coronary artery occlusion. American journal of physiology Heart and circulatory physiology. 2011;300(6):H2261-71.

48. Frangogiannis NG. The immune system and the remodeling infarcted heart: Cell biological insights and therapeutic opportunities. Journal of cardiovascular pharmacology. 2014;63(3):185-95.

49. Ghigo A, Franco I, Morello F, Hirsch E. Myocyte signalling in leucocyte recruitment to the heart. Cardiovascular research. 2014;102(2):270-80.

50. Hensley K, Robinson KA, Gabbita SP, Salsman S, Floyd RA. Reactive oxygen species, cell signaling, and cell injury. Free radical biology & medicine. 2000;28(10):1456-62.

51. Dewald O, Zymek P, Winkelmann K, Koerting A, Ren G, Abou-Khamis T, et al. Ccl2/monocyte chemoattractant protein-1 regulates inflammatory responses critical to healing myocardial infarcts. Circ Res. 2005;96(8):881-9.

52. Soehnlein O, Lindbom L. Phagocyte partnership during the onset and resolution of inflammation. Nature reviews Immunology. 2010;10(6):427-39.

53. Serhan CN. Novel lipid mediators and resolution mechanisms in acute inflammation: To resolve or not? The American journal of pathology. 2010;177(4):1576-91.

54. Bournazou I, Pound JD, Duffin R, Bournazos S, Melville LA, Brown SB, et al. Apoptotic human cells inhibit migration of granulocytes via release of lactoferrin. The Journal of clinical investigation. 2009;119(1):20-32.

55. Ponta H, Sherman L, Herrlich PA. Cd44: From adhesion molecules to signalling regulators. Nature reviews Molecular cell biology. 2003;4(1):33-45.

56. Shinde AV, Frangogiannis NG. Fibroblasts in myocardial infarction: A role in inflammation and repair. Journal of molecular and cellular cardiology. 2014;70:74-82.

57. Moore-Morris T, Guimaraes-Camboa N, Banerjee I, Zambon AC, Kisseleva T, Velayoudon A, et al. Resident fibroblast lineages mediate pressure overload-induced cardiac fibrosis. The Journal of clinical investigation. 2014;124(7):2921-34.

58. Gabbiani G. The myofibroblast in wound healing and fibrocontractive diseases. The Journal of pathology. 2003;200(4):500-3.

59. Dobaczewski M, Bujak M, Li N, Gonzalez-Quesada C, Mendoza LH, Wang XF, et al. Smad3 signaling critically regulates fibroblast phenotype and function in healing myocardial infarction. Circ Res. 2010;107(3):418-28.

60. Cairns JA, Walls AF. Mast cell tryptase stimulates the synthesis of type i collagen in human lung fibroblasts. The Journal of clinical investigation. 1997;99(6):1313-21.

61. Dell'Italia LJ, Husain A. Dissecting the role of chymase in angiotensin ii formation and heart and blood vessel diseases. Current opinion in cardiology. 2002;17(4):374-9.

62. Detillieux KA, Sheikh F, Kardami E, Cattini PA. Biological activities of fibroblast growth factor-2 in the adult myocardium. Cardiovascular research. 2003;57(1):8-19.

63. Zymek P, Bujak M, Chatila K, Cieslak A, Thakker G, Entman ML, et al. The role of platelet-derived growth factor signaling in healing myocardial infarcts. J Am Coll Cardiol. 2006;48(11):2315-23.

64. Xie Y, Chen J, Han P, Yang P, Hou J, Kang YJ. Immunohistochemical detection of differentially localized up-regulation of lysyl oxidase and down-regulation of matrix metalloproteinase-1 in rhesus monkey model of chronic myocardial infarction. Experimental biology and medicine (Maywood, NJ). 2012;237(7):853-9.

65. Traupe T, Gloekler S, Marchi SFd, Werner GS, Seiler C. Assessment of the human coronary collateral circulation. Circulation. 2010;122(12):1210-20.

66. Pouleur H. Diastolic dysfunction and myocardial energetics. European heart journal. 1990;11(suppl_C):30-4.

67. Pfeffer MA, Braunwald E. Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications. Circulation. 1990;81(4):1161-72.

68. Mann DL, Bogaev R, Buckberg GD. Cardiac remodelling and myocardial recovery: Lost in translation? European Journal of Heart Failure. 2010;12(8):789-96.

69. St John Sutton M, Lee D, Rouleau JL, Goldman S, Plappert T, Braunwald E, et al. Left ventricular remodeling and ventricular arrhythmias after myocardial infarction. Circulation. 2003;107(20):2577-82.

70. Weisman HF, Bush DE, Mannisi JA, Weisfeldt ML, Healy B. Cellular mechanisms of myocardial infarct expansion. Circulation. 1988;78(1):186-201.

71. Mukherjee R, Brinsa TA, Dowdy KB, Scott AA, Baskin JM, Deschamps AM, et al. Myocardial infarct expansion and matrix metalloproteinase inhibition. Circulation. 2003;107(4):618-25.

72. Picard MH, Wilkins GT, Gillam LD, Thomas JD, Weyman AE. Immediate regional endocardial surface expansion following coronary occlusion in the canine left ventricle: Disproportionate effects of anterior versus inferior ischemia. American heart journal. 1991;121(3 Pt 1):753-62.

73. Chistiakov DA, Orekhov AN, Bobryshev YV. Cardiac-specific mirna in cardiogenesis, heart function, and cardiac pathology (with focus on myocardial infarction). Journal of molecular and cellular cardiology. 2016;94:107-21.

74. van Rooij E, Olson EN. Micrornas: Powerful new regulators of heart disease and provocative therapeutic targets. The Journal of clinical investigation. 2007;117(9):2369-76.

75. He B, Xiao J, Ren A-J, Zhang Y-F, Zhang H, Chen M, et al. Role of mir-1 and mir-133a in myocardial ischemic postconditioning. J Biomed Sci. 2011;18(1):22-.

76. Dong S, Cheng Y, Yang J, Li J, Liu X, Wang X, et al. Microrna expression signature and the role of microrna-21 in the early phase of acute myocardial infarction. J Biol Chem. 2009;284(43):29514-25.

77. Bostjancic E, Zidar N, Glavac D. Microrna microarray expression profiling in human myocardial infarction. Disease markers. 2009;27(6):255-68.

78. Liu X, Zhang Y, Du W, Liang H, He H, Zhang L, et al. Mir-223-3p as a novel microrna regulator of expression of voltage-gated k+ channel kv4.2 in acute myocardial infarction. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology. 2016;39(1):102-14.

79. Fiedler J, Thum T. Micrornas in myocardial infarction. Arteriosclerosis, thrombosis, and vascular biology. 2013;33(2):201-5.

80. Kim GH. Microrna regulation of cardiac conduction and arrhythmias. Translational research : the journal of laboratory and clinical medicine. 2013;161(5):381-92.

81. Han J, Lee Y, Yeom K-H, Kim Y-K, Jin H, Kim VN. The drosha-dgcr8 complex in primary microrna processing. Genes Dev. 2004;18(24):3016-27.

82. Zhou SS, Jin JP, Wang JQ, Zhang ZG, Freedman JH, Zheng Y, et al. Mirnas in cardiovascular diseases: Potential biomarkers, therapeutic targets and challenges. Acta pharmacologica Sinica. 2018;39(7):1073-84.

83. Condorelli G, Latronico MVG, Cavarretta E. Micrornas in cardiovascular diseases: Current knowledge and the road ahead. Journal of the American College of Cardiology. 2014;63(21):2177-87.

84. Creemers EE, Tijsen AJ, Pinto YM, Rooij Ev. Circulating micrornas. Circulation Research. 2012;110(3):483-95.

85. Zhu H, Fan GC. Role of micrornas in the reperfused myocardium towards post-infarct remodelling. Cardiovascular research. 2012;94(2):284-92.

86. Liao C, Gui Y, Guo Y, Xu D. The regulatory function of microrna-1 in arrhythmias. Molecular bioSystems. 2016;12(2):328-33.

87. Yang B, Lin H, Xiao J, Lu Y, Luo X, Li B, et al. The muscle-specific microrna mir-1 regulates cardiac arrhythmogenic potential by targeting gja1 and kcnj2. Nature medicine. 2007;13(4):486-91.

88. Mitra M, Coller HA. Rnas that make a heart beat. Ann Transl Med. 2016;4(23):469-.

89. Lu Y, Zhang Y, Shan H, Pan Z, Li X, Li B, et al. Microrna-1 downregulation by propranolol in a rat model of myocardial infarction: A new mechanism for ischaemic cardioprotection. Cardiovascular research. 2009;84(3):434-41.

90. Shan ZX, Lin QX, Fu YH, Deng CY, Zhou ZL, Zhu JN, et al. Upregulated expression of mir-1/mir-206 in a rat model of myocardial infarction. Biochemical and biophysical research communications. 2009;381(4):597-601.

91. Li N, Zhou H, Tang Q. Mir-133: A suppressor of cardiac remodeling? Frontiers in Pharmacology. 2018;9(903).

92. Liu Y, Liang Y, Zhang JF, Fu WM. Microrna-133 mediates cardiac diseases: Mechanisms and clinical implications. Experimental cell research. 2017;354(2):65-70.

93. Bostjancic E, Zidar N, Stajner D, Glavac D. Microrna mir-1 is up-regulated in remote myocardium in patients with myocardial infarction. Folia biologica. 2010;56(1):27-31.

94. Li S, Xiao FY, Shan PR, Su L, Chen DL, Ding JY, et al. Overexpression of microrna-133a inhibits ischemia-reperfusion-induced cardiomyocyte apoptosis by targeting dapk2. Journal of human genetics. 2015;60(11):709-16.

95. Xu C, Hu Y, Hou L, Ju J, Li X, Du N, et al. Beta-blocker carvedilol protects cardiomyocytes against oxidative stress-induced apoptosis by up-regulating mir-133 expression. Journal of molecular and cellular cardiology. 2014;75:111-21.

96. Bostjancic E, Zidar N, Stajer D, Glavac D. Micrornas mir-1, mir-133a, mir-133b and mir-208 are dysregulated in human myocardial infarction. Cardiology. 2010;115(3):163-9.

97. D'Alessandra Y, Devanna P, Limana F, Straino S, Di Carlo A, Brambilla PG, et al. Circulating micrornas are new and sensitive biomarkers of myocardial infarction. European heart journal. 2010;31(22):2765-73.

98. Kuwabara Y, Ono K, Horie T, Nishi H, Nagao K, Kinoshita M, et al. Increased microrna-1 and microrna-133a levels in serum of patients with cardiovascular disease indicate myocardial damage. Circulation Cardiovascular genetics. 2011;4(4):446-54.

99. Wang GK, Zhu JQ, Zhang JT, Li Q, Li Y, He J, et al. Circulating microrna: A novel potential biomarker for early diagnosis of acute myocardial infarction in humans. European heart journal. 2010;31(6):659-66.

100. Chen Y, Zhao Y, Chen W, Xie L, Zhao Z-A, Yang J, et al. Microrna-133 overexpression promotes the therapeutic efficacy of mesenchymal stem cells on acute myocardial infarction. Stem Cell Res Ther. 2017;8(1):268-.

101. Chen S, Puthanveetil P, Feng B, Matkovich SJ, Dorn GW, 2nd, Chakrabarti S. Cardiac mir-133a overexpression prevents early cardiac fibrosis in diabetes. Journal of cellular and molecular medicine. 2014;18(3):415-21.

102. Sang H-Q, Jiang Z-M, Zhao Q-P, Xin F. Microrna-133a improves the cardiac function and fibrosis through inhibiting akt in heart failure rats. Biomedicine & Pharmacotherapy. 2015;71:185-9.

103. Castoldi G, Di Gioia CR, Bombardi C, Catalucci D, Corradi B, Gualazzi MG, et al. Mir-133a regulates collagen 1a1: Potential role of mir-133a in myocardial fibrosis in angiotensin ii-dependent hypertension. Journal of cellular physiology. 2012;227(2):850-6.

104. Ong S-B, Hernández-Reséndiz S, Crespo-Avilan GE, Mukhametshina RT, Kwek X-Y, Cabrera-Fuentes HA, et al. Inflammation following acute myocardial infarction: Multiple players, dynamic roles, and novel therapeutic opportunities. Pharmacol Ther. 2018;186:73-87.

105. Xiao Y, Zhao J, Tuazon JP, Borlongan CV, Yu G. Microrna-133a and myocardial infarction. Cell transplantation. 2019;28(7):831-8.

106. Muraoka N, Yamakawa H, Miyamoto K, Sadahiro T, Umei T, Isomi M, et al. Mir-133 promotes cardiac reprogramming by directly repressing snai1 and silencing fibroblast signatures. The EMBO journal. 2014;33(14):1565-81.

107. Bostjancic E, Brandner T, Zidar N, Glavac D, Stajer D. Down-regulation of mir-133a/b in patients with myocardial infarction correlates with the presence of ventricular fibrillation. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie. 2018;99:65-71.

108. van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS, et al. Dysregulation of microrna after myocardial infarction reveals a role of mir-29 in cardiac fibrosis. Proceedings of the National Academy of Sciences of the United States of America. 2008;105(35):13027-32.

109. Zhao W, Cheng L, Quek C, Bellingham SA, Hill AF. Novel mir-29b target regulation patterns are revealed in two different cell lines. Scientific Reports. 2019;9(1):17449.

110. Jing X, Yang J, Jiang L, Chen J, Wang H. Microrna-29b regulates the mitochondria-dependent apoptotic pathway by targeting bax in doxorubicin cardiotoxicity. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology. 2018;48(2):692-704.

111. Wang Y, Jin BJ, Chen Q, Yan BJ, Liu ZL. Microrna-29b upregulation improves myocardial fibrosis and cardiac function in myocardial infarction rats through targeting sh2b3. European review for medical and pharmacological sciences. 2019;23(22):10115-22.

112. Kriegel AJ, Liu Y, Fang Y, Ding X, Liang M. The mir-29 family: Genomics, cell biology, and relevance to renal and cardiovascular injury. Physiological genomics. 2012;44(4):237-44.

113. Zhang Y, Huang XR, Wei LH, Chung AC, Yu CM, Lan HY. Mir-29b as a therapeutic agent for angiotensin ii-induced cardiac fibrosis by targeting tgf-beta/smad3 signaling. Molecular therapy : the journal of the American Society of Gene Therapy. 2014;22(5):974-85.

114. Zhu J-N, Chen R, Fu Y-H, Lin Q-X, Huang S, Guo L-L, et al. Smad3 inactivation and mir-29b upregulation mediate the effect of carvedilol on attenuating the acute myocardium infarction-induced myocardial fibrosis in rat. PLOS ONE. 2013;8(9):e75557.

115. Sassi Y, Avramopoulos P, Ramanujam D, Gruter L, Werfel S, Giosele S, et al. Cardiac myocyte mir-29 promotes pathological remodeling of the heart by activating wnt signaling. Nature communications. 2017;8(1):1614.

116. Zhu ML, Yin YL, Ping S, Yu HY, Wan GR, Jian X, et al. Berberine promotes ischemia-induced angiogenesis in mice heart via upregulation of microrna-29b. Clinical and experimental hypertension (New York, NY : 1993). 2017;39(7):672-9.

117. Travers JG, Kamal FA, Robbins J, Yutzey KE, Blaxall BC. Cardiac fibrosis: The fibroblast awakens. Circulation research. 2016;118(6):1021-40.

118. Cai Y, Li Y. Upregulation of mir-29b-3p protects cardiomyocytes from hypoxia-induced apoptosis by targeting traf5. Cell Mol Biol Lett. 2019;24:27-.

119. van der Laan AM, Piek JJ, van Royen N. Targeting angiogenesis to restore the microcirculation after reperfused mi. Nature reviews Cardiology. 2009;6(8):515-23.

120. Issler O, Haramati S, Paul Evan D, Maeno H, Navon I, Zwang R, et al. Microrna 135 is essential for chronic stress resiliency, antidepressant efficacy, and intact serotonergic activity. Neuron. 2014;83(2):344-60.

121. Nagel R, le Sage C, Diosdado B, van der Waal M, Oude Vrielink JAF, Bolijn A, et al. Regulation of the adenomatous polyposis coli gene by the mir-135 family in colorectal cancer. Cancer Research. 2008;68(14):5795-802.

122. Chu Q, Li A, Chen X, Qin Y, Sun X, Li Y, et al. Overexpression of mir-135b attenuates pathological cardiac hypertrophy by targeting cacna1c. International journal of cardiology. 2018;269:235-41.

123. Li A, Yu Y, Ding X, Qin Y, Jiang Y, Wang X, et al. Mir-135b protects cardiomyocytes from infarction through restraining the nlrp3/caspase-1/il-1β pathway. International journal of cardiology. 2019.

124. Zhu H-J, Wang D-G, Yan J, Xu J. Up-regulation of microrna-135a protects against myocardial ischemia/reperfusion injury by decreasing txnip expression in diabetic mice. Am J Transl Res. 2015;7(12):2661-71.

125. Wang BF, Yoshioka J. The emerging role of thioredoxin-interacting protein in myocardial ischemia/reperfusion injury. Journal of cardiovascular pharmacology and therapeutics. 2017;22(3):219-29.

126. Wang S, Cheng Z, Chen X, Xue H. Microrna-135a protects against myocardial ischemia-reperfusion injury in rats by targeting protein tyrosine phosphatase 1b. Journal of Cellular Biochemistry. 2019;120(6):10421-33.

127. Wu Y, Liu Y, Pan Y, Lu C, Xu H, Wang X, et al. Microrna-135a inhibits cardiac fibrosis induced by isoproterenol via trpm7 channel. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie. 2018;104:252-60.

128. Duong E, Xiao J, Qi XY, Nattel S. Microrna-135a regulates sodium-calcium exchanger gene expression and cardiac electrical activity. Heart rhythm. 2017;14(5):739-48.

129. Mendell JT, Olson EN. Micrornas in stress signaling and human disease. Cell. 2012;148(6):1172-87.

130. Shah RU, Henry TD, Rutten-Ramos S, Garberich RF, Tighiouart M, Bairey Merz CN. Increasing percutaneous coronary interventions for st-segment elevation myocardial infarction in the united states: Progress and opportunity. JACC Cardiovascular interventions. 2015;8(1 Pt B):139-46.

131. Jortveit J, Govatsmark RE, Digre TA, Risoe C, Hole T, Mannsverk J, et al. Myocardial infarction in norway in 2013. Tidsskrift for den Norske laegeforening : tidsskrift for praktisk medicin, ny raekke. 2014;134(19):1841-6.

132. Windecker S, Kolh P, Alfonso F, Collet JP, Cremer J, Falk V, et al. 2014 esc/eacts guidelines on myocardial revascularization: The task force on myocardial revascularization of the european society of cardiology (esc) and the european association for cardio-thoracic surgery (eacts)developed with the special contribution of the european association of percutaneous cardiovascular interventions (eapci). European heart journal. 2014;35(37):2541-619.

133. Cannon CP, Gibson CM, Lambrew CT, Shoultz DA, Levy D, French WJ, et al. Relationship of symptom-onset-to-balloon time and door-to-balloon time with mortality in patients undergoing angioplasty for acute myocardial infarction. Jama. 2000;283(22):2941-7.

134. Nordmann AJ, Hengstler P, Harr T, Young J, Bucher HC. Clinical outcomes of primary stenting versus balloon angioplasty in patients with myocardial infarction: A meta-analysis of randomized controlled trials. The American journal of medicine. 2004;116(4):253-62.

135. Kastrati A, Dibra A, Spaulding C, Laarman GJ, Menichelli M, Valgimigli M, et al. Meta-analysis of randomized trials on drug-eluting stents vs. Bare-metal stents in patients with acute myocardial infarction. European heart journal. 2007;28(22):2706-13.

136. Indications for fibrinolytic therapy in suspected acute myocardial infarction: Collaborative overview of early mortality and major morbidity results from all randomised trials of more than 1000 patients. Fibrinolytic therapy trialists' (ftt) collaborative group. Lancet (London, England). 1994;343(8893):311-22.

137. O'Gara PT, Kushner FG, Ascheim DD, Casey DE, Jr., Chung MK, de Lemos JA, et al. 2013 accf/aha guideline for the management of st-elevation myocardial infarction: A report of the american college of cardiology foundation/american heart association task force on practice guidelines. J Am Coll Cardiol. 2013;61(4):e78-e140.

138. Elmariah S, Mauri L, Doros G, Galper BZ, O'Neill KE, Steg PG, et al. Extended duration dual antiplatelet therapy and mortality: A systematic review and meta-analysis. Lancet (London, England). 2015;385(9970):792-8.

139. Pfeffer MA, McMurray JJ, Velazquez EJ, Rouleau JL, Kober L, Maggioni AP, et al. Valsartan, captopril, or both in myocardial infarction complicated by heart failure, left ventricular dysfunction, or both. The New England journal of medicine. 2003;349(20):1893-906.

140. Makkar KM, Sanoski CA, Spinler SA. Role of angiotensin-converting enzyme inhibitors, angiotensin ii receptor blockers, and aldosterone antagonists in the prevention of atrial and ventricular arrhythmias. Pharmacotherapy. 2009;29(1):31-48.

141. Arnett DK, Blumenthal RS, Albert MA, Buroker AB, Goldberger ZD, Hahn EJ, et al. 2019 acc/aha guideline on the primary prevention of cardiovascular disease. A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. 2019;74(10):e177-e232.

142. Williams PT. Physical fitness and activity as separate heart disease risk factors: A meta-analysis. Medicine and science in sports and exercise. 2001;33(5):754-61.

143. Sofi F, Capalbo A, Cesari F, Abbate R, Gensini GF. Physical activity during leisure time and primary prevention of coronary heart disease: An updated meta-analysis of cohort studies. European Journal of Cardiovascular Prevention & Rehabilitation. 2008;15(3):247-57.

144. Sattelmair J, Pertman J, Ding EL, Kohl HW, 3rd, Haskell W, Lee IM. Dose response between physical activity and risk of coronary heart disease: A meta-analysis. Circulation. 2011;124(7):789-95.

145. Saint-Maurice PF, Troiano RP, Matthews CE, Kraus WE. Moderate-to-vigorous physical activity and all-cause mortality: Do bouts matter? Journal of the American Heart Association. 2018;7(6).

146. Willett WC, Sacks F, Trichopoulou A, Drescher G, Ferro-Luzzi A, Helsing E, et al. Mediterranean diet pyramid: A cultural model for healthy eating. The American Journal of Clinical Nutrition. 1995;61(6):1402S-6S.

147. Estruch R, Ros E, Salas-Salvadó J, Covas M-I, Corella D, Arós F, et al. Primary prevention of cardiovascular disease with a mediterranean diet supplemented with extra-virgin olive oil or nuts. New England Journal of Medicine. 2018;378(25):e34.

148. Wang DD, Li Y, Chiuve SE, Stampfer MJ, Manson JE, Rimm EB, et al. Association of specific dietary fats with total and cause-specific mortality. JAMA Internal Medicine. 2016;176(8):1134-45.

149. Jia X, Kohli P, Virani SS. Omega-3 fatty acid and cardiovascular outcomes: Insights from recent clinical trials. Current atherosclerosis reports. 2019;21(1):1.

150. Shahidi F, Ambigaipalan P. Omega-3 polyunsaturated fatty acids and their health benefits. Annual review of food science and technology. 2018;9:345-81.

151. Mozaffarian D, Wu JH. Omega-3 fatty acids and cardiovascular disease: Effects on risk factors, molecular pathways, and clinical events. J Am Coll Cardiol. 2011;58(20):2047-67.

152. Hu Y, Hu FB, Manson JE. Marine omega-3 supplementation and cardiovascular disease: An updated meta-analysis of 13 randomized controlled trials involving 127 477 participants. Journal of the American Heart Association. 2019;8(19):e013543.

153. Parikh M, Netticadan T, Pierce GN. Flaxseed: Its bioactive components and their cardiovascular benefits. American journal of physiology Heart and circulatory physiology. 2018;314(2):H146-h59.

1.8. Flaxseed and Cardiovascular diseases

Among all the dietary interventions being investigated for ischemic heart disease, flaxseed is of particular interest with regard to the thesis research pursued here. Flaxseed is a rich plant source of the omega-3 fatty acid – alpha-linolenic acid. In addition to this, flaxseed is also a good source of fiber and an especially rich source of the lignan – secoisolariciresinol diglucoside. The next section describes in detail the characteristics of flaxseed and its bioactive components. Moreover, it also summarizes the available evidence underpinning the importance of flaxseed as a potential dietary therapy for cardiovascular diseases and other chronic diseases.

1.8.1. Flaxseed: Its Bioactive Components and their Cardiovascular Benefits

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Abstract

Cardiovascular disease remains the leading cause of mortality and morbidity world-wide. The inclusion of functional foods and natural health products in the diet are gaining increasing recognition as integral components of lifestyle changes in the fight against cardiovascular disease. Several preclinical and clinical studies have shown the beneficial cardiovascular effects of dietary supplementation with flaxseed. The cardiovascular effects of dietary flaxseed have included an anti-hypertensive action, anti-atherogenic effects, a lowering of cholesterol, an anti-inflammatory action and an inhibition of arrhythmias. Its enrichment in the omega-3 fatty acid alpha-linolenic acid and the antioxidant lignan secoisolariciresinol diglucoside, as well as its high fiber content have been primarily implicated in these beneficial cardiovascular actions. Although not as well recognized, flaxseed is also composed of other potential bioactive compounds such as proteins, cyclolinopeptides and cyanogenic glycosides which may also produce biological actions. These compounds could also be responsible for the cardiovascular effects of flaxseed. This article will not only summarize the cardiovascular effects of dietary supplementation with flaxseed but also review its bioactive compounds in terms of their properties, biological effects and proposed

mechanisms of action. It will also discuss promising research directions for the future to identify additional health-related benefits of dietary flaxseed.

Introduction

Cardiovascular disease is responsible for 1 of every 3 deaths in the US (1). About 2,200 Americans die of cardiovascular disease each day, an average of 1 death every 40 seconds (17). Pharmacological drugs targeting specific pathways involved in cardiovascular pathologies have seen recent advancement with newer and more efficient drug targets emerging, but are limited in terms of their side effects and patient adherence. The potential of plant-based diets in the form of functional foods is increasingly being realized as an option to treat and/or prevent cardiovascular disease. Functional foods are specifically being studied in preclinical and clinical trials to understand more about their effects on cardiovascular health (147). Omega-3 (n-3) polyunsaturated fatty acids (PUFAs) have been regarded as cardioprotective by several clinical trials such as DART (21), GISSI-Prevenzione trial (11), GISSI-HF (10), JELIS (63), and DOIT (71). n-3 PUFAs have been reported to exhibit many beneficial effects against cardiovascular diseases and risk factors including hypertension, platelet aggregation, dyslipidemia, atherosclerosis, and arrhythmias (14). Fish is the major dietary source of n-3 PUFAs, moreover, studies have shown the association of fish oil consumption and significantly reduced cardiovascular disease risk factors (98). However, in spite of having cardiovascular protective effects, fish oil consumption is limited due to concerns regarding its taste, smell, potential toxins such as methylmercury, dioxins, and polychlorinated biphenyls, and allergies (98, 128). Flaxseed is the other alternative plant source of n-3 PUFAs. It has the highest amount of alpha-linolenic acid (ALA), which is an essential fatty acid that can be converted by biochemical pathways in the

body to long-chain n-3 PUFAs. These metabolites of ALA can also exert beneficial cardiovascular effects.

Flaxseed

Flaxseed, Linum usitatissimum, is also known as linseed. Flax is grown widely all over the world, with the highest production in Canada. The flax plant produces pale blue flowers and fruit capsules filled with small, brown seeds (25). The seeds can be industrially processed to obtain flax oil, as it is one of the richest plant sources of the n-3 polyunsaturated fatty acid (PUFA) – α-linolenic acid (17). The oil accounts for 32-45 % of seed mass, out of which 55-57% is ALA and 15-18% is linolenic acid which is a n-6 PUFA (116). Defatted flax meal represents 55-68 % of flaxseed mass and is left after oil extraction. The oil is typically used as animal feed. Because of fast polymerization properties of flax oil, it is also used in paints, varnishes, and inks. However, because flaxseed contains the highest amount of essential fatty acids from a plant source, it is increasingly used as a functional food. Flaxseed has many bioactive compounds besides ALA, including lignans, cyclic peptides, cyanogenic glycosides, flax proteins and soluble and insoluble fiber. Four common forms of flaxseed available for human consumption include whole flaxseed, ground flaxseed, flaxseed oil, and partially defatted flaxseed meal. The composition of each form of flaxseed is shown in Table 1.

Table 1. Nutritional composition of 4 common forms of flaxseed (per 100 g).

	Fat (g)	ALA (g)	Protein (g)	CHO (g)	Dietary fiber (g)	Lignan (mg)	Calories (Kcal)
Whole flaxseed	41.0	22.8	20.0	28.9	27.8	82-2600	450

Ground flaxseed	40.8	23.1	20.0	29.2	27.7	82-2600	450
Flaxseed oil	100.0	57.0	0.0	0.0	0.0	0	884
Partially defatted flaxseed meal	11.1	6.0	38.9	33.3	33.3	2500	389

Note: The nutritional composition was adapted from Bassett CM et al, 2009.

ALA, α-linolenic acid; CHO, carbohydrates (including sugars, starches, and total dietary fiber).

The seed of the flax plant is comprised of an embryo and two cotyledons surrounded by a shiny brown hull. Oil bodies called oleosomes are found in the endosperm and the cotyledons (134). The protein content of flaxseed is high in arginine (10-31) % (134). About 56-70% of the arginine is found in cotyledons and about 30% in the seed coat and endosperm. Flaxseed contains 20% soluble and 9% insoluble fibers which are present mainly in the hull. The soluble fiber includes acidic (composed of L-rhamnose (25.3%), L-galactose (11.7%), L-fructose (8.4%), and D-xylose (29.1%)) and neutral polysaccharides (L-arabinose (20%) and D-xylose/D-galactose (76%)) (6). Insoluble fiber is composed of cellulose (7-11%), lignin (2-7%), and acid detergent fiber (10-14%) (33).

The seed coat of flaxseed contains the richest source of a lignan phytoestrogen called secoisolariciresinol diglucoside (SDG). The flax lignan complex (FLC) isolated from flaxseed includes 34-38% SDG, 15-21% cinnamic acid glucoside, and 9.6-11% hydroxymethylglutaric acid by weight (116). In plant physiology, lignans are thought to play a protective role and are involved

in growth regulation. Once ingested, these plant lignans are metabolized by gut anaerobic microbes that convert them into the free bioactive mammalian lignans enterolactone and enterodiol (111).

The Biological Actions of Dietary Flaxseed

Flaxseed and its bioactive components have been extensively studied over the past two decades for their beneficial activities on cardiovascular risk factors and disease conditions. This includes dietary supplementation with flaxseed and its effects on inflammation (24), atherogenesis (44), lipid profile (146), platelet aggregation (14), arrhythmias (5), hypertension (25), oxidative stress (114), estrogen activity (14) and glycemic status (146). Some of the important cardiovascular effects will be discussed in detail below and are found in Table 2.

Table 2. Animal and clinical studies highlighting the cardiovascular effects of dietary flaxseed.

Reference	**Model**	**Component**	**Dose and duration**	**Effect**
Antihypertensive effects of flaxseed				
Talom et al (143)	SHR and WKY	Whole flax	20 %, 10-11 weeks	NS ↓ 4 mm Hg in SBP
Al-Bishri WM (1)	Wistar rats	Whole flax	10%, 8 weeks	S ↓ 12 mm Hg in SBP and 23 mm Hg in DBP
Rodriguez-Leyva D et al (129)	Peripheral artery disease (PAD) subjects	Whole flax	30 g, 24 weeks	S ↓ 10 mm Hg SBP and 7 mm Hg DBP

FLAX-PAD trials				
Ogawa A e al (102)	SHR	Flax oil (ALA)	10%, 4 weeks	S ↓ 11 mm Hg SBP
Sekine et al (132)	SHR	Flax oil (ALA)	1 mL, 4 h	S ↓ 14 mm Hg SBP
Begg DP et al (15)	Dams and offspring	Flax oil (ALA)	3%, 30 weeks	S ↓ 16 mm Hg SBP and 8 mm Hg DBP
Begg DP et al (16)	Dams and offspring	Flax oil (ALA)	3%, 24 weeks	S ↓ 16 mm Hg SBP and 8 mm Hg DBP
Barbeau PA et al (12)	Obese Zucker rats	Flax oil (ALA)	10%, 4 weeks	S ↓ 10 mm Hg MBP
Takeuchi H et al (142)	Healthy subjects	Flax oil (ALA)	14 g (2.6g ALA/day), 12 weeks	S ↓ 8 mm Hg SBP and 3 mm Hg DBP
Paschos GK et al (105)	Dyslipidemic subjects	Flax oil (ALA)	15 ml (8 g ALA), 12 weeks	S ↓ 10 mm Hg SBP and 8 mm Hg DBP
Cornish SM et al (32)	Metabolic syndrome subjects	Flax lignan (SDG)	543 mg/day SDG, 24 weeks	S ↓ 7 mm Hg SBP

Billinsky J et al (18)	Healthy subjects	Flax lignan (SDG)		↔
Udenigwe CC et al (148)	SHR	Flax protein a) FPI b) ACPF	200 mg/kg, 24 h	a) S ↓ 18.4 mm Hg at 6 h b) S ↓ 17.9 mm Hg at 2 h
Doyen A et al (41)	SHR	Flax protein FPI (KCl-F1)	200 mg/kg, 24 h	S ↓ 27 mm Hg SBP
Whelton SP et al (154)	T2DM subjects	Flax fiber	5 g, 12 weeks	↔
Lipid lowering and anti-atherosclerotic effects of flaxseed				
Dupasquier CM et al (44)	Hypercholesterolemic rabbits	Flax	10%; 16 weeks	S ↓ 50% in atherosclerosis
Edel Al et al (47) FLAX-PAD Trial	PAD subjects	Flax	30 g; 48 weeks	S ↓ 0.5 mmol/L TC S ↓0.4 mmol/L LDL
Lucas EA et al (91)	Ovariectomized Golden Syrian hamsters	Flax	7.5, 15, 22.5%; 17 weeks	S ↓ 0.9-1.2 mmol/L TC

				S ↓ 0.1-0.3 mmol/L non-HDL S ↓ ~80% atherosclerosis
Dupasquier CM et al (43)	LDL$^{-/-}$ mice	Flax	1%, 5%, 10%; 24 weeks	S ↓ 9-10 mmol/L TC S ↓ ~15% in atherosclerosis
Francis AA et al (54)	Hypercholesterolemic rabbits	Flax	10%; 14 weeks	S ↓ ~40% in atherosclerosis
Lee P et al (88)	Hypercholesterolemic rabbits	Flax oil	5%; 8 weeks	↔
Bassett CM et al (13)	LDL$^{-/-}$ mice	Flax oil	4.4%; 14 weeks	S ↓ 5.2 mmol/L TC S ↓ ~70% in atherosclerosis
Pan A (103) Meta-analysis	Healthy and hypercholesterolemic subjects	Flax oil	1-38 g ALA (median 12.4 g); 2-52 weeks (median 8.5 weeks)	↔

Prasad K (119)	Hypercholesterolemic rabbits	Flax lignan (SDG)	15 mg/kg; 8 weeks	S ↓ 22 mmol/L TC
Prasad K (122)	Hypercholesterolemic rabbits	Flax lignan complex	40 mg/kg; 16 weeks	S ↓ ~32% in atherosclerosis - Not effective in regression of established plaques
Hallund J et al (68)	Postmenopausal women	Flax lignan (SDG)	500 mg/kg; 6 weeks	↔
Bassett CM et al (13)	$LDL^{-/-}$ mice	Flax fiber	7%; 14 weeks	S ↓ 5.2 mmol/L TC S ↓ 0.44 mmol/L LDL
Jenkins DJ et al (76)	Hyperlipidemic subjects	Flax fiber	50 g PDFM/d, ~20 g fiber/d; 3 weeks	S ↓ 0.36 mmol/L TC S ↓ 0.44 mmol/L LDL
Thakur G et al (145)	T2DM	Flax fiber	6 g/d; 12 weeks	S ↓ 0.5 mmol/L TC & LDL-c
Kristensen M et al (84)	Healthy subjects	Flax fiber	3 drinks/d 5.2 g/10MJ	S ↓ 0.55 mmol/L TC

				S ↓ 0.48 mmol/L LDL-c
Anti-platelet effects of flaxseed				
Iancu RI et al (74)	Golden Syrian hamsters (Diabetic)	Flax	15%; 20 weeks	S ↓ 46.6% in platelet aggregation S ↓ 58% in platelet adhesiveness
Haliga RE et al (67)	Ovariectomized Wistar rats + high fat diet	Flax	15%; 36 weeks	NS ↓ 17% in platelet aggregation S ↓ 15% in platelet adhesiveness
Edel AL et al (46)	Healthy subjects	Flax	10, 20, 30, 40 g; 4 weeks	↔
Edel AL et a (47) FLAX-PAD Trial	Peripheral artery disease subjects	Flax	30 g; 48 weeks	S ↓ 5.5% in platelet aggregation

Vas Dias FW (152)	New Zealand white rabbits	Flax oil	60 g/kg; 8.5 weeks	S ↓ 4% in platelet aggregation
Holy EW et al (72)	Mice	Flax oil	7.3%; 2 weeks	S ↓ 10% in platelet aggregation
Austria JA et al (9)	Healthy subjects	Flax oil	6 g ALA; 12 weeks	↔
Patenaude A et al (108)	Healthy subjects	Flax oil	6 g ALA; 4 weeks	↔
Sano T et al (130)	$ApoE^{-/-}$ and $LDL^{-/-}$ mice	Flax lignan (SDG)	0.06%, 100 mg/kg; 8 weeks	↔
Sano T et al (130)	$ApoE^{-/-}$ and $LDL^{-/-}$ mice	Flax lignan (SDG)	5%, 8.3 g/kg; 8 weeks	S ↓ 25% in platelet aggregation
Flaxseed effects on cardiac function				
Ander BP et al (5)	New Zealand white rabbits	Flax	10%; 16 weeks	- Shorter QT interval compared to control

				S ↓ 78% in VF during ischemia S ↓66% in VF during reperfusion
Caligiuri SP etl (26) FLAX-PAD trial	PAD subjects	Flax	30 g; 48 weeks	↔
Xie N et al (155)	Diabetic rats	ALA	500 µg/kg; 4 weeks	S ↑ LV dp/dt
Ghule AE et al (61)	Aortic stenosis in rats	Flax lignan complex	400 mg/kg; 4 weeks	- Shorter ECG intervals - S ↑ LVEDP, LVSP, LVdp/dt
Penumathsa SV et al (109, 110)	Myocardial ischemic rats	Flax lignan (SDG)	20 mg/kg; 2 weeks	S ↑ LV ejection fraction and fractional shortening
Puukila S et al (124)	Monocrotaline model of pulmonary arterial hypertension in rats	Flax lignan (SDG)	25 mg/kg; 3 weeks	S ↓ RV hypertrophy, RVEDP, RVSP

↑ significant increase, ↓ significant decrease, ↔ no effect, *ACPF* Arginine-rich cationic peptide fraction, *ALA* alpha-linolenic acid, *ApoE*$^{-/-}$ apolipoprotein E knock-out, *D* day, *DBP* diastolic blood pressure, *FPI* flaxseed protein isolate, *HDL* high density lipoprotein cholesterol, *LDL* low density lipoprotein cholesterol, *LDL*$^{-/-}$ LDL receptor knock-out, *LV* left ventricle, *LVdp/dt* rate of rise of left ventricular pressure, *LVEDP* left ventricular end-diastolic pressure, *LVSP* left ventricular systolic pressure, *NS* not significant, *PAD* peripheral artery disease, *PDFM* partially defatted flaxseed meal, *RV* right ventricle, *RVEDP* right ventricle end-diastolic pressure, *RVSP* right ventricle systolic pressure, *S* significant, *SBP* systolic blood pressure, *SDG* secoisolariciresinol diglucoside, *SHR* spontaneously hypertensive rats, *T2DM* Type 2 Diabetes mellitus, *TC* total cholesterol, Values in % represents amount supplemented in diets, VF ventricular fibrillation, *WKY* Wistar-Kyoto rats.

Antihypertensive Effects of Dietary Flaxseed

***Whole flaxseed*:** Only two studies have been carried out in animals with whole flaxseed. Talom et al could not detect a significant change in systolic blood pressure (SBP) in spontaneously hypertensive rats (SHRs) given a flaxseed diet (144). On the contrary, another study reported a significant fall of ~ 12 mm Hg in SBP and ~ 23 mm Hg in diastolic blood pressure (DBP) after the administration of 8 weeks of a flaxseed supplemented diet (1). In humans, both observational and randomized controlled trials have shown the antihypertensive effects of flaxseed. The longest study, the FLAX-PAD Trial, a randomized, double-blinded, placebo-controlled year-long trial investigated the effect of ground flaxseed, 30 g in food, in hypertensive patients. A significant reduction of 10 mm Hg SBP and 7 mm Hg DBP was observed in the flax group of patients. An

even higher reduction of 15 mm Hg in SBP was observed in a patient subgroup that entered the trial with elevated blood pressure at baseline (129). This drop in blood pressure produced by flaxseed in hypertensive patients was more than the average decrease observed with antihypertensive medications taken at standard doses (87). Because brachial blood pressure is not always considered a good marker to assess the effect of antihypertensive compounds, effect of flaxseed on central blood pressure was also checked. A drop of 3.4 and 4.9 mm Hg in central SBP from baseline was observed at intervals of 6 months and 12 months, respectively, in the group of patients that consumed 30 g flaxseed in food compared to placebo. Central diastolic blood pressure exhibited 1.3 and 2.6 mm Hg fall at 6 and 12 months respectively (26).

***Flax oil rich in ALA*:** Two studies examining the effect of dietary flax oil enriched in ALA have exhibited a significant decrease in SBP (102, 132). In addition, offspring from rats fed flaxseed during pregnancy also demonstrated a significant reduction in blood pressure compared to offspring from dams not fed flaxseed (15, 16). Recently, flaxseed oil administered to obese Zucker rats demonstrated a 6 % reduction in mean arterial blood pressure (MAP), and a 25 % reduction in total peripheral resistance (TPR) (12). In the INTERMAP Trial, a large epidemiological study of 4680 men and women, a significant inverse relationship was observed between dietary ALA and blood pressure (149). Twelve weeks of daily consumption of 14 g of oil enriched with 2.6 g ALA resulted in ~10 and 3 mm Hg reduction in SBP and DBP respectively, in pre-hypertensive patients (142). Flaxseed oil was found to reduce SBP, DBP and MAP by 10, 8 and 8 mm Hg respectively in dyslipidemic patients after ingestion of 15 ml flax oil containing 8 g ALA/day in a meal for 12 weeks (105).

Flax lignans and SDG: SDG in doses of 3, 5 and 10 mg/kg administered intravenously in rats produced a dose dependent reduction in SBP (15-40 %), DBP (24-48 %), and MAP (22-43 %)

(113). However, it is important to recognize that this type of intervention is entirely unphysiological. Ingested SDG is not present in the circulation, but is metabolized in the gut into its metabolites enterodiol (END) and enterolactone (ENL). Ingestion of the flax lignan SDG (543 mg/day, orally in tablet form, for 24 weeks) by metabolic syndrome patients induced a significant decrease of 7 mm Hg DBP (32). However, a randomized controlled trial in a healthy population for 6 months with Beneflax, a commercially available source of SDG that provides 543 mg of SDG in one tablet, showed no difference between treatment groups (18). These negative results in a normotensive population coincides with results in normotensive patients fed milled flaxseed as well (40, 76, 129, 138).

Flax protein: A flaxseed protein isolate (FPI) given at a dose of 200 mg/kg body weight to spontaneously hypertensive rats exhibited a delayed hypotensive effect starting 4 h after administration with a maximum reduction of 18.4 mmHg in SBP at 6 hours. The delayed effect may be due to the time required to metabolize the large proteins in the intestine before absorption could take place. In the same study, these investigators generated peptide fractions through hydrolysis of the FPI and separated it by electrodialysis and ultrafiltration to obtain cationic peptides. These peptides were rich in arginine residues. This arginine-rich cationic peptide fraction (ACPF) produced a significant decrease in SBP of 17.9 mmHg after 2 h of administration. This was compared against the amino acid form of arginine which showed only a 10.3 mmHg reduction in SBP. Moreover, the peptide fraction produced a long-lasting effect of up to 6 h. The could be explained by a more efficient absorption of peptides compared to free arginine. Thus, in summary, both the FPI and the ACPF exhibit a hypotensive effect, but the FPI produces a slow and sustained effect while the ACPF results in a faster less prolonged action (148). An antihypertensive effect has also been demonstrated using a fraction of the FPI, called KCl-F1 (32). A large 27 mmHg

reduction in SBP was observed after 4 h of administration to spontaneously hypertensive rats (41). Recently a fraction sequence GVKVEGDGGLVRRDEI with a predominant compound sequence of a 13-amino acid – GFPGRLDHWCASE, was identified (136). This sequence has the highest antioxidant activity and could also be responsible for the hypotensive effect seen with flax protein. This study has implications to advance our understanding of the bioactive molecular components within the FPI (136).

Flaxseed fiber: A meta-analysis of 25 randomized controlled clinical trial reported reduction of SBP and DBP with fiber doses of <=7.1 g/day orally and significant reduction of 3.4 mm Hg in SBP and 1.97 mm Hg in DBP with doses 7.2 to 18.9 g/day. However, this effect does not increase by increasing the fiber dose above 19 g/day (154). A same trend of statistically insignificant fall in SBP, DBP and MAP was also seen in a randomized controlled trial with hypertensive patients consuming 5 g/day of flaxseed gum, orally in wheat flour chapattis, for 12 weeks (145). An increase in sample size may be required to define the action of flaxseed gum conclusively.

Mechanism: Several studies have suggested that ALA may be the responsible bioactive that produces antihypertensive action of flaxseed (Figure 8) (12, 15, 16, 102, 132, 149). The mechanism may involve its action on oxylipin concentration. Oxylipins are oxygenated metabolites of polyunsaturated fatty acids (58). They have been implicated in the pathogenesis of hypertension because of their role in controlling inflammation and vascular tone. Polyunsaturated fatty acids (PUFAs) cleaved from membrane phospholipids by cytosolic phospholipase A_2 initiates the biosynthesis of oxylipins (28, 58). PUFAs can be metabolized by a) cyclooxygenase, b) lipoxygenase, and c) cytochrome P450 (CYP450) (58). Epoxygenase CYP450 catalyzes formation of epoxy-eicosatrienoic acid (EpETrE) oxylipins from PUFAs (58). EpETrE are further

metabolized to dihydroxy-eicosatrienoic acid (DiHETrE) by soluble epoxide hydrolase (sHE) (28). EpETrE are endothelial-derived hyperpolarizing factors that are associated with vasodilation through activation of nitric oxide synthase (23). But because of metabolism of EpETrE to DiHETrE by sHE, there occurs a loss of vasodilation (27). Moreover, sHE also leads to production of inflammatory and cytotoxic oxylipins particularly dihyroxyoctadecenoic acids (DiHOMES) (23). Flaxseed exerts its antihypertensive action via inhibition of sHE activity as evidenced by inverse relationship observed between ALA and % sHE activity (23).

An anti-inflammatory action may prevent the imbalance between vascular tone regulators contributing to blood pressure control. A reduction in peripheral blood mononuclear cell production of interleukin-6, interleukin-1 and tumor necrosis factor-alpha in hypercholesterolemic patients fed an ALA-enriched diet for 6 weeks (161). Consumption of ground flaxseed for 4 weeks reduced pro-inflammatory oxylipins in the plasma of older adults (24). Additionally, reactive oxygen species are known to play a pathogenic role in hypertension (97). SDG metabolites may lower BP through their antioxidant effects (38). Activation of guanylate cyclase and angiotensin I inhibition are also proposed mechanisms for the BP lowering effects of SDG (113, 121). Inhibition of the angiotensin-converting enzyme and renin could also be the possible mechanism of the anti-hypertensive action of peptides (24). Finally, ground flaxseed and flaxseed peptides are rich in arginine content, and as arginine is converted in the vascular endothelium to nitric oxide and citrulline, they may also exert an anti-hypertensive action by increasing the availability of nitric oxide and thereby causing vasodilation.

Lipid-Lowering and Anti-atherosclerotic Effects of Flaxseed

Whole flaxseed: Several animal studies have shown variable effects of flaxseed on serum lipid profile. Whereas one study demonstrated an increase in total cholesterol (TC) and LDL cholesterol with a flaxseed supplemented diet (114), most show a decrease (43, 85, 126). Dupasquier et al reported no changes in circulating cholesterol levels in rabbits, either in presence or absence of additional cholesterol in the diet (44). The majority of the human studies have reported that flaxseed reduces TC by 6-11 % and LDL cholesterol by 9-18 % in normolipidemic subjects (31, 34, 35), and TC by 5-17 % and LDL by 4-10% in hypercholesterolemic patients (76, 93, 107). Results from the FLAX-PAD Trial reported a reduction of 15% and 11%, respectively, in LDL cholesterol and TC at 1 and 6 month of the trial in subjects fed a diet supplemented with 30 g flaxseed compared to the placebo. However, at 12 months these differences were no longer significant. Furthermore, significant differences observed at 1 and 6 months were only within the flaxseed group and not between-treatment groups (47). This is consistent with other studies in hemodialysis patients (82), in hyperlipidemic patients (76, 94), and in postmenopausal women (107). Flaxseed consumption did not interfere and was additive to the cholesterol lowering effects of cholesterol lowering medications like statins (47). Ground flaxseed, 38-40 g/day in food, can also reduce the levels of atherogenic lipoproteins Lp(a) and apolipoproteins A-1 and B after 12 weeks of dietary intervention (19, 92).

Most animal studies have demonstrated no changes in serum high density lipoprotein cholesterol (HDL) and triglyceride levels (TG) (43, 85, 91, 114). These results are consistent with human studies as well. A meta-analysis of 28 studies concluded that flaxseed produces no significant changes in HDL and TG levels (103). The same lack of action of flaxseed on HDL and TG levels was also observed in an interventional clinical trial (47).

Dupasquier et al reported a reduction in atherosclerosis in hypercholesterolemic rabbits fed a flaxseed supplemented diet (44). A dose dependent reduction in atherosclerosis was also observed in ovariectomized Golden Syrian hamsters (91). In LDL receptor knockout ($LDL^{-/-}$) mice, flaxseed supplementation to the diet produced a dose dependent (1%, 5%, and 10% flaxseed diet) suppression in atherosclerotic plaque formation (43). Furthermore, it exerted an anti-inflammatory and anti-proliferative action. Expression of IL-6 proinflammatory cytokine, macrophage marker mac-3, vascular cell adhesion molecule VCAM-1, and proliferation cell nuclear antigen (PCNA) levels were significantly reduced in the flaxseed supplemented group compared to the cholesterol group alone (43). The effects of flaxseed on regression of established atherosclerotic plaques have also been studied (54). Four weeks of 1% cholesterol feeding in rabbits followed by 8 weeks of regular chow produced stabilized atherosclerotic plaques. Dietary flaxseed administered for 14 weeks after plaque stabilization exhibited a significant ~40% reduction in the extent of lesion area. The impaired vascular reactivity that accompanies atherosclerosis was also improved in both contraction and endothelium-dependent vessel relaxation in the presence of a flaxseed supplemented diet (54).

Flax oil rich in ALA: Lee and Prasad reported no effect of flaxseed oil on plasma lipids in animals (88). However, in $LDL^{-/-}$ mice, flaxseed oil lowered circulating cholesterol levels (13). Clinical trials with flax oil (2-20 g/day) produced no beneficial effect on TC, LDL cholesterol, HDL cholesterol, or TG levels after 4-12 weeks of intervention (79, 101, 103). Moreover, two studies suggested that flax oil may cause more harm by increasing TC (8.3%) and decreasing HDL cholesterol (8.3%) levels after 4-26 weeks of intervention (70, 101). However, flax oil rich in ALA was observed to prevent the initiation of atherosclerotic development induced by administration of trans fatty acids from industrially hydrogenated vegetable sources in $LDL^{-/-}$ mice (13). This

would suggest that flax oil (ALA) exerts an anti-atherogenic action by a mechanism other than lipid lowering (13).

Flax lignan and SDG: A series of studies have demonstrated the beneficial effects of flax lignan complex and SDG on the plasma lipid profile (57, 110, 115, 118-120, 122, 123). SDG and its aglycone form secoisolariciresinol (SECO) reduced hepatic cholesterol levels in a dose dependent manner in diet-induced hypercholesterolemic and hypertriglyceridemic female rats (53). Dietary supplementation with 500 mg/day of SDG in healthy postmenopausal women induced no changes in serum lipid levels after 6 weeks (68). However, Zhang et al (159) reported a dose dependent (300 and 600 mg/day SDG in tablet) reduction in TC (22%) and LDL cholesterol (24%) during 8 weeks of treatment in hypercholesterolemic adults. Flax lignan given at a dose of 0.41 g in nutrition bar also significantly reduced oxidized LDL cholesterol by 25% in healthy men and women (3). Both animal and human studies have shown no effect on TG (57, 110, 118, 119, 159).

An 8-week diet supplemented with delipidated flaxseed and cholesterol to rabbits resulted in a suppression of aortic atherosclerosis (123). SDG (20 mg/kg body weight) suppressed the atherosclerotic plaque progression by 73% in hypercholesterolemic rabbits, whereas a flax lignan complex (FLC) was only 31% effective. This suggests that for an anti-atherogenic action, SDG is the main bioactive in FLC and other components in FLC may reduce the full effect of SDG. For regression of atherosclerosis, SDG and FLC both were found to be ineffective (116, 119, 122).

Flax fiber: Very few animal studies have shown the effect of flax fiber (FF) on serum lipid. Bassett and co-workers reported a significant reduction in serum cholesterol levels in $LDL^{-/-}$ mice fed a partially defatted flaxseed meal (PDFM) high in fibre content (13). PDFM fed to human subjects at a dose of 50 g/day reduced TC by 4.6% and LDL cholesterol by 7.6% after 3 weeks

(76). Flaxseed gum containing soluble fibre at a dosage of 5 g/day in wheat flour chapattis for three months reduced TC and LDL cholesterol by 10% and 16%, respectively, in type II diabetic patients (145). Kristensen reported that flax fibre (FF) drink, (5.2 g/10MJ) 3/day, lowered TC by 12% and LDL cholesterol by 15%. In the same study, FF significantly increased the faecal excretion of fat by 50% (84). Others have also suggested that the high fibre content of flaxseed was responsible for its LDL cholesterol lowering action (47). Human trials with PDFM have reported no effect on TG levels (47, 76, 84, 145). Though it has lipid lowering potential, FF was found to exert no beneficial effect on hypercholesterolemic atherosclerosis in $LDL^{-/-}$ mice (13).

Mechanism: Several mechanisms have been proposed for the anti-atherogenic activity of flaxseed (Figure 8). Flaxseed decreased the expression of IL-6, VCAM-1 and PCNA. IL-6 is involved in atherogenic smooth muscle cell and fibroblast proliferation, LDL oxidation, monocyte and macrophage activation (75). VCAM-1 is involved in monocyte recruitment and infiltration into the arterial wall and differentiation into macrophages (43). PCNA is a cell proliferation marker and cell proliferation is thought to be an important component of atherosclerosis (157). This suggests that flaxseed exerts its anti-atherogenic effects through anti-inflammatory and anti-proliferative actions (89, 141). ALA is metabolized to the longer chain fatty acids eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA). DPA inhibited cyclooxygenase I activity, and decreased cellular TNF-α activity, whereas EPA and DHA activated peroxisome proliferator-activated receptors (PPARs) – γ and α, which are known to be involved in various anti-inflammatory and anti-atherogenic activities (95). By incorporating n-3 fatty acids into the cell membrane, this may reduce the oxidative stress by scavenging reactive oxygen species and consequently decreasing the activation of redox sensitive proinflammatory transcription factor NF-kB (36). ALA can also cause cholesterol efflux from macrophages through inhibition of

stearoyl CoA desaturase (SCD)-1 activity (158). The anti-atherogenic action of SDG may involve a scavenging of the hydroxyl radical (·OH) and its ability to lower lipid levels (117). In addition, a metabolite of SDG, ENL, is a phase 2 protein inducer (77). Induction of the expression of this protein can decrease oxidative stress (153). Flaxseed fiber may exert its lipid lowering effect by creating satiety and thereby reducing caloric intake (7), decreasing transit time (50), increasing the rate of bile acid excretion and reducing bile acid reabsorption, and via increased faecal excretion of cholesterol (7, 51, 140). Fibers may also act through the formation of small chain fatty acids (SCFAs) e.g. acetate, propionate. These SCFAs can influence multiple metabolic pathways including fatty acid synthesis, oxidation, lipolysis and cholesterol synthesis (37, 52). Increased levels of propionate inhibit the conversion of acetate to lipids (22, 37) and 3-hydroxy-3-methyglutaryl-CoA reductase (37), thus, decreasing hepatic cholesterol synthesis.

Antiplatelet Effects of Flaxseed

Whole flaxseed: Only two studies have investigated the effects of whole flaxseed on platelet aggregation in animal studies. One of them reported significant reduction in platelet aggregation after 20 weeks of flaxseed diet supplementation (15% flaxseed diet) to diabetic Golden Syrian hamsters (74). The other study also demonstrated an anti-aggregation effect on platelets in rats given a flaxseed supplemented high fat diet (67). In contrast, clinical trials in humans have not found any changes in platelet aggregation with flaxseed after 1 to 12 months of treatment (9, 39, 108). Edel et al also reported no changes in the rate and extent of platelet aggregation by increasing the dose of flaxseed from 10-40 g/day in food (46). A large randomized, placebo-controlled trial, the FLAX-PAD trial, exhibited only an isolated but significant inhibition of thrombin-induced platelet aggregation by flaxseed in patients suffering from peripheral artery

disease but most of the results examining the effects of dietary flaxseed on platelet aggregation were negative (47).

Flax oil rich in ALA: Flaxseed oil supplemented in a rabbit diet (60 g/kg) demonstrated a significant lowering of platelet aggregation after 60 days compared to control (152). Mice fed a cholesterol diet rich supplemented with flax oil (7.3% flax oil supplemented diet) resulted in a significant inhibition of thrombin- and collagen-induced platelet aggregation compared to control (72). Again, in contrast to the animal data, most of the human studies with flax oil have detected no effect on platelet aggregation (9, 55, 79, 80, 108). Only Allman et al have reported a decrease in the collagen-induced platelet aggregation response was induced by flaxseed oil. However, with a sample size of only 11 healthy individuals, these results may require a larger sample size to draw any firm conclusions in view of the conflicting negative results (2).

Flax fiber, lignan and SDG: T Sano et al reported a reduction in thrombus formation in apolipoprotein E and $LDL^{-/-}$ mice fed a PDFM supplemented diet (130). However, SDG induced no change in thrombus formation in the same species of mice (130).

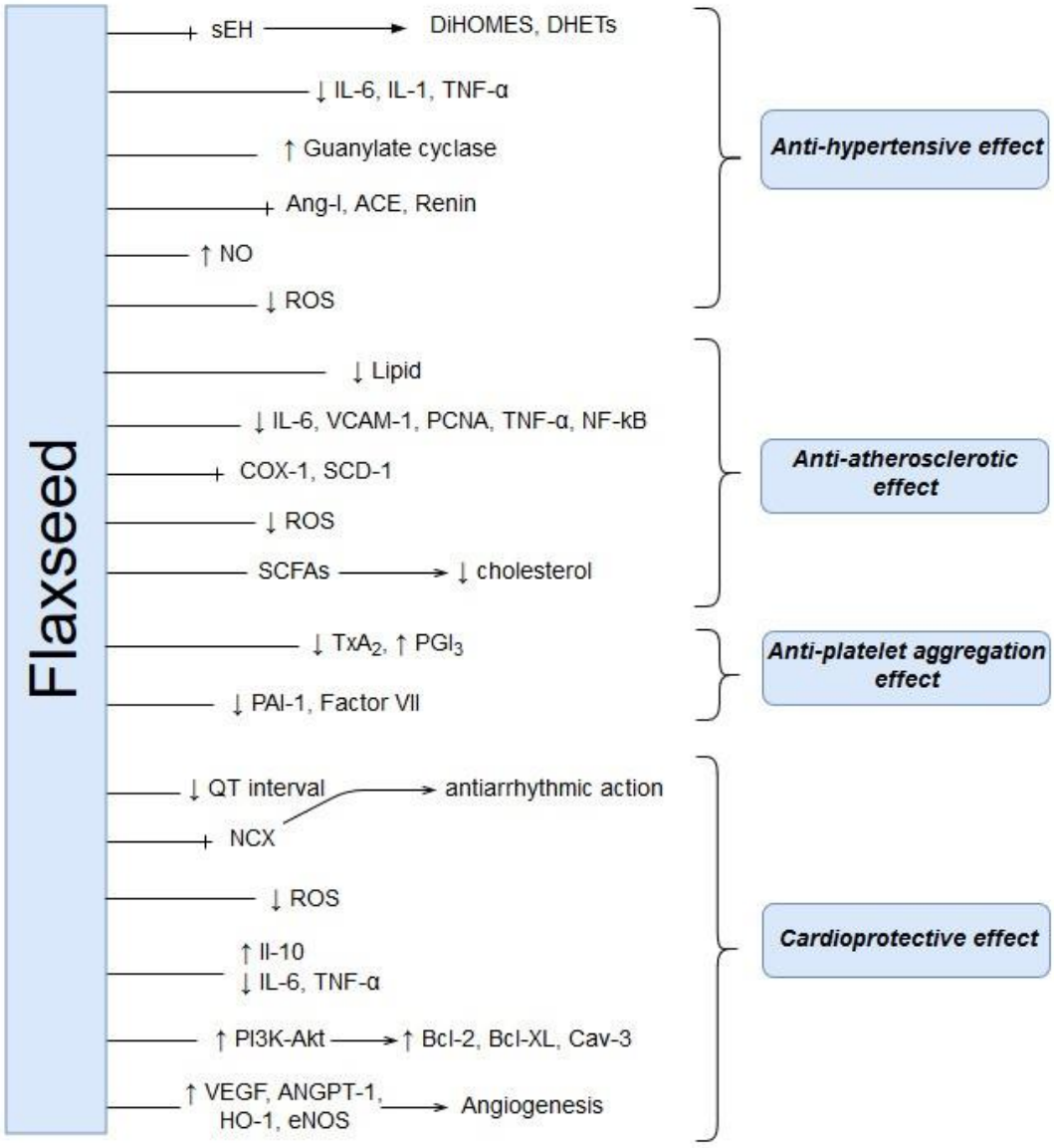


Figure 8. Proposed mechanism of action of flaxseed.

Mechanism: The modest effects observed on platelet aggregation could have been induced by the ALA content of the flaxseed supplemented diet. ALA may cause changes in the fatty acid composition of platelet lipids by increasing EPA and DHA content in the membrane (Figure 8). The simultaneous release of EPA with arachidonic acid during aggregation may cause inhibition of membrane released arachidonic acid metabolism to aggregatory metabolites in platelets (100,

152). This may promote production of anti-aggregatory prostacyclin PGI3 and inhibition of pro-aggregatory species TxA2 (45). B) Changes in G-protein coupled receptors or in the leucine-rich repeat family of receptors may also have been involved (47). Alternatively, flaxseed fiber could have decreased thrombus formation via a reduced plasminogen activator inhibitor-1 activity (139) or coagulation factor VII (96).

Effects of Flaxseed on Cardiac Function

Whole flaxseed: Rabbits were fed a flaxseed supplemented diet in the absence or presence of cholesterol enrichment in the diet as well (5). Isolated hearts from these animals were then subjected to an ischemia and reperfusion (IR) challenge. The hearts from the animals fed the flaxseed intervention exhibited a significantly lower incidence of arrhythmias. Specifically, ventricular fibrillation (VF) was observed in only 6% of hearts from flaxseed diet group during ischemia compared to cholesterol fed group which exhibited 28% VF. During reperfusion flaxseed fed group showed 19% VF compared to 64% VF in cholesterol fed group (5). The flaxseed supplemented diet also resulted in a shorter QT interval compared to the prolonged interval in cholesterol fed-group (5).

In clinical trials, dietary flaxseed has exhibited no effect on cardiac function. Over 12 months' intervention with flaxseed in the FLAX-PAD Trial, no significant changes were observed on heart rate, left ventricular dp/dt, ejection duration, central diastolic duration, and the central Buckberg subendocardial viability ratio (an index of myocardial oxygen supply and demand) (26).

Flax oil rich in ALA: ALA (500 µg/kg) administered in the diet for 4 weeks to rats subjected to IR exhibited improved left ventricular developed pressure compared to a diabetic control group not administered ALA (155). ALA also reduced infarct size, apoptosis, and TNF-α and IL-6 concentrations (155). The dietary intervention also increased plasma IL-10

concentrations and the expression of phosphatidylinositol-3 kinases (PI3K) and Akt phosphorylation (155).

Recently Ganguly et al (59) reported the cardioprotective effect of ALA in ischemia/reperfusion condition. Oxidized phospholipid, especially oxidized phosphatidylcholine (OxPC) species, produced by oxidation from free radicals have pro-inflammatory and pro-apoptotic activities. Adult rat cardiomyocytes were treated with ALA for 24 h before exposing them to ischemia and IR conditions for 1 h each. Besides increased cell death, two OxPC species, 1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine (POVPC) and 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine (PGPC) were found to be significantly increased after IR condition in adult rat cardiomyocytes. ALA pre-treatment for 24 h at 400 μM resulted in a significant reduction in apoptosis. Furthermore, a signification decrease was observed in the levels of POVPC and PGPC in the cardiomyocytes with ALA treatment (60).

Flax lignan and SDG: FLC delivered at a concentration of 400 mg/kg significantly prevented prolongation of ECG intervals (QT, ST, RR, and PR) and increased heart rate and amplitude of the QRS complex compared to control animals in a rat model of aortic stenosis-induced cardiac hypertrophy. FLC treatment also resulted in an improvement of left ventricular end diastolic pressure (LVEDP) and systolic pressure (LVSP) (61).

Penumathsa et al (109, 110) reported that SDG ingested at a dosage of 20 mg/kg for 2 weeks in an IR study reduced the myocardial infarct size and improved left ventricular ejection fraction and fractional shortening (109, 110). Puukila and colleagues reported that SDG (25mg/kg) provided protection against right ventricular (RV) damage and dysfunction in a monocrotaline-induced model of pulmonary arterial hypertension by decreasing RV hypertrophy and reducing RV end diastolic pressure (RVEDP) and RV systolic pressure (124).

Mechanism: QT prolongation is associated with polymorphic tachycardia and torsade de pointes (Figure 8) (8, 106). Flaxseed fed rabbits exhibited a shorter QT interval suggesting that flaxseed may produce its antiarrhythmic action by shortening the QT interval and in turn reducing the action potential duration (5). No study has revealed the main bioactive behind the antiarrhythmic effect. However, ALA may be the most likely bioactive responsible for this action based upon the following four lines of evidence. First, primary action of n-3 PUFA is on the phospholipid bilayer of the myocardial cells especially in the micro domains where fast, voltage-dependent sodium channels are present (83). During ischemia, an increase in intracellular Ca^{2+} activates phospholipase $A_{2,}$ which then liberates n-3 fatty acids from the cardiac membrane (62, 150). N-3 fatty acids may shift the voltage required to change sodium channels from inactive to active, thus this may decrease the activity of sodium channels in cardiomyocytes and increase the threshold required for depolarization (49, 83). Second, EPA and DHA also regulate the activity of L-type calcium channels, leading to a reduction in free cytosolic calcium ion. This stabilizes myocyte electrical excitability to prevent fatal arrhythmia (49). Third, incorporation of n-3 fatty acids in cardiac cell membrane may lower vulnerability to arrhythmias by producing variety of eicosanoids (30). Fourth, increased Na^{+}/Ca^{2+} exchanger (NCX) activity is arrhythmogenic and NCX is responsible for inward current associated with delayed afterdepolarizations or oscillatory afterpotentials (48). ALA and EPA were observed to inhibit NCX activity in HEK293 cells (4). This suggests that antiarrhythmic potential of ALA is mediated through inhibition of increased NCX activity.

ALA has been suggested to improve cardiac function via increasing the expression of the anti-inflammatory cytokine IL-10 and decreasing the expression of the proinflammatory cytokines IL-6 and TNF-α in both serum and myocardium (155). ALA also decreased the production of

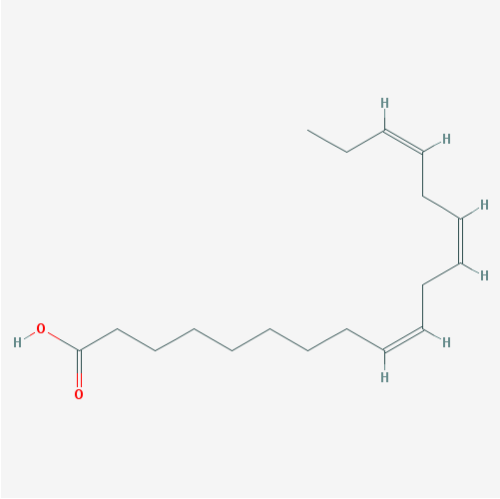
reactive oxygen species and increased the levels of cardiac antioxidant enzymes (155). ALA also upregulated the expression of the anti-apoptotic proteins Bcl-2 and Bcl-X_L in cardiomyocytes at the same time as increasing the expression of caveolin-3 (Cav-3) (29). ALA increased the internalization of TNF receptor 1 (TNFR1) and thus its pro-apoptotic effects are prevented by the enhanced Cav-3 expression (29). These antiapoptotic actions of ALA may be mediated through a PI3K-Akt signaling pathway (29, 155). Alternatively, exposure of the cells to ALA may alter the fatty acid composition of the myocardial cell membrane and reduce the production of cardiotoxic oxidized phospholipid species (23). POVPC and PGPC can initiate apoptotic stress response by activating acidic sphingomyelinases, caspase-3, and mitogen-activated protein kinase in smooth muscle cells. So, it can be suggested that in cardiomyocytes also these two OxPC species can cause apoptosis in a similar manner. ALA incorporates itself in the phospholipid membrane of cardiomyocytes and decreases production of OxPC during IR. It also reduces DNA fragmentation and decreases caspase-3 activation thereby inhibiting apoptosis (60).

SDG increased the expression of vascular endothelial growth factor (VEGF), angiopoietin-1, and phosphorylated endothelial nitric oxide synthase (e-NOS) in the myocardium (109, 110). VEGF is responsible for stimulation of blood vessel growth through induction of mitosis in endothelial progenitor cells and it may also enhance endothelial cell survival (109). eNOS has been suggested to be a downstream mediator of VEGF, and is also thus involved in the process of neovascularization (99). Angiopoietin-1 increases endothelial cell survival by inhibiting apoptosis. This is mediated through several pathways, including phosphatidylinositol 3-kinase/Akt activation and upregulation of surviving protein (69, 104). HO-1 is a stress-inducible protein, which on degradation releases CO that can activate the guanylate cyclase-cGMP pathway. This results in vasodilation and inhibition of smooth muscle cell proliferation and platelet activation (42).

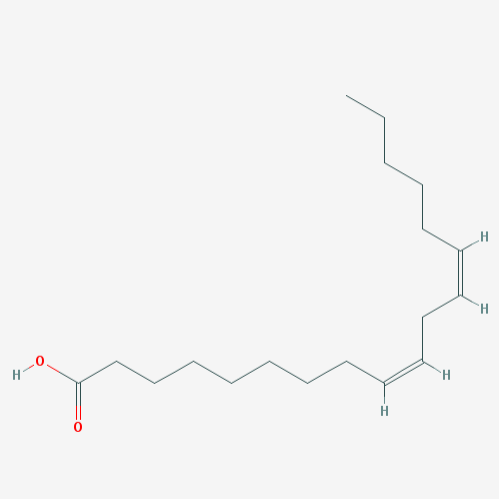
Increased expression induced by SDG of these angiogenic proteins identified above may restore vascular responsiveness in ischemic tissue resulting in improved myocardial perfusion and cardiac function. SDG may thereby correct the imbalance between myocardial oxygen supply and demand which causes myocyte necrosis, apoptosis and abnormal collagen accumulation in the myocardium (61).

Effects of Other Bioactives Within Flaxseed

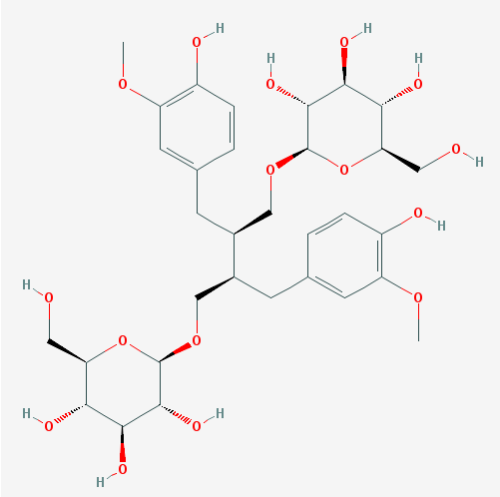
Cyanogenic glycosides: Flaxseed contains nitrogenous secondary plant metabolites called cyanogenic glycosides (CGs). With a content of 250-550 mg/g dry weight of seed (135), CGs are glycosides of cyanohydrins and include linamarin, linustatin, lotaustralin, neolinustatin, and amygdalin (Figure 9) (134, 135, 156). Valine, leucine, isoleucine, phenylalanine, and tyrosine are the primary amino acid precursors for aglycones of CGs (156). Upon ingestion, the glycone portion is removed, liberating an unstable cyanohydrin. This metabolic process is mediated through intestinal β-glucosidase-induced hydrolysis of glucosidic bonds. Cyanohydrin is further degraded to hydrogen cyanide (HCN) which is toxic to the respiratory, nervous and endocrine systems (135). Although this may initially be considered a significant health concern, it is not for several reasons. First, the adult human body has the ability to detoxify up to 100 mg of cyanide/day. A person would need to consume more than 1 kg of flaxseed each day for cyanide toxicity to manifest itself (116). This is not realistic. Secondly, cooking destroys cyanide (116) and even 50 g/day of flaxseed does not increase urinary thiocyanate levels (34). Thus, the toxicity of flaxseed from CGs is not a realistic health threat.



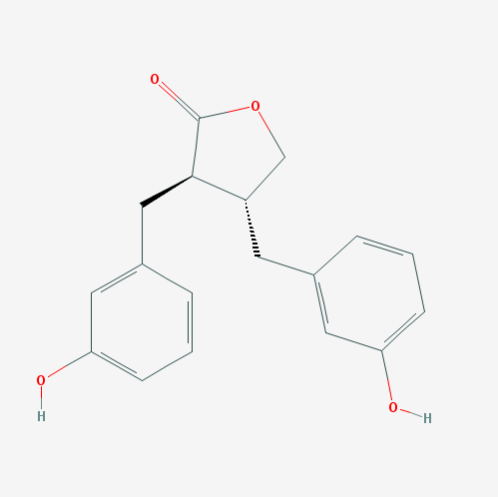
Alpha-Linolenic acid (ALA)



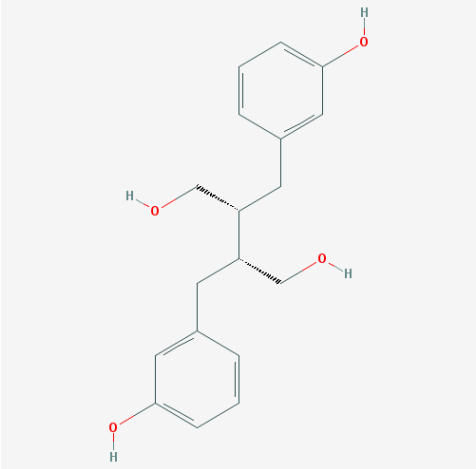
Linoleic acid (LA)



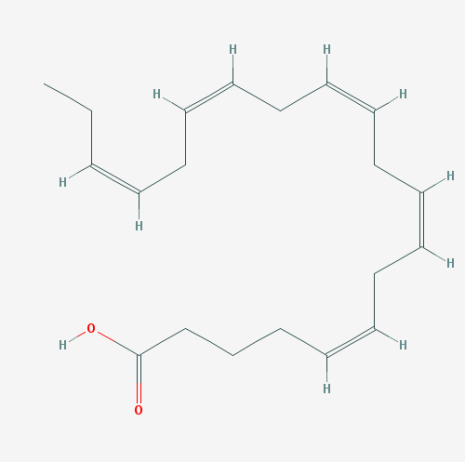
Secoisolariciresinol diglucoside (SDG)



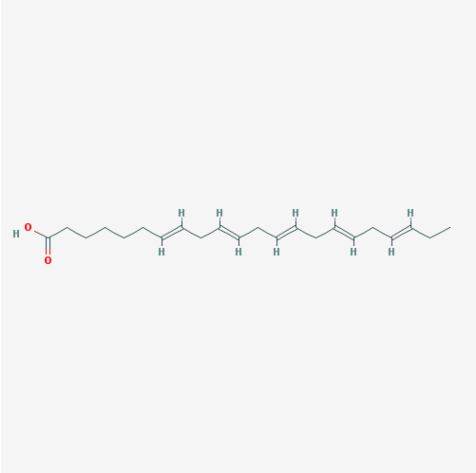
Enterolactone (ENL)



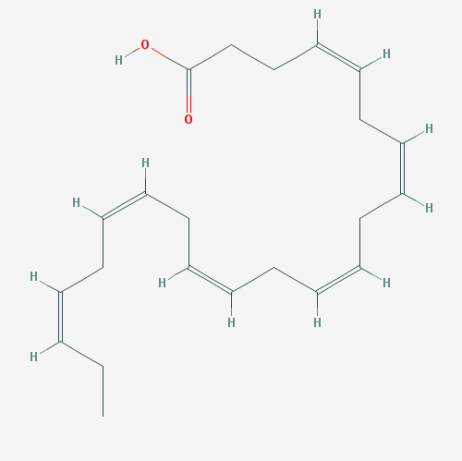
Enterodiol (END)



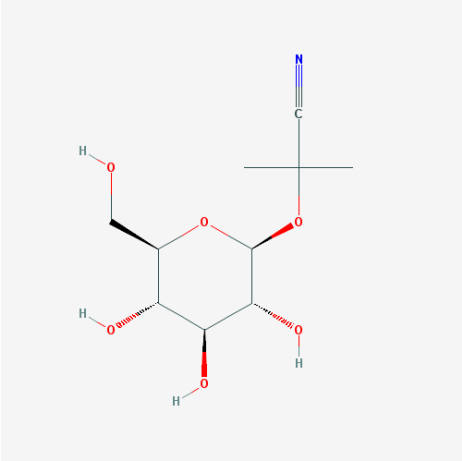
Eicosapentaenoic acid (EPA)



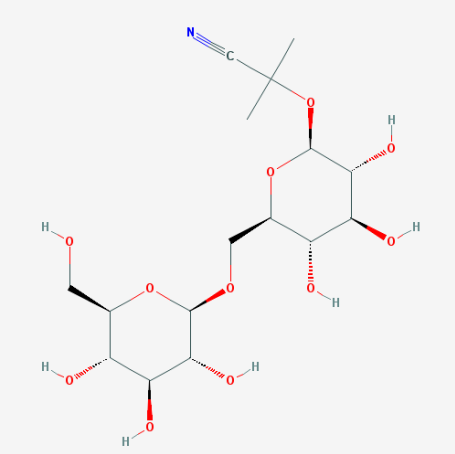
Docosapentaenoic acid (DPA)



Docosahexaenoic acid (DHA)



Linamarin



Linustatin

Figure 9. Chemical structures of some important flax compounds.

A recent study isolated and characterized five new CGs from flaxseed: linustatins A-E. These new isolates were found to moderately inhibit aldose reductase activity, in addition to modest inhibitions of α-glucosidase, dipeptidyl peptidase IV (DPP-IV) and fructose bisphosphatase (FBPase) activities. α-glucosidase (131), DPP-IV (38, 137) and FBPase (151, 160) are all involved in glucose homeostasis. Thus, these effects may have implications for diabetes which is a major risk factor for cardiovascular disease. Linustatins A-E may have to be evaluated to check their effects in a diabetes model. Moreover, in view of the efficacy of flaxseed in

improving glucose homeostasis (73, 127), it is unlikely that these compounds are present in the concentrations necessary to constitute a serious threat to the diabetic condition.

Cyclolinopeptides: Flaxseed contains cyclic hydrophobic peptides of eight or nine amino acid residues known as cyclolinopeptides (CLs) (66, 78, 133). To date, 25 CLs have been discovered (78) with molecular weights around 960-1100 Da (86). Most of the CLs contain the same amino acid sequence but differ in the oxidation state of the methionine residue (78). CLs are hydrophobic in nature and after seed processing they are found dissolved in oil with a total concentration of ~270 μg/g of dry seed weight (66). The first CL to be identified from flax oil was cyclolinopeptide A (CLA). Subsequently discovered CLs were then assigned the next alphabet in the series (65).

CLs are biologically active. Kessler et al reported an inhibition of cholate uptake into hepatocytes by CLA (81). Gorski et al demonstrated an immunosuppressive effect of CLA and this effect, which is identical to cyclosporine A, was mediated through an inhibition of interleukin 1-α and interleukin-2 (64, 134). CLB and CLE also exhibited an immunosuppressive activity but it was modest compared to CLA (112). CLs have added value to flax oil and they should be now investigated more for their anti-inflammatory and hepatoprotective effect in cardiometabolic syndrome.

Flax gum: Soluble flaxseed gum, also called mucilage, is a series of water-soluble polysaccharides from the hulls of flaxseed (20, 90). In vitro assays such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and a β-carotene bleaching test have demonstrated the powerful antioxidant activity of soluble flaxseed gum (20). In addition to polysaccharides, oligosaccharides from flaxseed exhibit biological activity as well. Oligosaccharides are short polymers of 2-20 monosaccharide units interconnected by

glycosidic bonds (56). These oligosaccharides have several physiological functions such as regulation of gastrointestinal function, liver protection, antitumor, reducing the risk of cardiovascular disease (90, 125). Recently Liang et al reported a method of H_20_2 oxidation to degrade flaxseed gum to obtain a novel oligosaccharide (90). This novel flaxseed oligosaccharide is an acidic pyranoligosaccharide with a molecular weight of 1047 Da. The monosaccharide composition of flaxseed oligosaccharide includes rhamnose, fucose, xylose, mannose, arabinose, glucose and galactose. The flaxseed oligosaccharide exhibited a strong potential to scavenge hydroxyl radicals (90). The proposed antioxidant mechanism for the flaxseed oligosaccharide involves $OH^{\cdot}$ inducing abstraction of the hydrogen atom bonded to a carbon on the carbohydrate. The resultant carbon-centered radical then reacts with $OH^{\cdot}$ to form carbohydrate-OH complexes (90). It may be of value to conduct further studies to assess the antioxidant activity of flax gum in cardiovascular diseases that may have a component of oxidation like atherosclerosis.

Conclusion

Flaxseed is one of the richest sources of the essential fatty acid ALA and lignan SDG. Recently discovered new components of flax have also shown biological effects. While cyclolinopeptides have immunosuppressive effect, cyanogenic glycosides improve glucose homeostasis and thus may indirectly affect cardiovascular system. Flax fiber/gum by reducing caloric intake may produce antiatherogenic activity. SDG restores vascular function by increasing neovascularization and thereby improves cardiac function. It also has powerful antioxidant activity. ALA/flax oil has shown antihypertensive activity by altering pro-inflammatory oxylipin concentrations, antiatherogenic activity by reducing inflammatory cytokine production, antiplatelet action through inhibition of anti-aggregatory prostacyclin, anti-arrhythmic effects by decreasing ventricular fibrillation, and effect on cardiac cell survival by upregulating antiapoptotic

proteins. With knowledge gained so far, it can be mentioned that ALA/ flax oil could be the most beneficial bioactive of the flaxseed for the cardiovascular effects. However, it should be noted that other bioactives are not very well studied in multiple cardiac conditions as flax oil has been. So further future investigations may challenge what we currently believe, or it may reaffirm the cardioprotective ability of ALA. More preclinical studies as well as larger trials and clinical trials are needed to identify a comprehensive list of bioactives from flaxseed.

References

1. Al-Bishri WM. Favorable effects of flaxseed supplemented diet on liver and kidney functions in hypertensive Wistar rats. *J Oleo Sci* 62: 709-715, 2013.

2. Allman MA, Pena MM, and Pang D. Supplementation with flaxseed oil versus sunflowerseed oil in healthy young men consuming a low fat diet: effects on platelet composition and function. *Eur J Clin Nutr* 49: 169-178, 1995.

3. Almario RU, and Karakas SE. Lignan content of the flaxseed influences its biological effects in healthy men and women. *J Am Coll Nutr* 32: 194-199, 2013.

4. Ander BP, Hurtado C, Raposo CS, Maddaford TG, Deniset JF, Hryshko LV, Pierce GN, and Lukas A. Differential sensitivities of the NCX1.1 and NCX1.3 isoforms of the Na+–Ca2+ exchanger to α-linolenic acid. *Cardiovasc Res* 73: 395-403, 2007.

5. Ander BP, Weber AR, Rampersad PP, Gilchrist JS, Pierce GN, and Lukas A. Dietary flaxseed protects against ventricular fibrillation induced by ischemia-reperfusion in normal and hypercholesterolemic Rabbits. *J Nutr* 134: 3250-3256, 2004.

6. Anderson E, and Lowe HJ. The composition of flaxseed mucilage. *J Biol Chem* 168: 289-297, 1947.

7. Anderson JW, Baird P, Davis RH, Jr., Ferreri S, Knudtson M, Koraym A, Waters V, and Williams CL. Health benefits of dietary fiber. *Nutr Rev* 67: 188-205, 2009.

8. Antzelevitch C, Shimizu W, Yan GX, and Sicouri S. Cellular basis for QT dispersion. *J Electrocardiol* 30 Suppl: 168-175, 1998.

9. Austria JA, Richard MN, Chahine MN, Edel AL, Malcolmson LJ, Dupasquier CMC, and Pierce GN. Bioavailability of Alpha-Linolenic Acid in Subjects after Ingestion of Three Different Forms of Flaxseed. *J Am Coll Nutr* 27: 214-221, 2008.

10. Bang HO, Dyerberg J, and Hjoorne N. The composition of food consumed by Greenland Eskimos. *Acta Med Scand* 200: 69-73, 1976.

11. Bang HO, Dyerberg J, and Nielsen Aon. PLASMA LIPID AND LIPOPROTEIN PATTERN IN GREENLANDIC WEST-COAST ESKIMOS. *The Lancet* 297: 1143-1146.

12. Barbeau PA, Holloway TM, Whitfield J, Baechler BL, Quadrilatero J, van Loon LJC, Chabowski A, and Holloway GP. alpha-Linolenic acid and exercise training independently, and additively, decrease blood pressure and prevent diastolic dysfunction in obese Zucker rats. *J Physiol* 2017.

13. Bassett CM, McCullough RS, Edel AL, Patenaude A, LaVallee RK, and Pierce GN. The alpha-linolenic acid content of flaxseed can prevent the atherogenic effects of dietary trans fat. *Am J Physiol Heart Circ Physiol* 301: H2220-2226, 2011.

14. Bassett CM, Rodriguez-Leyva D, and Pierce GN. Experimental and clinical research findings on the cardiovascular benefits of consuming flaxseed. *Appl Physiol Nutr Metab* 34: 965-974, 2009.

15. Begg DP, Sinclair AJ, Stahl LA, Garg ML, Jois M, and Weisinger RS. Dietary Protein Level Interacts With ω-3 Polyunsaturated Fatty Acid Deficiency to Induce Hypertension. *Am J Hypertens* 23: 125-128, 2010.

16. Begg DP, Sinclair AJ, Stahl LA, Premaratna SD, Hafandi A, Jois M, and Weisinger RS. Hypertension induced by omega-3 polyunsaturated fatty acid deficiency is alleviated by alpha-linolenic acid regardless of dietary source. *Hypertens Res* 33: 808-813, 2010.

17. Benjamin EJ, Blaha MJ, Chiuve SE, Cushman M, Das SR, Deo R, de Ferranti SD, Floyd J, Fornage M, Gillespie C, Isasi CR, Jiménez MC, Jordan LC, Judd SE, Lackland D, Lichtman JH, Lisabeth L, Liu S, Longenecker CT, Mackey RH, Matsushita K, Mozaffarian D, Mussolino ME,

Nasir K, Neumar RW, Palaniappan L, Pandey DK, Thiagarajan RR, Reeves MJ, Ritchey M, Rodriguez CJ, Roth GA, Rosamond WD, Sasson C, Towfighi A, Tsao CW, Turner MB, Virani SS, Voeks JH, Willey JZ, Wilkins JT, Wu JH, Alger HM, Wong SS, and Muntner P. Heart Disease and Stroke Statistics—2017 Update: A Report From the American Heart Association. *Circulation* 135: e146-e603, 2017.

18. Billinsky J, Glew RA, Cornish SM, Whiting SJ, Thorpe LU, Alcorn J, Paus-Jenssen L, Hadjistavropoulos T, and Chilibeck PD. No evidence of hypoglycemia or hypotension in older adults during 6 months of flax lignan supplementation in a randomized controlled trial: a safety evaluation. *Pharm Biol* 51: 778-782, 2013.

19. Bloedon LT, Balikai S, Chittams J, Cunnane SC, Berlin JA, Rader DJ, and Szapary PO. Flaxseed and cardiovascular risk factors: results from a double blind, randomized, controlled clinical trial. *J Am Coll Nutr* 27: 65-74, 2008.

20. Bouaziz F, Koubaa M, Barba FJ, Roohinejad S, and Chaabouni SE. Antioxidant Properties of Water-Soluble Gum from Flaxseed Hulls. *Antioxidants* 5: 26, 2016.

21. Burr ML, Fehily AM, Gilbert JF, Rogers S, Holliday RM, Sweetnam PM, Elwood PC, and Deadman NM. Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART). *Lancet* 2: 757-761, 1989.

22. Byrne CS, Chambers ES, Morrison DJ, and Frost G. The role of short chain fatty acids in appetite regulation and energy homeostasis. *Int J Obes (Lond)* 39: 1331-1338, 2015.

23. Caligiuri SP, Aukema HM, Ravandi A, Guzman R, Dibrov E, and Pierce GN. Flaxseed consumption reduces blood pressure in patients with hypertension by altering circulating oxylipins via an alpha-linolenic acid-induced inhibition of soluble epoxide hydrolase. *Hypertension* 64: 53-59, 2014.

24. Caligiuri SP, Aukema HM, Ravandi A, and Pierce GN. Elevated levels of pro-inflammatory oxylipins in older subjects are normalized by flaxseed consumption. *Exp Gerontol* 59: 51-57, 2014.

25. Caligiuri SP, Edel AL, Aliani M, and Pierce GN. Flaxseed for hypertension: implications for blood pressure regulation. *Curr Hypertens Rep* 16: 499, 2014.

26. Caligiuri SP, Rodriguez-Leyva D, Aukema HM, Ravandi A, Weighell W, Guzman R, and Pierce GN. Dietary Flaxseed Reduces Central Aortic Blood Pressure Without Cardiac Involvement but Through Changes in Plasma Oxylipins. *Hypertension* 68: 1031-1038, 2016.

27. Caligiuri SPB, Aukema HM, Ravandi A, Lavallee R, Guzman R, and Pierce GN. Specific plasma oxylipins increase the odds of cardiovascular and cerebrovascular events in patients with peripheral artery disease. *Can J Physiol Pharmacol* 95: 961-968, 2017.

28. Caligiuri SPB, Parikh M, Stamenkovic A, Pierce GN, and Aukema HM. Dietary Modulation of Oxylipins in Cardiovascular Disease and Aging. *Am J Physiol Heart Circ Physiol* ajpheart.00201.02017, 2017.

29. Carotenuto F, Minieri M, Monego G, Fiaccavento R, Bertoni A, Sinigaglia F, Vecchini A, Carosella L, and Di Nardo P. A diet supplemented with ALA-rich flaxseed prevents cardiomyocyte apoptosis by regulating caveolin-3 expression. *Cardiovasc Res* 100: 422-431, 2013.

30. Charnock JS. Omega-3 polyunsaturated fatty acids and ventricular fibrillation: the possible involvement of eicosanoids. *Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA)* 61: 243-247, 1999.

31. Clark WF, Parbtani A, Huff MW, Spanner E, de Salis H, Chin-Yee I, Philbrick DJ, and Holub BJ. Flaxseed: a potential treatment for lupus nephritis. *Kidney Int* 48: 475-480, 1995.

32. Cornish SM, Chilibeck PD, Paus-Jennsen L, Biem HJ, Khozani T, Senanayake V, Vatanparast H, Little JP, Whiting SJ, and Pahwa P. A randomized controlled trial of the effects of flaxseed lignan complex on metabolic syndrome composite score and bone mineral in older adults. *Appl Physiol Nutr Metab* 34: 89-98, 2009.

33. Cui W, Mazza G, Oomah BD, and Biliaderis CG. Optimization of an Aqueous Extraction Process for Flaxseed Gum by Response Surface Methodology. *LWT - Food Science and Technology* 27: 363-369, 1994.

34. Cunnane SC, Ganguli S, Menard C, Liede AC, Hamadeh MJ, Chen ZY, Wolever TM, and Jenkins DJ. High alpha-linolenic acid flaxseed (Linum usitatissimum): some nutritional properties in humans. *Br J Nutr* 69: 443-453, 1993.

35. Cunnane SC, Hamadeh MJ, Liede AC, Thompson LU, Wolever TM, and Jenkins DJ. Nutritional attributes of traditional flaxseed in healthy young adults. *Am J Clin Nutr* 61: 62-68, 1995.

36. De Caterina R, and Massaro M. Omega-3 fatty acids and the regulation of expression of endothelial pro-atherogenic and pro-inflammatory genes. *J Membr Biol* 206: 103-116, 2005.

37. den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud DJ, and Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J Lipid Res* 54: 2325-2340, 2013.

38. Dicker D. DPP-4 Inhibitors. *Impact on glycemic control and cardiovascular risk factors* 34: S276-S278, 2011.

39. Dodin S, Cunnane SC, Masse B, Lemay A, Jacques H, Asselin G, Tremblay-Mercier J, Marc I, Lamarche B, Legare F, and Forest JC. Flaxseed on cardiovascular disease markers in

healthy menopausal women: a randomized, double-blind, placebo-controlled trial. *Nutrition* 24: 23-30, 2008.

40. Dodin S, Lemay A, Jacques H, Legare F, Forest JC, and Masse B. The effects of flaxseed dietary supplement on lipid profile, bone mineral density, and symptoms in menopausal women: a randomized, double-blind, wheat germ placebo-controlled clinical trial. *J Clin Endocrinol Metab* 90: 1390-1397, 2005.

41. Doyen A, Udenigwe CC, Mitchell PL, Marette A, Aluko RE, and Bazinet L. Anti-diabetic and antihypertensive activities of two flaxseed protein hydrolysate fractions revealed following their simultaneous separation by electrodialysis with ultrafiltration membranes. *Food Chem* 145: 66-76, 2014.

42. Duckers HJ, Boehm M, True AL, Yet S-F, San H, Park JL, Clinton Webb R, Lee M-E, Nabel GJ, and Nabel EG. Heme oxygenase-1 protects against vascular constriction and proliferation. *Nat Med* 7: 693-698, 2001.

43. Dupasquier CM, Dibrov E, Kneesh AL, Cheung PK, Lee KG, Alexander HK, Yeganeh BK, Moghadasian MH, and Pierce GN. Dietary flaxseed inhibits atherosclerosis in the LDL receptor-deficient mouse in part through antiproliferative and anti-inflammatory actions. *Am J Physiol Heart Circ Physiol* 293: H2394-2402, 2007.

44. Dupasquier CM, Weber AM, Ander BP, Rampersad PP, Steigerwald S, Wigle JT, Mitchell RW, Kroeger EA, Gilchrist JS, Moghadasian MM, Lukas A, and Pierce GN. Effects of dietary flaxseed on vascular contractile function and atherosclerosis during prolonged hypercholesterolemia in rabbits. *Am J Physiol Heart Circ Physiol* 291: H2987-2996, 2006.

45. Dyerberg J, Bang HO, Stoffersen E, Moncada S, and Vane JR. Eicosapentaenoic acid and prevention of thrombosis and atherosclerosis? *Lancet* 2: 117-119, 1978.

46. Edel AL, Patenaude AF, Richard MN, Dibrov E, Austria JA, Aukema HM, Pierce GN, and Aliani M. The effect of flaxseed dose on circulating concentrations of alpha-linolenic acid and secoisolariciresinol diglucoside derived enterolignans in young, healthy adults. *Eur J Nutr* 55: 651-663, 2016.

47. Edel AL, Rodriguez-Leyva D, Maddaford TG, Caligiuri SP, Austria JA, Weighell W, Guzman R, Aliani M, and Pierce GN. Dietary flaxseed independently lowers circulating cholesterol and lowers it beyond the effects of cholesterol-lowering medications alone in patients with peripheral artery disease. *J Nutr* 145: 749-757, 2015.

48. Elias CL, Lukas A, Shurraw S, Scott J, Omelchenko A, Gross GJ, Hnatowich M, and Hryshko LV. Inhibition of Na⁺/Ca²⁺ exchange by KB-R7943: transport mode selectivity and antiarrhythmic consequences. *American Journal of Physiology - Heart and Circulatory Physiology* 281: H1334-H1345, 2001.

49. Endo J, and Arita M. Cardioprotective mechanism of omega-3 polyunsaturated fatty acids. *J Cardiol* 67: 22-27, 2016.

50. Erkkila AT, and Lichtenstein AH. Fiber and cardiovascular disease risk: how strong is the evidence? *J Cardiovasc Nurs* 21: 3-8, 2006.

51. Eussen S, Klungel O, Garssen J, Verhagen H, van Kranen H, van Loveren H, and Rompelberg C. Support of drug therapy using functional foods and dietary supplements: focus on statin therapy. *Br J Nutr* 103: 1260-1277, 2010.

52. Fechner A, Kiehntopf M, and Jahreis G. The formation of short-chain fatty acids is positively associated with the blood lipid-lowering effect of lupin kernel fiber in moderately hypercholesterolemic adults. *J Nutr* 144: 599-607, 2014.

53. Felmlee MA, Woo G, Simko E, Krol ES, Muir AD, and Alcorn J. Effects of the flaxseed lignans secoisolariciresinol diglucoside and its aglycone on serum and hepatic lipids in hyperlipidaemic rats. *Br J Nutr* 102: 361-369, 2009.

54. Francis AA, Deniset JF, Austria JA, LaVallee RK, Maddaford GG, Hedley TE, Dibrov E, and Pierce GN. Effects of dietary flaxseed on atherosclerotic plaque regression. *Am J Physiol Heart Circ Physiol* 304: H1743-1751, 2013.

55. Freese R, and Mutanen M. Alpha-linolenic acid and marine long-chain n-3 fatty acids differ only slightly in their effects on hemostatic factors in healthy subjects. *Am J Clin Nutr* 66: 591-598, 1997.

56. Fry SC, Aldington S, Hetherington PR, and Aitken J. Oligosaccharides as signals and substrates in the plant cell wall. *Plant Physiol* 103: 1-5, 1993.

57. Fukumitsu S, Aida K, Ueno N, Ozawa S, Takahashi Y, and Kobori M. Flaxseed lignan attenuates high-fat diet-induced fat accumulation and induces adiponectin expression in mice. *Br J Nutr* 100: 669-676, 2008.

58. Gabbs M, Leng S, Devassy JG, Monirujjaman M, and Aukema HM. Advances in Our Understanding of Oxylipins Derived from Dietary PUFAs. *Advances in Nutrition: An International Review Journal* 6: 513-540, 2015.

59. Ganguly R, Hasanally D, Stamenkovic A, Maddaford TG, Chaudhary R, Pierce GN, and Ravandi A. Alpha linolenic acid decreases apoptosis and oxidized phospholipids in cardiomyocytes during ischemia/reperfusion. 2017.

60. Ganguly R, Hasanally D, Stamenkovic A, Maddaford TG, Chaudhary R, Pierce GN, and Ravandi A. Alpha linolenic acid decreases apoptosis and oxidized phospholipids in cardiomyocytes during ischemia/reperfusion. *Mol Cell Biochem* 2017.

61. Ghule AE, Kandhare AD, Jadhav SS, Zanwar AA, and Bodhankar SL. Omega-3-fatty acid adds to the protective effect of flax lignan concentrate in pressure overload-induced myocardial hypertrophy in rats via modulation of oxidative stress and apoptosis. *Int Immunopharmacol* 28: 751-763, 2015.

62. Gluck T, and Alter P. Marine omega-3 highly unsaturated fatty acids: From mechanisms to clinical implications in heart failure and arrhythmias. *Vascul Pharmacol* 82: 11-19, 2016.

63. Gonzalez M, Sealls W, Jesch ED, Brosnan MJ, Ladunga I, Ding X, Black PN, and DiRusso CC. Defining a relationship between dietary fatty acids and the cytochrome P450 system in a mouse model of fatty liver disease. *Physiol Genomics* 43: 121-135, 2011.

64. Górski A, Kasprzycka M, Nowaczyk M, Wieczoreck Z, Siemion IZ, Szelejewski W, and Kutner A. Cyclolinopeptide: a novel immunosuppressive agent with potential anti-lipemic activity. *Transplant Proc* 33: 553.

65. Gui B, Shim YY, Datla RS, Covello PS, Stone SL, and Reaney MJ. Identification and quantification of cyclolinopeptides in five flaxseed cultivars. *J Agric Food Chem* 60: 8571-8579, 2012.

66. Gui B, Shim YY, and Reaney MJ. Distribution of cyclolinopeptides in flaxseed fractions and products. *J Agric Food Chem* 60: 8580-8589, 2012.

67. Haliga RE, Iancu RI, Butcovan D, and Mocanu V. Flaxseed Prevents Leukocyte and Platelet Adhesion to Endothelial Cells in Experimental Atherosclerosis by Reducing sVCAM-1 and vWF. *The Scientific World Journal* 2013: 6, 2013.

68. Hallund J, Ravn-Haren G, Bugel S, Tholstrup T, and Tetens I. A lignan complex isolated from flaxseed does not affect plasma lipid concentrations or antioxidant capacity in healthy postmenopausal women. *J Nutr* 136: 112-116, 2006.

69. Harfouche R, Hassessian HM, Guo Y, Faivre V, Srikant CB, Yancopoulos GD, and Hussain SN. Mechanisms which mediate the antiapoptotic effects of angiopoietin-1 on endothelial cells. *Microvasc Res* 64: 135-147, 2002.

70. Harper CR, Edwards MC, and Jacobson TA. Flaxseed oil supplementation does not affect plasma lipoprotein concentration or particle size in human subjects. *J Nutr* 136: 2844-2848, 2006.

71. Hjerkinn EM, Abdelnoor M, Breivik L, Bergengen L, Ellingsen I, Seljeflot I, Aase O, Ole Klemsdal T, Hjermann I, and Arnesen H. Effect of diet or very long chain omega-3 fatty acids on progression of atherosclerosis, evaluated by carotid plaques, intima-media thickness and by pulse wave propagation in elderly men with hypercholesterolaemia. *Eur J Cardiovasc Prev Rehabil* 13: 325-333, 2006.

72. Holy EW, Forestier M, Richter EK, Akhmedov A, Leiber F, Camici GG, Mocharla P, Lüscher TF, Beer JH, and Tanner FC. Dietary α-Linolenic Acid Inhibits Arterial Thrombus Formation, Tissue Factor Expression, and Platelet Activation. *Arterioscler Thromb Vasc Biol* 31: 1772-1780, 2011.

73. Hutchins AM, Brown BD, Cunnane SC, Domitrovich SG, Adams ER, and Bobowiec CE. Daily flaxseed consumption improves glycemic control in obese men and women with pre-diabetes: a randomized study. *Nutr Res* 33: 367-375, 2013.

74. Iancu RI, Haliga R, Luca V, Stitt PA, and Mocanu V. [Modulation of the platelet function by diet supplemented with flaxseed and vitamin E in diabetic hamsters]. *Rev Med Chir Soc Med Nat Iasi* 110: 962-967, 2006.

75. Ikeda U, Ikeda M, Oohara T, Oguchi A, Kamitani T, Tsuruya Y, and Kano S. Interleukin 6 stimulates growth of vascular smooth muscle cells in a PDGF-dependent manner. *Am J Physiol* 260: H1713-1717, 1991.

76. Jenkins DJ, Kendall CW, Vidgen E, Agarwal S, Rao AV, Rosenberg RS, Diamandis EP, Novokmet R, Mehling CC, Perera T, Griffin LC, and Cunnane SC. Health aspects of partially defatted flaxseed, including effects on serum lipids, oxidative measures, and ex vivo androgen and progestin activity: a controlled crossover trial. *Am J Clin Nutr* 69: 395-402, 1999.

77. Juurlink BH. Therapeutic potential of dietary phase 2 enzyme inducers in ameliorating diseases that have an underlying inflammatory component. *Can J Physiol Pharmacol* 79: 266-282, 2001.

78. Kaneda T, Yoshida H, Nakajima Y, Toishi M, Nugroho AE, and Morita H. Cyclolinopeptides, cyclic peptides from flaxseed with osteoclast differentiation inhibitory activity. *Bioorg Med Chem Lett* 26: 1760-1761, 2016.

79. Kaul N, Kreml R, Austria JA, Richard MN, Edel AL, Dibrov E, Hirono S, Zettler ME, and Pierce GN. A comparison of fish oil, flaxseed oil and hempseed oil supplementation on selected parameters of cardiovascular health in healthy volunteers. *J Am Coll Nutr* 27: 51-58, 2008.

80. Kelley DS, Nelson GJ, Love JE, Branch LB, Taylor PC, Schmidt PC, Mackey BE, and Iacono JM. Dietary alpha-linolenic acid alters tissue fatty acid composition, but not blood lipids, lipoproteins or coagulation status in humans. *Lipids* 28: 533-537, 1993.

81. Kessler H, Klein M, Müller A, Wagner K, Bats JW, Ziegler K, and Frimmer M. Conformational Prerequisites for the in vitro Inhibition of Cholate Uptake in Hepatocytes by Cyclic Analogues of Antamanide and Somatostatin. *Angewandte Chemie International Edition in English* 25: 997-999, 1986.

82. Khalatbari Soltani S, Jamaluddin R, Tabibi H, Mohd Yusof BN, Atabak S, Loh SP, and Rahmani L. Effects of flaxseed consumption on systemic inflammation and serum lipid profile in

hemodialysis patients with lipid abnormalities. *Hemodialysis international International Symposium on Home Hemodialysis* 17: 275-281, 2013.

83. Khoueiry G, Abi Rafeh N, Sullivan E, Saiful F, Jaffery Z, Kenigsberg DN, Krishnan SC, Khanal S, Bekheit S, and Kowalski M. Do omega-3 polyunsaturated fatty acids reduce risk of sudden cardiac death and ventricular arrhythmias? A meta-analysis of randomized trials. *Heart Lung* 42: 251-256, 2013.

84. Kristensen M, Jensen MG, Aarestrup J, Petersen KE, Søndergaard L, Mikkelsen MS, and Astrup A. Flaxseed dietary fibers lower cholesterol and increase fecal fat excretion, but magnitude of effect depend on food type. *Nutr Metab (Lond)* 9: 8, 2012.

85. Kritchevsky D, Tepper SA, and Klurfeld DM. Influence of flaxseed on serum and liver lipids in rats. *Journal of Nutritional Biochemistry* 2: 133-134.

86. Lao YW, Mackenzie K, Vincent W, and Krokhin OV. Characterization and complete separation of major cyclolinopeptides in flaxseed oil by reversed-phase chromatography. *Journal of separation science* 37: 1788-1796, 2014.

87. Law MR, Wald NJ, Morris JK, and Jordan RE. Value of low dose combination treatment with blood pressure lowering drugs: analysis of 354 randomised trials. *BMJ* 326: 1427, 2003.

88. Lee P, and Prasad K. Effects of flaxseed oil on serum lipids and atherosclerosis in hypercholesterolemic rabbits. *J Cardiovasc Pharmacol Ther* 8: 227-235, 2003.

89. Ley K, and Huo Y. VCAM-1 is critical in atherosclerosis. *J Clin Invest* 107: 1209-1210, 2001.

90. Liang S, Liao W, Ma X, Li X, and Wang Y. H2O2 oxidative preparation, characterization and antiradical activity of a novel oligosaccharide derived from flaxseed gum. *Food Chem* 230: 135-144, 2017.

91. Lucas EA, Lightfoot SA, Hammond LJ, Devareddy L, Khalil DA, Daggy BP, Smith BJ, Westcott N, Mocanu V, Soung DY, and Arjmandi BH. Flaxseed reduces plasma cholesterol and atherosclerotic lesion formation in ovariectomized Golden Syrian hamsters. *Atherosclerosis* 173: 223-229, 2004.

92. Lucas EA, Wild RD, Hammond LJ, Khalil DA, Juma S, Daggy BP, Stoecker BJ, and Arjmandi BH. Flaxseed improves lipid profile without altering biomarkers of bone metabolism in postmenopausal women. *J Clin Endocrinol Metab* 87: 1527-1532, 2002.

93. Mandaşescu S, Mocanu V, Dăscaliţa A-M, Haliga R, Nestian I, Stitt PA, and Luca V. Flaxseed supplementation in hyperlipidemic patients. *Rev Med Chir Soc Med Nat Iasi* 109: 502-506, 2005.

94. Mandasescu S, Mocanu V, Dascalita AM, Haliga R, Nestian I, Stitt PA, and Luca V. Flaxseed supplementation in hyperlipidemic patients. *Rev Med Chir Soc Med Nat Iasi* 109: 502-506, 2005.

95. Marx N, Duez H, Fruchart JC, and Staels B. Peroxisome proliferator-activated receptors and atherogenesis: regulators of gene expression in vascular cells. *Circ Res* 94: 1168-1178, 2004.

96. Mennen LI, Witteman JC, den Breeijen JH, Schouten EG, de Jong PT, Hofman A, and Grobbee DE. The association of dietary fat and fiber with coagulation factor VII in the elderly: the Rotterdam Study. *Am J Clin Nutr* 65: 732-736, 1997.

97. Montezano AC, and Touyz RM. Molecular mechanisms of hypertension--reactive oxygen species and antioxidants: a basic science update for the clinician. *Can J Cardiol* 28: 288-295, 2012.

98. Mozaffarian D, and Wu JH. Omega-3 fatty acids and cardiovascular disease: effects on risk factors, molecular pathways, and clinical events. *J Am Coll Cardiol* 58: 2047-2067, 2011.

99. Murohara T, Asahara T, Silver M, Bauters C, Masuda H, Kalka C, Kearney M, Chen D, Symes JF, Fishman MC, Huang PL, and Isner JM. Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J Clin Invest* 101: 2567-2578, 1998.

100. Needleman P, Whitaker MO, Wyche A, Watters K, Sprecher H, and Raz A. Manipulation of platelet aggregation by prostaglandins and their fatty acid precursors: pharmacological basis for a therapeutic approach. *Prostaglandins* 19: 165-181, 1980.

101. Nestel PJ, Pomeroy SE, Sasahara T, Yamashita T, Liang YL, Dart AM, Jennings GL, Abbey M, and Cameron JD. Arterial compliance in obese subjects is improved with dietary plant n-3 fatty acid from flaxseed oil despite increased LDL oxidizability. *Arterioscler Thromb Vasc Biol* 17: 1163-1170, 1997.

102. Ogawa A, Suzuki Y, Aoyama T, and Takeuchi H. Dietary alpha-linolenic acid inhibits angiotensin-converting enzyme activity and mRNA expression levels in the aorta of spontaneously hypertensive rats. *J Oleo Sci* 58: 355-360, 2009.

103. Pan A, Yu D, Demark-Wahnefried W, Franco OH, and Lin X. Meta-analysis of the effects of flaxseed interventions on blood lipids. *Am J Clin Nutr* 90: 288-297, 2009.

104. Papapetropoulos A, Fulton D, Mahboubi K, Kalb RG, O'Connor DS, Li F, Altieri DC, and Sessa WC. Angiopoietin-1 inhibits endothelial cell apoptosis via the Akt/survivin pathway. *J Biol Chem* 275: 9102-9105, 2000.

105. Paschos GK, Magkos F, Panagiotakos DB, Votteas V, and Zampelas A. Dietary supplementation with flaxseed oil lowers blood pressure in dyslipidaemic patients. *Eur J Clin Nutr* 61: 1201-1206, 2007.

106. Passman R, and Kadish A. Polymorphic ventricular tachycardia, long Q-T syndrome, and torsades de pointes. *Med Clin North Am* 85: 321-341, 2001.

107. Patade A, Devareddy L, Lucas EA, Korlagunta K, Daggy BP, and Arjmandi BH. Flaxseed reduces total and LDL cholesterol concentrations in Native American postmenopausal women. *Journal of women's health (2002)* 17: 355-366, 2008.

108. Patenaude A, Rodriguez-Leyva D, Edel AL, Dibrov E, Dupasquier CM, Austria JA, Richard MN, Chahine MN, Malcolmson LJ, and Pierce GN. Bioavailability of alpha-linolenic acid from flaxseed diets as a function of the age of the subject. *Eur J Clin Nutr* 63: 1123-1129, 2009.

109. Penumathsa SV, Koneru S, Thirunavukkarasu M, Zhan L, Prasad K, and Maulik N. Secoisolariciresinol diglucoside: relevance to angiogenesis and cardioprotection against ischemia-reperfusion injury. *J Pharmacol Exp Ther* 320: 951-959, 2007.

110. Penumathsa SV, Koneru S, Zhan L, John S, Menon VP, Prasad K, and Maulik N. Secoisolariciresinol Diglucoside Induces Neovascularization Mediated Cardioprotection against Ischemic-Reperfusion Injury In Hypercholesterolemic Myocardium. *J Mol Cell Cardiol* 44: 170-179, 2008.

111. Peterson J, Dwyer J, Adlercreutz H, Scalbert A, Jacques P, and McCullough ML. Dietary lignans: physiology and potential for cardiovascular disease risk reduction. *Nutr Rev* 68: 571-603, 2010.

112. Picur B, Cebrat M, Zabrocki J, and Siemion IZ. Cyclopeptides of Linum usitatissimum. *Journal of Peptide Science* 12: 569-574, 2006.

113. Prasad K. Antihypertensive Activity of Secoisolariciresinol Diglucoside (SDG) Isolated from Flaxseed: Role of Guanylate Cyclase. *Int J Angiol* 13: 7-14, 2004.

114. Prasad K. Dietary flax seed in prevention of hypercholesterolemic atherosclerosis. *Atherosclerosis* 132: 69-76, 1997.

115. Prasad K. Flax Lignan Complex Slows Down the Progression of Atherosclerosis in Hyperlipidemic Rabbits. *J Cardiovasc Pharmacol Ther* 14: 38-48, 2009.

116. Prasad K. Flaxseed and cardiovascular health. *J Cardiovasc Pharmacol* 54: 369-377, 2009.

117. Prasad K. Hydroxyl radical-scavenging property of secoisolariciresinol diglucoside (SDG) isolated from flax-seed. *Mol Cell Biochem* 168: 117-123, 1997.

118. Prasad K. Hypocholesterolemic and antiatherosclerotic effect of flax lignan complex isolated from flaxseed. *Atherosclerosis* 179: 269-275, 2005.

119. Prasad K. Reduction of serum cholesterol and hypercholesterolemic atherosclerosis in rabbits by secoisolariciresinol diglucoside isolated from flaxseed. *Circulation* 99: 1355-1362, 1999.

120. Prasad K. Regression of hypercholesterolemic atherosclerosis in rabbits by secoisolariciresinol diglucoside isolated from flaxseed. *Atherosclerosis* 197: 34-42, 2008.

121. Prasad K. Secoisolariciresinol Diglucoside (SDG) Isolated from Flaxseed, an Alternative to ACE Inhibitors in the Treatment of Hypertension. *The International Journal of Angiology : Official Publication of the International College of Angiology, Inc* 22: 235-238, 2013.

122. Prasad K. A study on regression of hypercholesterolemic atherosclerosis in rabbits by flax lignan complex. *J Cardiovasc Pharmacol Ther* 12: 304-313, 2007.

123. Prasad K, Mantha SV, Muir AD, and Westcott ND. Reduction of hypercholesterolemic atherosclerosis by CDC-flaxseed with very low alpha-linolenic acid. *Atherosclerosis* 136: 367-375, 1998.

124. Puukila S, Fernandes RO, Turck P, Carraro CC, Bonetto JH, de Lima-Seolin BG, da Rosa Araujo AS, Bello-Klein A, Boreham D, and Khaper N. Secoisolariciresinol diglucoside attenuates

cardiac hypertrophy and oxidative stress in monocrotaline-induced right heart dysfunction. *Mol Cell Biochem* 2017.

125. Qiang X, YongLie C, and QianBing W. Health benefit application of functional oligosaccharides. *Carbohydrate Polymers* 77: 435-441, 2009.

126. Ratnayake WMN, Behrens WA, Fischer PWF, L'Abbé MR, Mongeau R, and Beare-Rogers JL. Chemical and nutritional studies of flaxseed (variety Linott) in rats. *J Nutr Biochem* 3: 232-240, 1992.

127. Rhee Y, and Brunt A. Flaxseed supplementation improved insulin resistance in obese glucose intolerant people: a randomized crossover design. *Nutr J* 10: 44-44, 2011.

128. Rodriguez-Leyva D, Dupasquier CM, McCullough R, and Pierce GN. The cardiovascular effects of flaxseed and its omega-3 fatty acid, alpha-linolenic acid. *Can J Cardiol* 26: 489-496, 2010.

129. Rodriguez-Leyva D, Weighell W, Edel AL, LaVallee R, Dibrov E, Pinneker R, Maddaford TG, Ramjiawan B, Aliani M, Guzman R, and Pierce GN. Potent antihypertensive action of dietary flaxseed in hypertensive patients. *Hypertension* 62: 1081-1089, 2013.

130. Sano T, Oda E, Yamashita T, Shiramasa H, Ijiri Y, Yamashita T, and Yamamoto J. Antithrombic and anti-atherogenic effects of partially defatted flaxseed meal using a laser-induced thrombosis test in apolipoprotein E and low-density lipoprotein receptor deficient mice. *Blood Coagul Fibrinolysis* 14: 707-712, 2003.

131. Scheen AJ. Is there a role for alpha-glucosidase inhibitors in the prevention of type 2 diabetes mellitus? *Drugs* 63: 933-951, 2003.

132. Sekine S, Sasanuki S, Aoyama T, and Takeuchi H. Lowering systolic blood pressure and increases in vasodilator levels in SHR with oral alpha-linolenic acid administration. *J Oleo Sci* 56: 341-345, 2007.

133. Sharav O, Shim YY, Okinyo-Owiti DP, Sammynaiken R, and Reaney MJ. Effect of cyclolinopeptides on the oxidative stability of flaxseed oil. *J Agric Food Chem* 62: 88-96, 2014.

134. Shim YY, Gui B, Arnison PG, Wang Y, and Reaney MJT. Flaxseed (Linum usitatissimum L.) bioactive compounds and peptide nomenclature: A review. *Trends in Food Science & Technology* 38: 5-20, 2014.

135. Shim YY, Olivia CM, Liu J, Boonen R, Shen J, and Reaney MJT. Secoisolariciresinol Diglucoside and Cyanogenic Glycosides in Gluten-free Bread Fortified with Flaxseed Meal. *J Agric Food Chem* 64: 9551-9558, 2016.

136. Silva FGDe, Hernández-Ledesma B, Amigo L, Netto FM, and Miralles B. Identification of peptides released from flaxseed (Linum usitatissimum) protein by Alcalase® hydrolysis: Antioxidant activity. *LWT - Food Science and Technology* 76, Part A: 140-146, 2017.

137. Singh AK, Jatwa R, Purohit A, and Ram H. Synthetic and phytocompounds based dipeptidyl peptidase-IV (DPP-IV) inhibitors for therapeutics of diabetes. *Journal of Asian natural products research* 1-10, 2017.

138. Stuglin C, and Prasad K. Effect of flaxseed consumption on blood pressure, serum lipids, hemopoietic system and liver and kidney enzymes in healthy humans. *J Cardiovasc Pharmacol Ther* 10: 23-27, 2005.

139. Sundell IB, and Ranby M. Oat husk fiber decreases plasminogen activator inhibitor type 1 activity. *Haemostasis* 23: 45-50, 1993.

140. Surampudi P, Enkhmaa B, Anuurad E, and Berglund L. Lipid Lowering with Soluble Dietary Fiber. *Current atherosclerosis reports* 18: 75, 2016.

141. Szekanecz Z, Shah MR, Pearce WH, and Koch AE. Human atherosclerotic abdominal aortic aneurysms produce interleukin (IL)-6 and interferon-gamma but not IL-2 and IL-4: the possible role for IL-6 and interferon-gamma in vascular inflammation. *Agents Actions* 42: 159-162, 1994.

142. Takeuchi H, Sakurai C, Noda R, Sekine S, Murano Y, Wanaka K, Kasai M, Watanabe S, Aoyama T, and Kondo K. Antihypertensive effect and safety of dietary alpha-linolenic acid in subjects with high-normal blood pressure and mild hypertension. *J Oleo Sci* 56: 347-360, 2007.

143. Talom RT, Judd SA, McIntosh DD, and McNeill JR. High flaxseed (linseed) diet restores endothelial function in the mesenteric arterial bed of spontaneously hypertensive rats. *Life Sci* 64: 1415-1425, 1999.

144. Talom RT, Judd SA, McIntosh DD, and McNeill JR. High flaxseed (linseed) diet restores endothelial function in the mesenteric arterial bed of spontaneously hypertensive rats. *Life Sci* 64: 1415-1425, 1999.

145. Thakur G, Mitra A, Pal K, and Rousseau D. Effect of flaxseed gum on reduction of blood glucose and cholesterol in type 2 diabetic patients. *Int J Food Sci Nutr* 60 Suppl 6: 126-136, 2009.

146. Tomaz Pacheco J, Beltrame Daleprame J, and Teles Boaventura G. Impact of dietary flaxseed (linum usitatissimum) supplementation on biochemical profile in healthy rats. *Nutr Hosp* 26: 798-802, 2011.

147. Tomé-Carneiro J, and Visioli F. Polyphenol-based nutraceuticals for the prevention and treatment of cardiovascular disease: Review of human evidence. *Phytomedicine* 23: 1145-1174, 2016.

148. Udenigwe CC, Adebiyi AP, Doyen A, Li H, Bazinet L, and Aluko RE. Low molecular weight flaxseed protein-derived arginine-containing peptides reduced blood pressure of spontaneously hypertensive rats faster than amino acid form of arginine and native flaxseed protein. *Food Chem* 132: 468-475, 2012.

149. Ueshima H, Stamler J, Elliott P, Chan Q, Brown IJ, Carnethon MR, Daviglus ML, He K, Moag-Stahlberg A, Rodriguez BL, Steffen LM, Van Horn L, Yarnell J, and Zhou B. Food omega-3 fatty acid intake of individuals (total, linolenic acid, long-chain) and their blood pressure: INTERMAP study. *Hypertension* 50: 313-319, 2007.

150. Van der Vusse GJ, Reneman RS, and van Bilsen M. Accumulation of arachidonic acid in ischemic/reperfused cardiac tissue: possible causes and consequences. *Prostaglandins Leukot Essent Fatty Acids* 57: 85-93, 1997.

151. van Poelje PD, Potter SC, and Erion MD. Fructose-1, 6-bisphosphatase inhibitors for reducing excessive endogenous glucose production in type 2 diabetes. *Handb Exp Pharmacol* 279-301, 2011.

152. Vas Dias FW, Gibney MJ, and Taylor TG. The effect of polyunsaturated fatty acids of the n-3 and n-6 series on platelet aggregation and platelet and aortic fatty acid composition in rabbits. *Atherosclerosis* 43: 245-257, 1982.

153. Wang W, Liu LQ, Higuchi CM, and Chen H. Induction of NADPH:Quinone Reductase by Dietary Phytoestrogens in Colonic Colo205 Cells. *Biochem Pharmacol* 56: 189-195, 1998.

154. Whelton SP, Hyre AD, Pedersen B, Yi Y, Whelton PK, and He J. Effect of dietary fiber intake on blood pressure: a meta-analysis of randomized, controlled clinical trials. *J Hypertens* 23: 475-481, 2005.

155. Xie N, Zhang W, Li J, Liang H, Zhou H, Duan W, Xu X, Yu S, Zhang H, and Yi D. alpha-Linolenic acid intake attenuates myocardial ischemia/reperfusion injury through anti-inflammatory and anti-oxidative stress effects in diabetic but not normal rats. *Arch Med Res* 42: 171-181, 2011.

156. Yang Q-Y, Song L, Zhang J-F, Shen Z-F, Liu Q, Liu S-N, Zheng W-S, and Yao C-S. Cyanogenetic glycosides and simple glycosides from the linseed meal. *Fitoterapia* 106: 78-83, 2015.

157. Zettler ME, Merchant MA, and Pierce GN. Augmented cell cycle protein expression and kinase activity in atherosclerotic rabbit vessels. *Exp Clin Cardiol* 15: e139-e144, 2010.

158. Zhang J, Kris-Etherton PM, Thompson JT, Hannon DB, Gillies PJ, and Heuvel JP. Alpha-linolenic acid increases cholesterol efflux in macrophage-derived foam cells by decreasing stearoyl CoA desaturase 1 expression: evidence for a farnesoid-X-receptor mechanism of action. *J Nutr Biochem* 23: 400-409, 2012.

159. Zhang W, Wang X, Liu Y, Tian H, Flickinger B, Empie MW, and Sun SZ. Dietary flaxseed lignan extract lowers plasma cholesterol and glucose concentrations in hypercholesterolaemic subjects. *Br J Nutr* 99: 1301-1309, 2008.

160. Zhang Y, Xie Z, Zhou G, Zhang H, Lu J, and Zhang WJ. Fructose-1,6-bisphosphatase regulates glucose-stimulated insulin secretion of mouse pancreatic beta-cells. *Endocrinology* 151: 4688-4695, 2010.

161. Zhao G, Etherton TD, Martin KR, Gillies PJ, West SG, and Kris-Etherton PM. Dietary alpha-linolenic acid inhibits proinflammatory cytokine production by peripheral blood mononuclear cells in hypercholesterolemic subjects. *Am J Clin Nutr* 85: 385-391, 2007.

1.8.2. Dietary Flaxseed: What We Know and Don't Know About Its Effects on Cardiovascular Disease

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Abstract

Flaxseed is composed of a unique combination of bioactive components that appear to generate, either through an isolated or synergistic action, a significant beneficial effect on the cardiovascular system. With a significant increase in the generation of data on the dietary impact of flaxseed on the cardiovascular system, a review of where we stand – what we know and what we still need to understand about these effects on the heart and the vasculature – was thought to be of value and the rationale for this paper. For example, although we now know how to deliver the bioactives most efficiently (oil versus ground seed versus whole seed) but we do not know how different foods can influence that delivery. Further, we know flaxseed has anti-arrhythmic, anti-atherogenic, anti-hypertensive, and cholesterol-lowering actions in animal studies and some selected human trials but much more needs to be learned, particularly in human trials. These results have justified further commitment of resources to the initiation of human trials. Because of the impact of nutrition on many chronic diseases, this may not only be true for the effects of flaxseed on cardiovascular disease but may be just as relevant for many other disease conditions.

Key words: diet; cardiovascular disease; nutrition; heart disease; flaxseed; linseed; alpha linolenic acid

An introduction to flaxseed

Linum usitatissimum, more familiarly known as flaxseed, is an oilseed crop grown throughout most of the world (Caligiuri et al, 2014). It contains three bioactive compounds beneficial in the treatment and potentially for the prevention of cardiovascular disease. First, it is one of the richest sources of the plant omega-3 (n-3) fatty acid, alpha-linolenic acid (ALA; C18:3n-3) (Gebauer et al, 2006). Second, flaxseed also contains a potent antioxidant (Prasad, 2000), the lignan secoisolariciresinol diglucoside (SDG), in larger quantities than any other source [Edel et al, 2015a]. SDG is not bioactive itself but is converted in the colon by gut microbiota into its primary metabolites enterolactone (ENL) and enterodiol (END). ENL and END possess weak estrogenic action in addition to acting as antioxidants (Carreau et al, 2008). Finally, flaxseed is also enriched in soluble and insoluble fibers. Cardiovascular disease is thought to have components of inflammation, oxidation and hyperlipidemia as key causative factors in its development (Parikh et al, 2018). Providing the body with the anti-inflammatory action of ALA (Dupasquier et al, 2007), the anti-oxidative ability of lignans (Prasad, 2000) and the cholesterol-lowering capacity of dietary fiber (Edel et al, 2015b) may, therefore, represent the primary mechanisms whereby flaxseed can induce its beneficial health-related actions on the cardiovascular system. In view of the increasing prevalence of cardiovascular disease globally, gaining a better understanding of: a) how we can introduce flaxseed into the diet, b) what effects flaxseed has on the cardiovascular system, and c) identifying the gaps in our current knowledge to direct future research in this area, has great value and is the purpose of this manuscript.

The introduction of flaxseed into the diet: What we know and what we don't know

How do we eat flaxseed to provide its bioactive components optimally to the body?

Flaxseed stability is influenced by the form of flaxseed studied. Whole flaxseed is stable for long periods of time (months and even years) due to its hard seed coat that acts as a barrier to oxidative degradation (Edel et al, 2015a). Temperatures up to 350^0C did not influence ALA content and baking had no effect on oxidative stability of the flaxseed (Edel et al, 2015a). Grinding (milling) the flaxseed breaks up the seed coat barrier and increases its susceptibility to oxidation. However, the integrity of ground flaxseed was unchanged when kept in plastic bags for 4 to 20 months at room temperature (Malcolmson et al, 2000; Przybylski and Daun, 2001). Flax oil is the most sensitive form of flaxseed to oxidation. However, if stored at refrigeration temperatures in dark bottles under routine usage conditions, flax oil is stable for up to 6 months (Edel et al, 2015a). At room temperature storage conditions, the flax oil is very sensitive to oxidation and can oxidize within a week (Edel et al, 2015a). It is important to note that the individual components of flaxseed, its oil and SDG content, may also be protected from oxidation when incorporated into functional foods (Edel et al, 2015a). This is a concept that the food processing industry must address as they endeavor to develop new foods containing flax.

The form in which flaxseed is ingested will also influence the bioavailability of the bioactives within flaxseed. Flaxseed is best consumed in form of flax oil in order to provide ALA to the body [Figure 10]. Ground (milled) flaxseed will also provide significant amounts of ALA to the body. However, flaxseed ingested in the form of whole seed delivers very little ALA to the circulation [Figure 10]. However, since flax oil is not tolerated as well as ground flaxseed (Austria et al. 2008), the best form of flaxseed to practically deliver ALA to the body is ground flaxseed (Austria et al, 2008).

The age of the population ingesting flaxseed was also studied as a variable for influencing bioavailability. Would an elderly population tolerate the high fiber content of flax as well as a younger counterpart? We now know the answer to that question. Older people (aged 45-69 years) can tolerate ground flaxseed as well as younger subjects (18-29 years) (Figure 11) (Patenaude et al. 2009). The older subjects also absorbed ALA (Patenaude et al. 2009) and converted the SDG into enterolignans (Edel et al. 2015a) as well as the younger participants.

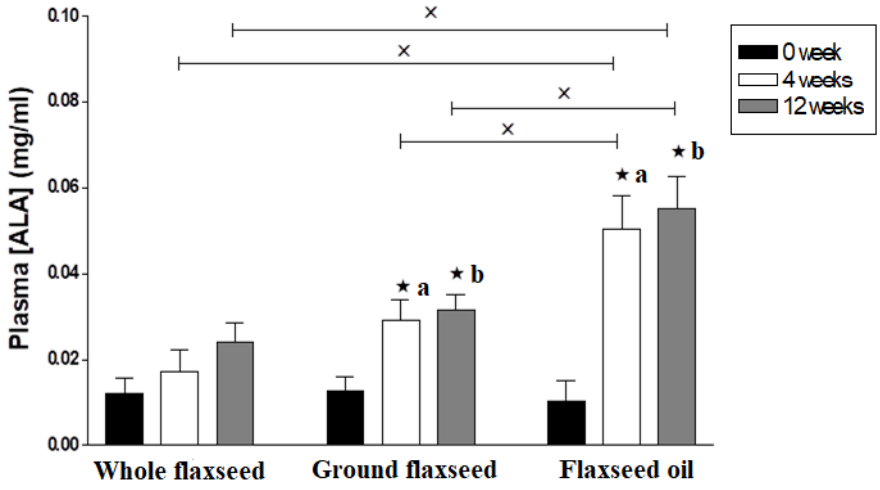


Figure 10. Plasma alpha-linolenic acid (ALA) of subjects ingesting whole, ground flaxseed or flaxseed oil after 0, 4, and 12 weeks, adapted from Austria et al. (2008). **[a]** At 4 weeks, there was a significantly higher concentration of ALA in plasma from subjects fed ground flaxseed in comparison to whole flaxseed ($P < 0.05$) and in those fed flaxseed oil in comparison to both the other groups ($P = 0.004$). **[b]** At 12 weeks, there was a significantly higher concentration of ALA in plasma from subjects fed ground flaxseed in comparison to whole flaxseed ($P < 0.05$) and in those fed flaxseed oil in comparison to both the other groups ($P = 0.002$).

The type of food into which ground flaxseed is incorporated also influences its acceptance in the North American diet. For example, flax bagels and muffins are well accepted but when ground flaxseed was included in buns, tea biscuits and pasta, these choices were not as popular (Figure 12). The flavor of the food product was also important in influencing ingestion of flaxseed (Austria et al. 2016). The incorporation of ground flaxseed into a variety of different foods ingested by cultures outside of North America is yet another challenge. For example, the Indian diet incorporates flaxseed into laddoos and parathas but many other baked and dessert options exist. Other cultures will look to different ways to incorporate the nutty goodness of flaxseed into their own traditional dishes.

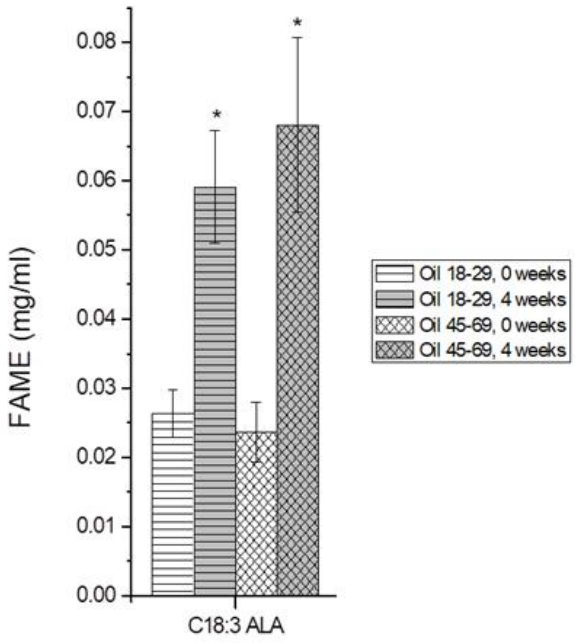


Figure 11. Plasma n-3 fatty acid concentrations following dietary supplementation with ground flaxseed and flaxseed oil for two age groups: 18–29 and 45–69 years, adapted from Patenaude et al. (2009). ALA, α-linolenic acid. *p<0.05 compared with 0 weeks in the same flaxseed source and age group; a P < 0.05 compared with ground flaxseed in the same age group.

What do we still not know about flaxseed ingestion in our diet?

1. Is ALA and lignan bioavailability different when flaxseed is incorporated into different foods or beverages?

2. Is ALA and lignan bioavailability and stability different when the flaxseed is in a roasted form?

3. Is organic flaxseed nutritionally superior to non-organic flaxseed?

4. Is flaxseed tolerated and the bioactives absorbed well in children?

5. Do different ethnic populations react differently to dietary flaxseed?

6. How can flaxseed be incorporated into culturally acceptable foods for different countries of the world?

These six questions still require additional research in order to provide answers.

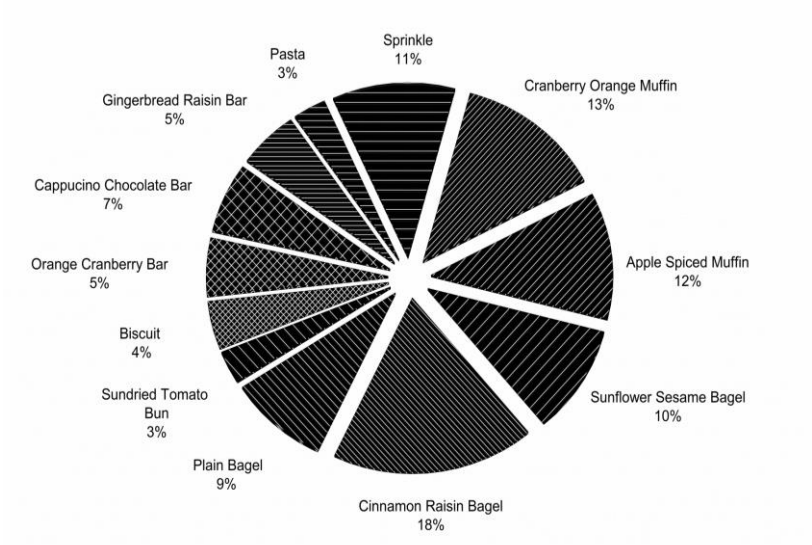


Figure 12. Individual food preferences in terms of total flax-supplemented foods delivered to patients during the one-year FLAXPAD trial (Austria et al. 2016). Values represent a % of the total foods ordered.

The biological actions of dietary flaxseed on the cardiovascular system

What do we know about the effects of dietary flaxseed on the heart and vasculature?

No epidemiological data have been produced on the ingestion of flaxseed. However, one of its primary bioactives, ALA, has been studied in epidemiological investigations. Epidemiological studies have long suggested that the ingestion of ALA has cardioprotective effects. It is important to emphasize that these studies draw indirect evidence by association, as do all epidemiological studies. Despite this limitation, these works remain powerful because they identify potential sources of cause-and-effect relationships and because of the large sample sizes studied in many of these investigations. For example, the NHLBI Family Heart Study examined 4584 subjects and found a significant reduction in the risk for heart disease by 40% for linoleic acid and 70% for ALA (Djousse et al. 2001). The Health Professionals Follow-Up Study compiled data from 51,529 health professionals and found a 1% increase in ALA intake (as a % of energy) resulted in a 40% lower risk of non-fatal coronary heart disease (Ascherio, et al. 1996). The Nurse's Health Study gave a 1,116-item food frequency questionnaire to 76,283 women. It had a 10-year follow-up period. They found that a higher intake of ALA in the form of an oil/vinegar salad dressing for 5-6 times per week resulted in a significantly reduced risk of fatal coronary heart disease (Hu et al. 1999). The MRFIT Study followed 12,000 men aged 35-57 years old for 6-8 years and concluded that a higher intake of ALA resulted in a decreased risk of death from cardiovascular disease, coronary heart disease and all causes of death combined (Dolecek, et al. 1992). They also reported that an increase in the ALA content of the plasma phospholipid fraction of as little as 0.06% was associated with a 28% reduction in the risk of stroke (Simon et al. 1995). The Finnish Dietary Intervention Study showed an increased ALA consumption was associated with a decreased coronary heart disease mortality and a >60% decrease in the incidence of stroke

(Vartiainen, et al. 1994). The Cardiovascular Health Study found a higher intake of ALA was associated with a lower risk of fatal ischemic heart disease (Lemaitre et al. 2003). The Costa Rica Study found an inverse relationship between ALA content in adipose tissue and the risk of non-fatal acute myocardial infarctions (Baylin et al. 2003).

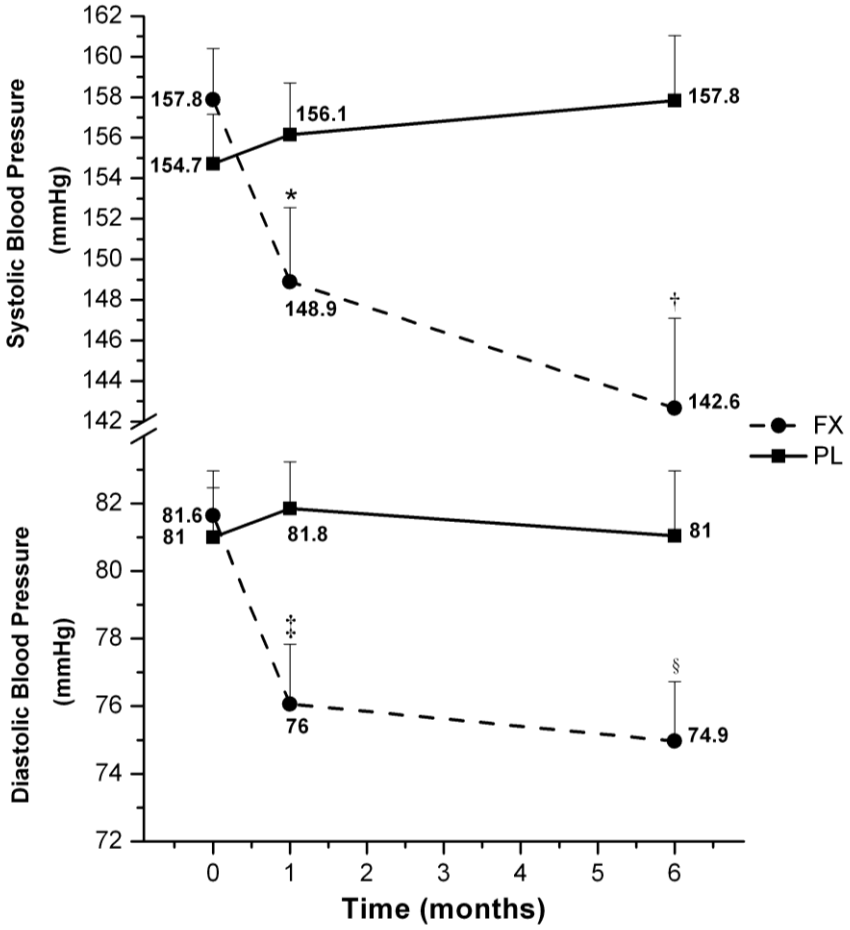


Figure 13. Mean systolic and diastolic blood pressure at baseline, 1 month, and 6 months for placebo (PL) and flaxseed (FX) groups (Rodriguez-Leyva et al. 2013). **P*=0.04, flaxseed vs placebo for systolic blood pressure at 6 months; †*P*=0.004, flaxseed vs placebo for diastolic blood pressure at 6 months.

Interventional studies have also demonstrated powerful results linking ALA, and in some cases, flaxseed with cardiovascular outcomes. The Lyon Diet Heart Study was a randomized secondary prevention trial with over 600 men and women enrolled to consume a typical western style diet or a Mediterranean diet enriched in ALA from canola margarine. They found a 75% reduction in non-fatal myocardial infarctions, a 70% reduction in total death and a 61% reduction in cancer risk in the experimental group without a change in serum cholesterol levels. The results were attributed to a consumption of ALA (de Lorgeril et al. 1999). The FlaxPAD Trial involved 110 subjects with peripheral artery disease who ingested 30 grams of ground flaxseed or a placebo mixture incorporated into a variety of foods including bagels, muffins, pasta, tea biscuits, snack bars and buns. They found a dramatic 15 mmHg reduction in systolic and a 7 mmHg drop in diastolic blood pressures in hypertensive subjects over a 6-month ingestion period (Figure 13) (Rodriguez-Leyva et al. 2013). Significant decreases in blood pressure were observed in as little as one month after the initiation of the dietary intervention (Rodriguez-Leyva et al. 2013). Central blood pressure was also reduced without changes in cardiac hemodynamic performance (Caligiuri et al. 2016). The effects were attributed to the ALA content of the diet and its effects on oxylipin metabolism (Caligiuri et al. 2014). ALA was shown to inhibit soluble epoxide hydrolase, an enzyme that converts vasodilatory oxylipins into oxylipins incapable of vasodilation (Caligiuri et al. 2014). This enzyme also metabolizes other oxylipins into pro-inflammatory oxylipins (Caligiuri et al. 2014). The inhibition of the activity of soluble epoxide hydrolase was suggested to be a critical mechanism for the anti-hypertensive action of the dietary flaxseed (Caligiuri et al. 2014). However, the delivery of other vasoactive components within dietary flaxseed (like L-arginine) is also considered to be a contributing factor in the anti-hypertensive action of flaxseed (Parikh et al. 2018). Although these results were predicted to induce a dramatic reduction in the incidence of

strokes and myocardial infarctions, the actual number of these cardiovascular events was too low in the FlaxPAD Trial to achieve statistical significance (Rodriguez-Leyva et al. 2013) despite a trend for a lower incidence of significant cardiovascular events in the flax group as opposed to the placebo group (Rodriguez-Leyva et al. 2013). At the same time as reducing blood pressure, flaxseed ingestion also induced a significant decrease in total and LDL cholesterol levels (Edel et al. 2015b). It did not interfere with the cholesterol-lowering capacity of conventional cholesterol-lowering drugs like statins (Figure 14) (Edel et al. 2015b).

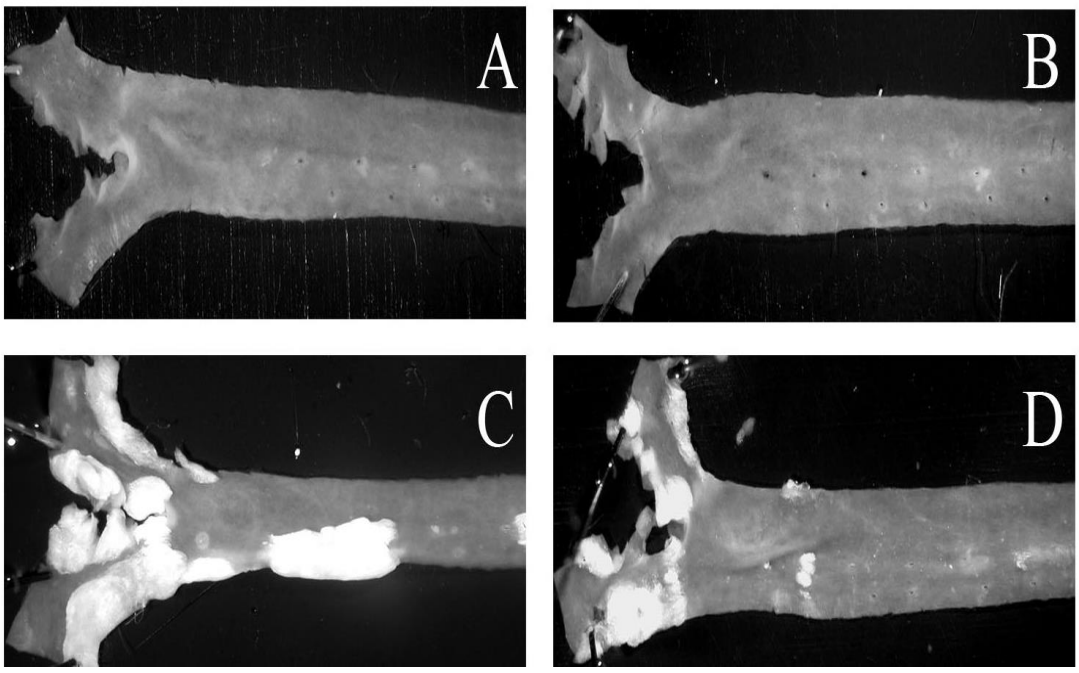


Figure 14. Dietary flaxseed helps prevent cholesterol induced atherosclerotic development (Dupasquier et al. 2007). Representative images of atherosclerotic development on the luminal surface of aortas obtained from LDLrKO mice fed a regular chow diet (*A*), 10% ground flaxseed supplemented chow diet (*B*), atherogenic chow diet supplemented with 2% cholesterol (*C*), or 10% ground flaxseed supplemented atherogenic chow diet (*D*).

The cholesterol-lowering effects of flaxseed may represent an important mechanism to explain the anti-atherogenic effects of dietary flaxseed shown in animal studies. Ingestion of flaxseed inhibited the development of atherosclerosis in rabbits (Dupasquier et al. 2006) and mice fed an atherogenic diet (Figure 14) (Dupasquier et al. 2007). In rabbits, flaxseed did not lower circulating cholesterol levels but still decreased atherogenesis (Dupasquier et al. 2006). This suggested strongly that the cholesterol-lowering action of flaxseed was not the only mechanism through which atherosclerosis was inhibited. Further work provided evidence that the mechanism likely involved an anti-inflammatory action through ALA (Dupasquier et al. 2007). The anti-atherogenic action of dietary flaxseed was observed not only in the presence of cholesterol-supplemented diets but in conditions of elevated trans fats in the diet as well (Bassett et al. 2011).

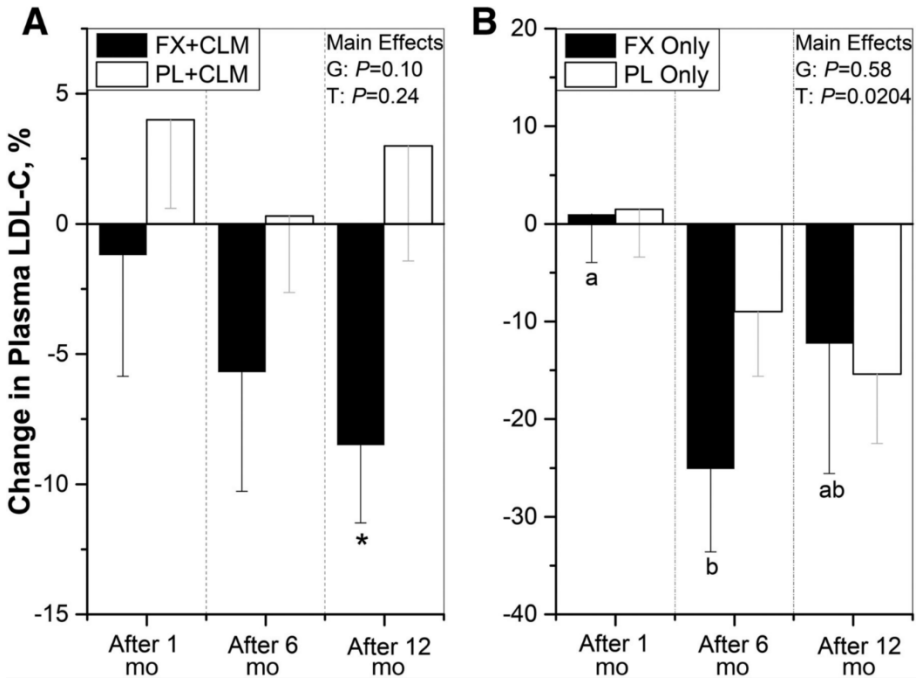


Figure 15. Mean percent change from baseline in plasma LDL-C in patients with PAD in FX or PL subgroups consuming the diet plus CLMs (A) or just the diet (B) after 1, 6, and 12 months (Edel et al. 2015b). Values are represented as means (SEMs). Different from placebo, *P < 0.05. Within a group, labeled means without a common letter differ, P ≤ 0.017. CLM, cholesterol-

lowering medication; FX, milled flaxseed treatment; G, group; LDL-C, LDL cholesterol; PAD, peripheral artery disease; PL, placebo; T, time.

Most anti-atherogenic studies examine the effects of a dietary intervention on growing plaques. However, in a clinical setting, physicians and patients are commonly faced with an established atherosclerotic plaque that causes clinical symptoms and want to know if the plaque can be reduced which would relieve some of the clinical symptoms. Francis and co-workers (2013) created an animal model in which the atherosclerotic plaque was already established, stable and no longer growing, and only then introduced the flaxseed supplementation to the animal's diet. They demonstrated that dietary flaxseed was capable of regressing established atherosclerosis plaque formation (Francis et al. 2013).

As expected, although this animal data provides mechanistic information and new avenues for the use of dietary flaxseed, this remains to be tested in human trials.

Ingestion of flaxseed may also lower the incidence of life-threatening arrhythmias. Rabbits were fed a regular chow diet, or a flaxseed supplemented diet, or a cholesterol-supplemented diet, or a flaxseed and cholesterol supplemented diet. In isolated heart preparations isolated from these from rabbits exposed to both experimental ischemia and ischemia/reperfusion, the incidence of ventricular tachycardia and fibrillation was reduced significantly in hearts from rabbits fed the flaxseed supplemented diets, even if cholesterol was also present in the diet (Ander et al. 2004).

Examining the effects of dietary flaxseed in other pathological conditions like obesity have also produced positive results relevant to cardiovascular disease. For example, flaxseed oil has been associated with lower plasma levels of the inflammatory marker CRP, reduced carotid intima-media thickness and thus a lower cardiovascular disease risk in obese subjects (De Oliveira et al, 2017). Insulin resistance which occurs as a consequence of obesity has been attributed to the

secretion of pro-inflammatory cytokines. Flaxseed oil ingested in a mouse model of obesity demonstrated strong anti-inflammatory and immunomodulatory activity (Bashir et al, 2015). The mechanism proposed for its ability to improve insulin sensitivity was suggested to be a reduction in the levels of TNF-alpha, IFN-gamma and IL-4 levels (Bashir et al, 2015). The higher amount of polyunsaturated fatty acids rather than saturated fat in obese subjects consuming flaxseed was thought to be the critical factor in generating these positive results.

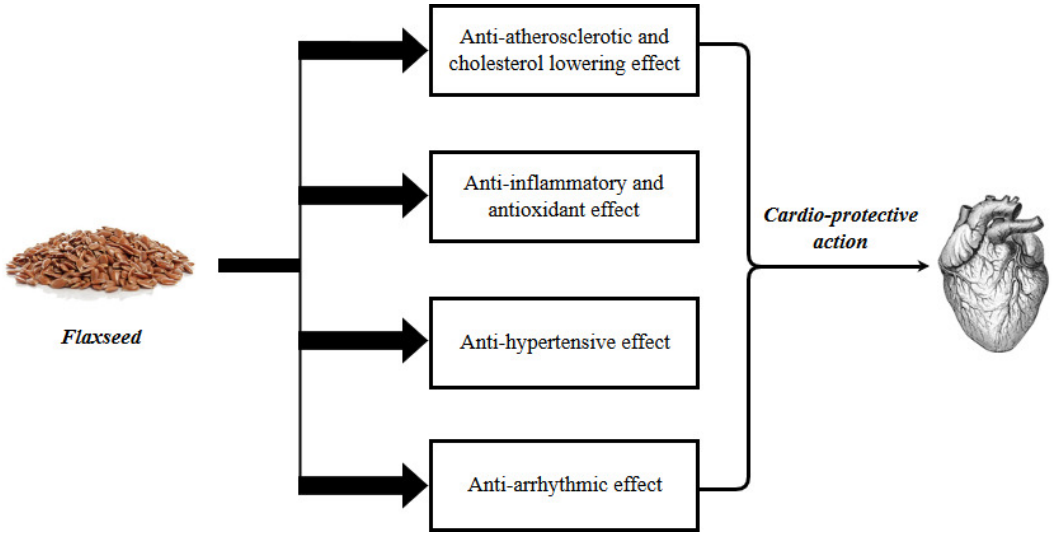


Figure 16. Schematic of the biological actions of dietary flaxseed that have demonstrated cardioprotective effects.

A schematic summarizing the known biological effects of dietary flaxseed that provide demonstrated cardioprotective action is depicted in Figure 16.

What do we still not know about the effects of dietary flaxseed on cardiovascular disease?

1. Can flaxseed inhibit the incidence of arrhythmias after an experimental myocardial infarction? Can it reduce infarct size?
2. What is the bioactive responsible?
3. Can flaxseed inhibit human atrial fibrillation?

4. Is flaxseed cardioprotective in human conditions of ischemia/reperfusion or myocardial infarctions?

5. Can flaxseed lower blood pressure in newly diagnosed hypertensive patients?

6. Can flaxseed replace anti-hypertensive medication?

7. What is the optimal anti-hypertensive dose of flaxseed?

8. Does flax reduce myocardial infarctions/stroke associated with hypertension?

9. What is the mechanism of action?

10. Can flaxseed inhibit atherosclerosis/CHD in humans?

11. What is the bioactive responsible?

12. Can dietary flaxseed prevent/delay CHD in humans?

13. Can dietary flaxseed regress atherosclerosis/CHD in humans?

14. Does flaxseed interact with any other functional food components or cardiovascular drugs that may alter its biological actions?

The opportunities for the use of dietary flaxseed in other diseases related to cardiovascular disease

Although we likely know more about the cardiovascular actions of flaxseed than any other disease condition, many opportunities exist to expand the usage of dietary flaxseed as a strategy to treat other diseases related to heart disease.

(1) The cardiotoxic effects of many drugs like doxorubicin and trastuzumab used to treat breast cancer are well known. Flaxseed is already used extensively in animal studies to treat a variety of cancers including breast cancer (Mason and Thomson, 2014). The possibility exists that dietary flaxseed through one or more of its bioactive components may delay or prevent the onset of cardiotoxicity induced by these anti-cancer drugs.

(2) The critical role that omega-3 fatty acids obtained from marine sources play in brain development is well known (Lauritzen et al, 2016). Will ALA have the same impact? Can it treat a variety of cardiovascular-related brain disorders like stroke? Stroke is an interesting and potentially viable target for flaxseed therapy. Polyunsaturated fatty acids from marine sources have shown positive effects when administered in experimental conditions of stroke (Jiang et al, 2018). It is possible that the ALA content of flaxseed will have beneficial effects as well.

(3) The influence of the microbiome on human and animal health is receiving increasing attention for its influence on cardiovascular function (Ettinger et al, 2014). The impact of dietary flaxseed on the gut microbiome in healthy and diseased populations is also unknown. It may be very different in the two populations.

(4) The economic impact of using flaxseed to replace conventional drugs for cardiovascular disease needs to be carefully analyzed by economists to provide reliable data of its value to governments and the lay public. The same type of data is required for the impact on the agricultural sector if flaxseed is employed as a viable strategy in the medical field (Brown et al, 2018).

Conclusions for the future research directions for dietary flaxseed.

The basic question facing those in the nutrition/cardiovascular disease field is: Is it time to implement dietary flaxseed as a strategy to control human cardiovascular disease? As a general postulate, dietary strategies, nutritional supplements and life style changes can be as effective as pharmaceutical approaches for controlling cardiovascular disease (Caligiuri et al. 2017). In addition, these interventions can prevent as well as treat cardiovascular diseases. The ASCEND trial recently reported that omega-3 fatty acid supplements had only a modest or no benefit in the risk for serious cardiovascular events. This study is significantly different than the flaxseed/ALA results based on several points. First, the ASCEND Trial studied the effects of fish derived omega-

3 supplements, not a plant-based source like the ALA in flaxseed. Secondly, the ASCEND Trial used a relatively low dose of omega-3 fish oil (~1g) as opposed to the 6 g of ALA used in the FlaxPAD Trial (Rodriguez-Leyva et al, 2013). Third, the ASCEND Trial studied cardiovascular disease in a diabetic population and ALA has not been used to measure its effects in this population. Finally, mortality caused by arrhythmias were not separately analyzed in the ASCEND Trial but coupled with other causes of death from coronary heart disease. Strong evidence exists supporting the potential anti-arrhythmic effect of omega-3 PUFAs and their role in prevention of sudden cardiac death. There may have been significant differences between groups if the study had examined the different causes of death in diabetic patients. In view of the preceding discussion, at the very least, the animal and human data should make a compelling case to justify further research on the efficacy of dietary flaxseed in different types of cardiovascular diseases. However, there are many other arguments favouring the use of nutritional interventions like flaxseed as viable treatment strategies for cardiovascular disease. For example, pharmaceutical strategies are only partly effective. Adherence to drugs is a continuing struggle for patients. Flaxseed offers the unique opportunity to control different aspects of cardiovascular disease and its risk factors simultaneously. For example, flaxseed can decrease blood pressure and cholesterol levels. No single drug can achieve both of these effects. Preliminary cost analyses would suggest that dietary interventions are typically less expensive than medication. Flaxseed is consistent with this (Caligiuri et al. 2017). Whereas medication in the form of pills, tablets or capsules can be tasteless or taste badly, flaxseed has a pleasant nutty taste that would encourage its use, not discourage it. Pharmaceuticals are well known to have significant side-effects, many of which are undesirable. Flaxseed ingestion can induce some side-effects, but these can be relatively minor and controllable (Brown et al. 2018). Most importantly, dietary interventions like flaxseed can provide important

alternative strategies to control cardiovascular disease or its risk factors in third world countries that cannot afford medication for the population at large. Indeed, flaxseed may be the only option available to many economically disadvantaged countries. Finally, the increased usage of flaxseed in the medical field can stimulate the flax industry and the agricultural economy. New food processing options would be required, more acreage devoted to flax, and an expected increased price for the commodity would all stimulate the flax industry and improve the economic picture of the farmer and the food processor right through to the marketplace (Brown et al. 2018).

Much has been accomplished in the last two decades to help us understand flaxseed as a potential strategy to control cardiovascular disease. Clearly, much more remains to be done, particularly with the implementation of human trials. Research using flaxseed as an intervention in many pathologies including cardiovascular disease remains an attractive avenue for enterprising researchers in the future.

Acknowledgements

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References

1. Ajith, TA. A recent update on the effects of omega-3 fatty acids in Alzheimer's disease. 2018. Curr. Clin. Pharmacol. Aug 7. doi: 10.2174/1574884713666180807145648.
2. Ander, BP., Weber, AR., Rampersad, P., Gilchrist, JSC., Pierce, GN. and Lukas, A. 2004. Dietary flaxseed protects against ventricular fibrillation induced by ischemia-reperfusion in normal and hypercholesterolemic rabbits. J. Nutr. 134:3250-3256.
3. Ascherio, A., Rimm, EB., Giovannucci, EL., Spiegelman, D., Stampfer, M., Willett, WC. 1996. Dietary fat and risk of coronary heart disease in men: cohort follow-up study in the United States. Br. Med. J. 313:84-90.
4. Austria, JA., Richard, MN., Chahine, MN., Edel, AL., Malcolmson, LJ., Dupasquier, CMC. et al. 2008. Bioavailability of alpha linolenic acid in subjects after ingestion of three different forms of flaxseed. J. Am. Coll. Nutr. 27:214-221.
5. Austria, JA., Aliani, M., Malcolmson, LJ., Dibrov, E., Blackwood, EP., Maddaford, TG., et al. 2016. Daily food choices over one year when patient diets are supplemented with milled flaxseed. J. Funct. Foods. 26:772-780.
6. Bashir, S., Ali, S., and Khan, F. 2015. Partial reversal of obesity-induced insulin resistance owing to anti-inflammatory immunomodulatory potential of flaxseed oil. Immunol Invest 44(5):451-69.
7. Bassett, CMC., McCullough, RS., Edel, AL., Patenaude, A., La Vallee, R. and Pierce, GN. 2011. The alpha linolenic acid content of flaxseed can prevent the atherogenic effects of dietary trans fat. Am. J. Physiol. 301:H2220-H2226.
8. Baylin, A., Kabagambe, EK., Ascherio, A., Spiegelman, D., Campos, H. 2003. Adipose tissue alpha-linolenic acid and nonfatal acute myocardial infarction in Costa Rica. Circulation.

107(12): 1586-1591.

9. Brown, L., Caligiuri, SPB., Brown, D. and Pierce, GN. 2018. Clinical trials using functional foods provide unique challenges. J. Funct. Foods. 45:233-238.
10. Caligiuri, SPB., Aukema, HM., Ravandi, A., Guzman, R., Dibrov, E. and Pierce, GN. 2014. Flaxseed consumption reduces blood pressure in hypertensive patients by altering circulating oxylipins via an alpha linolenic acid-induced inhibition of soluble epoxide hydrolase. Hypertension. 64:53-59.
11. Caligiuri, SPB., Rodriguez-Leyva, D., Aukema, H., Ravandi, A.; Weighell, W., Guzman, R. et al. 2016. Dietary flaxseed reduces central aortic blood pressure without cardiac involvement but through changes in plasma oxylipins. Hypertension. 68:1031-1038.
12. Caligiuri, SPB., and Pierce, GN. 2017. A review of the relative efficacy of dietary, nutritional supplements, lifestyle and drug therapies in the management of hypertension. Crit Rev Food Sci Nutr. 57:3508-3527.
13. De Lorgeril, M., Salen, P., Martin, JL., Monjaud, I., Delaye, J., Mamelle, N. 1999. Mediterranean diet, traditional risk factors, and the rate of cardiovascular complications after myocardial infarction: final report of the Lyon Diet Heart Study. Circulation 99(6):779-785.
14. De Oliveira, P.A., Kovacs, C., Moreira, P., Magnoni, D., Saleh, M.H., Faintuch, J. 2017. Unsaturated fatty acids improve atherosclerosis markers in obese and overweight non-diabetic elderly patients. Obesity Surg 27(10):2663-71.
15. Djousse, L., Pankow, JS., Eckfeldt, JH., Folsom AR., Hopkins, PN., Province, MA., et al. 2001. Relation between dietary linolenic acid and coronary artery disease in the National Heart, Lung, and Blood Institute Family Heart Study. Am. J. Clin. Nutr. 74(5):612-619.
16. Dolecek, TA.1992. Epidemiological evidence of relationships between dietary

polyunsaturated fatty acids and mortality in the multiple risk factor intervention trial. Proc. Soc. Exp. Biol. Med. 200:177-182.

17. Dupasquier, CM., Weber, AM., Ander, BP., Rampersad, PP., Steigerwald, S., Wigle, JT., et al. 2006. Effects of dietary flaxseed on vascular contractile function and atherosclerosis during prolonged hypercholesterolemia in rabbits. Am. J. Physiol. Heart Circ. Physiol. 291(6):H2987-H2996.
18. Dupasquier, CM., Dibrov, E., Kneesh, AL., Cheung, PK., Lee, KG., Alexander, HK., et al. 2007. Dietary flaxseed inhibits atherosclerosis in the LDL receptor-deficient mouse in part through antiproliferative and anti-inflammatory actions. Am. J. Physiol. Heart Circ. Physiol. 293(4):H2394-H2402.
19. Edel, AL., Pierce, G.N. and Aliani, M. 2015a. Age dependency in the metabolism of flaxseed lignans by healthy adults. J. Funct. Foods. 17:948-957.
20. Edel, AL., Rodriguez-Leyva, D., Maddaford, TG., Caligiuri, SPB., Austria, JA., Weighell, W., et al. 2015b. Dietary flaxseed independently lowers circulating cholesterol and lowers it beyond the effects of cholesterol-lowering medications alone in patients with peripheral arterial disease. J. Nutr. 145:749-757.
21. Ettinger G., MacDonald K., Reid G., and Burton JP. 2014. The influence of the human microbiome and probiotics on cardiovascular health. Gut Microbes 5(6):719-28.
22. Francis, AA., Austria, JA., La Vallee, RK., Deniset, JF., Maddaford, GG., Dibrov, E. et al. 2013. The effects of dietary flaxseed on atherosclerotic plaque regression. Am. J. Physiol. Heart Circ. Physiol. 304:H1743-H1751.
23. Hu, FB., Stampfer, MJ., Manson, JE., Rimm, EB., Wold, A., Colditz, GA., et al. 1999. Dietary intake of alpha-linolenic acid and risk of fatal ischemic heart disease among women. Am. J.

Clin. Nutr. 69(5): 890-897.

24. Lauritzen L., Brambilla P., Mazzocchi A., Harsløf LB., Ciappolino V., and Agostoni C. 2016. DHA effects in brain development and function. Nutrients 4;8(1). pii: E6. doi: 10.3390/nu8010006.
25. Lemaitre, RN., King, IB., Mozaffarian, D., Kuller, LH., Tracy, RP., Siscovick, DS. 2003. n-3 polyunsaturated fatty acids, fatal ischemic heart disease, and nonfatal myocardial infarction in older adults: the Cardiovascular Health Study. Am. J. Clin. Nutr. 77(2):319-325.
26. Malcolmson, LJ, Przybylski, R., and Daun, JK. 2000. Storage stability of milled flaxseed. J. Am. Oil Chem. Soc. 77: 235-238.
27. Mason JK., and Thompson LU. 2014. Flaxseed and its lignan and oil components: Can they play a role in reducing the risk of and improving the treatment of breast cancer? Appl. Physiol. Nutr. Metab. 39(6):663-78.
28. Parikh, M., Netticadan, T. and Pierce, GN. 2018. Flaxseed: Its bioactive components and their cardiovascular effects. Am. J. Physiol. 314: H146-H159.
29. Patenaude, A., Rodriguez-Leyva, D., Edel, AL., Dibrov, E., Dupasquier, CMC., Austria, JA., et al. 2009. Bioavailability of alpha linolenic acid from flaxseed diets as a function of the age of the subject. Eur. J. Clin. Nutr. 63: 1123-1129.
30. Przybylski, R., and Daun, JK. 2001. Additional data on the storage stability of milled flaxseed. J. Am. Oil Chem. Soc. 78: 105-106.
31. Rodriguez-Leyva, D., Weighell, W., Edel, AL., La Vallee, R., Dibrov, E., Pinneker, R., et al. 2013. Potent anti-hypertensive action of dietary flaxseed in hypertensive patients. Hypertension. 62:1081-1089.
32. Simon, JA., Fong, J., Bernert, JT., and Browner, WS. 1995. Serum fatty acids and the risk of

stroke. Stroke. 26:778-782.

33. The ASCEND Study Collaborative Group. 2018. Effects of n−3 fatty acid supplements in diabetes mellitus. New Eng. J. Med. 379:1540-1550.

34. Vartiainen, E., Puska, P., Pekkanen, J., Toumilehto, J., and Jousilahti, P. 1994. Changes in risk factors explain changes in mortality from ischemic heart disease in Finland. Br. Med. J. 309:23-27.

1.8.3. Dietary Flaxseed as a Strategy for Improving Human Health

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Abstract

Flaxseed is a rich source of the omega-3 fatty acid, alpha linolenic acid, the lignan secoisolariciresinol diglucoside, and fiber. These compounds provide bioactivity of value to the health of animals and humans through their anti-inflammatory action, anti-oxidative capacity and lipid modulating properties. The characteristics of ingesting flaxseed or its bioactive components are discussed in this article. The benefits of administering flaxseed or these individual bioactive components on health and disease are also discussed in this review. Specifically, the current evidence on the benefits or limitations of dietary flaxseed in a variety of cardiovascular diseases, in cancer, gastro-intestinal health, brain development and function, as well as hormonal status in menopausal women is a comprehensive topic for discussion.

Introduction

Dietary flaxseed has an impressive and growing research literature supporting its use in a variety of health conditions. Whereas in the late 20th century little was known of the health-related benefits of flaxseed as well as how to consume flaxseed to achieve any health benefits, today is an entirely different time. Research information on the effects of dietary flaxseed has increased dramatically. We now know what diseases flaxseed can treat or prevent, the health impacts dietary flaxseed can provide, the bioactives within flaxseed that provide these health-related effects in many cases, and the forms of flaxseed that are required to provide these bioactives to the body. Data on the impact of dietary flaxseed on disease has been translated, albeit in limited amounts, from animal research studies to human trials. Many of these trials have been conducted with the highest standards of control available in order to provide solid, reliable information on its value to the general public and, more specifically, to those in a disease compromised condition. These data have sparked the food processing industry to create a greater variety of foods that contain flaxseed, giving the public an increased opportunity to incorporate flaxseed into their everyday diet. With this growing availability of flaxseed in the marketplace and the increasing awareness of the public to its impact on human health when supplemented into the diet on a daily basis, it would be expected that flaxseed will also grow in the coming years in economic importance to the farmer, the food processer and the retailer in the marketplace (1).

We now know that environmental factors like diet can have a profound effect on the maintenance of health and the appearance of disease (2). It is also increasingly apparent that the treatment of disease is not only achieved through pharmacological therapy but through dietary interventions as well. The purpose of this review is to provide a broad summary of the highlights

of the research that has supported the growth of flaxseed as a commodity with significance in the fields of health and medicine.

Flaxseed and Its Use in the Diet

The main bioactive compounds in flaxseed include alpha-linolenic acid (ALA), lignans, and fiber. Four common forms of flaxseed available for human consumption include whole flaxseed, ground flaxseed, flaxseed oil and partially defatted flaxseed meal (3). A new form available in the market is flax "milk" (Pizzey Ingredients Inc, Manitoba, Canada). An alternative to "milks" like almond milk, flax milk is finely milled flaxseed mixed with filtered water and other minor compounds. Flax milk is high in ALA and is an excellent alternative to diary milk, as it has no cholesterol or lactose. It is suitable for people allergic to soy, nuts, and gluten, as it contains more health benefits than almond milk.

However, no matter how good a food is for the general public to consume, if that food does not have acceptable taste, texture, appearance, colour and aroma qualities, the majority of people will not eat it. Flaxseed has several characteristics that could negatively affect its flavor profile and are cause for concern. The two most important are the potential for the very high omega-3 fatty acid content in flaxseed to become rancid through oxidation, and the propensity for bitterness. The high content of alpha linolenic acid (ALA) renders it highly susceptible to oxidation. The oxidation and subsequent rancidity will lead to off flavours and a musty aroma that would be rejected in taste tests. The antioxidant content in flaxseed provided in turn by its secoisolariciresinol diglucoside (SDG) content is of great value in curtailing any oxidation process. In addition, flaxseed has been described as possessing a "nice nutty smell and aroma" [4] and is potentially ideal for the incorporation into a variety of foods. In research studies, flaxseed has been

incorporated successfully into snack bars, muffins, bagels, bread, buns, tea biscuits, cinnamon rolls and pasta [5,6]. The concentration of flaxseed in the food will, of course, influence its flavor characteristics. Flaxseed has been incorporated into foods for human consumption from 5-28% of total ingredients by weight before baking [7]. The amount of flaxseed ingested daily over an extended period of time has been as much as 40 to 50 g [8]. Clinical trials having patients ingesting a food each day for up to one year have been successfully completed with drop-out rates of approximately 20% in both placebo and flax groups [5]. This demonstrates that it is not the flaxseed that induces non-compliance *per se*, but instead a great number of different flax-containing foods are needed to allow variety in the daily choices if one is to maintain subjects in a trial with an extended duration.

The most common foods in which to have flaxseed are baked goods. The process of baking even up to 178^{o}C for two hours does not alter the composition or content of ALA in a baked muffin, for example [9]. The addition of flavouring to the baked good also makes it possible to disguise any less than optimal flavor profile induced by the bitterness of flaxseed or any minor rancidity. Flavourings like banana and nuts, banana chocolate chip, cinnamon apple raisin, cinnamon raisin, cinnamon spice, gingerbread raisin, cappuccino chocolate chip, sunflower sesame and cranberry orange have been used in research studies [7,10]. The public will have preferences to these flavourings and matrices for the flaxseed. In a study of the daily preferences of the ingestion of food products containing 30g of milled flaxseed over one year, bagels were consumed > muffins > snack bars > sprinkles > tea biscuits > pasta > sundried tomato buns [11]. Cinnamon raisin flavourings were preferred > cranberry orange > apple spiced > sunflower sesame [11]. Recently, a company (Pizzey's Ingredients, Manitoba) has developed a flax "milk", much the same as

almond “milk” on the market. This provides an even more convenient way to consume flaxseed and preserve it as well.

Each of these different food matrices can influence the stability of the flaxseed. Processing of the seed, storage temperature and duration as well as the form of flaxseed (milled flaxseed *versus* whole seed *versus* flax oil) will also influence the stability of the product. Milling, grinding or crushing flaxseed will destroy the hard protective seed coat on flaxseed exposing the ALA and SDG to oxidation [12]. However, this process is required to render these bioactive ingredients bioavailable [12]. ALA is more bioavailable to the body if it is in oil or milled form [13]. The incorporation of milled flaxseed into baked products may actually protect the ALA and SDG from degradation [12]. Cooler storage temperatures and a shortened storage duration, particularly for flax oil, will also result in a better preservation of the ALA and SDG [12].

Recently, a concern was raised about the presence of components in flaxseed that may have undesirable effects that could affect the bioavailability and bioaccessibility of essential nutrients [14]. These include the presence of protease inhibitors, phytic acids, linatine and cyanogenic glycosides in flaxseed. However, there are no deleterious effects reported of these components in human studies. The concentrations of these components delivered through dietary flaxseed may be below that needed to induce any biological actions. Nevertheless, it is prudent to take into account the raised concern. Plant bio-breeding and/or food processing methods may be considered to reduce the levels of these components in flaxseed.

Dietary Flaxseed and Cardiovascular Disease

The effects of flaxseed on parameters of cardiovascular disease have become one of the most intensely studied areas with regard to the health-related benefits of dietary flaxseed (Figure 17).

In animal models of heart disease, dietary flaxseed has decreased the progression of atherosclerosis induced by a high dietary cholesterol or a high dietary trans fat content [15-17], likely via an anti-inflammatory action provided through its ALA content (15,16,18). Depending upon the animal species, flaxseed may (mice and rats) or may not (rabbits) lower circulating cholesterol levels (15,16,18). Flaxseed can also lower circulating trans fats levels (17). In a stable, established atherosclerotic plaque, supplementation of the diet with flaxseed can also regress atherosclerosis (19).

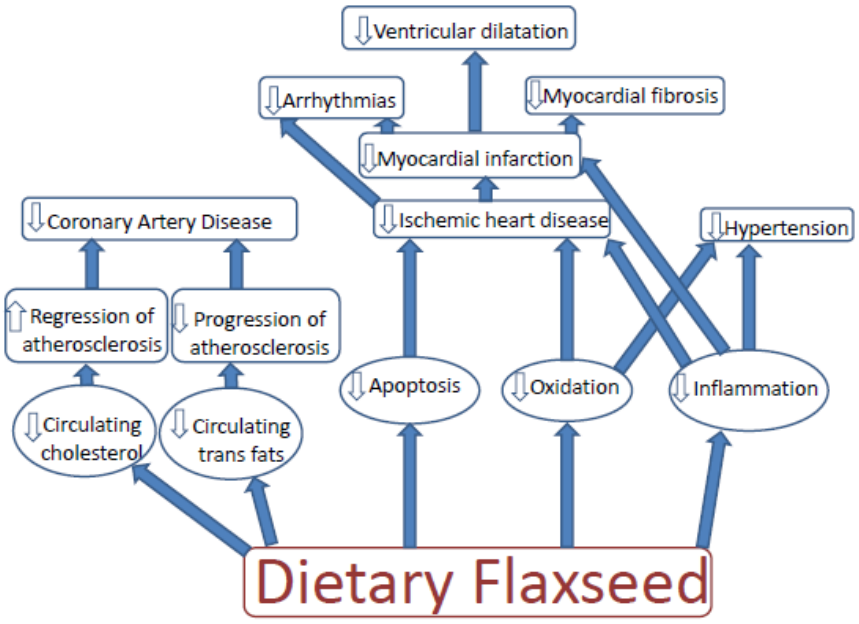


Figure 17. The effect of dietary flaxseed on the various types of cardiovascular disease. arrows going up denote an increase in a specific parameter whereas arrows going down denote a decrease in a specific parameter.

Ischemic reperfusion challenge to isolated hearts can induce arrhythmias. Rabbits on a flaxseed supplemented diet prior to being subjected to the ischemic reperfusion insult are protected from ventricular fibrillation (20). If rats are placed on a flaxseed supplemented diet prior to and after the induction of a myocardial infarction, they exhibit smaller infarcts, less arrhythmias and a

reduction in left ventricular dilation (18). The mechanism once again is likely to involve the ALA content of flaxseed (18). ALA has shown a capacity to alter arrhythmogenic ionic currents in the heart (21). However, the metabolism of SDG into enterolignans within the circulation may also have a cardioprotective role (18,22).

In humans exhibiting symptoms of cardiovascular disease, dietary flaxseed has displayed powerfully protective effects. The most impressive involves the decrease in both systolic and diastolic blood pressure in patients with peripheral arterial disease (PAD) (5). Both brachial and central blood pressures were significantly reduced by dietary flaxseed in this trial (5,23). In the double-blinded, placebo-controlled, randomized FlaxPAD Trial (24), PAD patients fed 30g of milled flaxseed every day for 6 months exhibited significant decreases in both systolic and diastolic blood pressure (5). As shown in Figure 18, not all patients responded to the flaxseed diet with decreases in systolic blood pressure (SBP). In the placebo group, fifteen subjects showed a decrease in SBP over the 6 months whereas twenty-one exhibited an increase in SBP. In the flax-fed group, twenty-seven subjects exhibited a decrease in SBP over the 6 months and twelve had an increase in SBP over this time. The SBP in two subjects was unchanged over this time period. The average drop in the flax group was substantial (10 mm Hg) and statistically significant. This hypotensive effect was shown as early as 1 month after commencing the flaxseed in the diet and maintained for up to 1 year (5). The decrease was sufficient to predict a 50% decrease in the incidence of myocardial infarctions and strokes (5). This action was shown to be associated with a significant change in lipid metabolism such that the generation of oxylipins with less vasodilatory and more pro-inflammatory action was blocked (25). This action was suggested to be due to the ALA content of the flaxseed (24). Alternatively, in animal studies, flaxseed protein hydrolysates and flax oil decreased blood pressure in the spontaneously hypertensive rat in acute or chronic

conditions, respectively (26,27). As well, a flaxseed lignan concentrate reduced blood pressure in the deoxycorticosterone acetate (DOCA-salt) induced renal hypertensive rats (28) and the high fat, high sugar-fed rat (29).

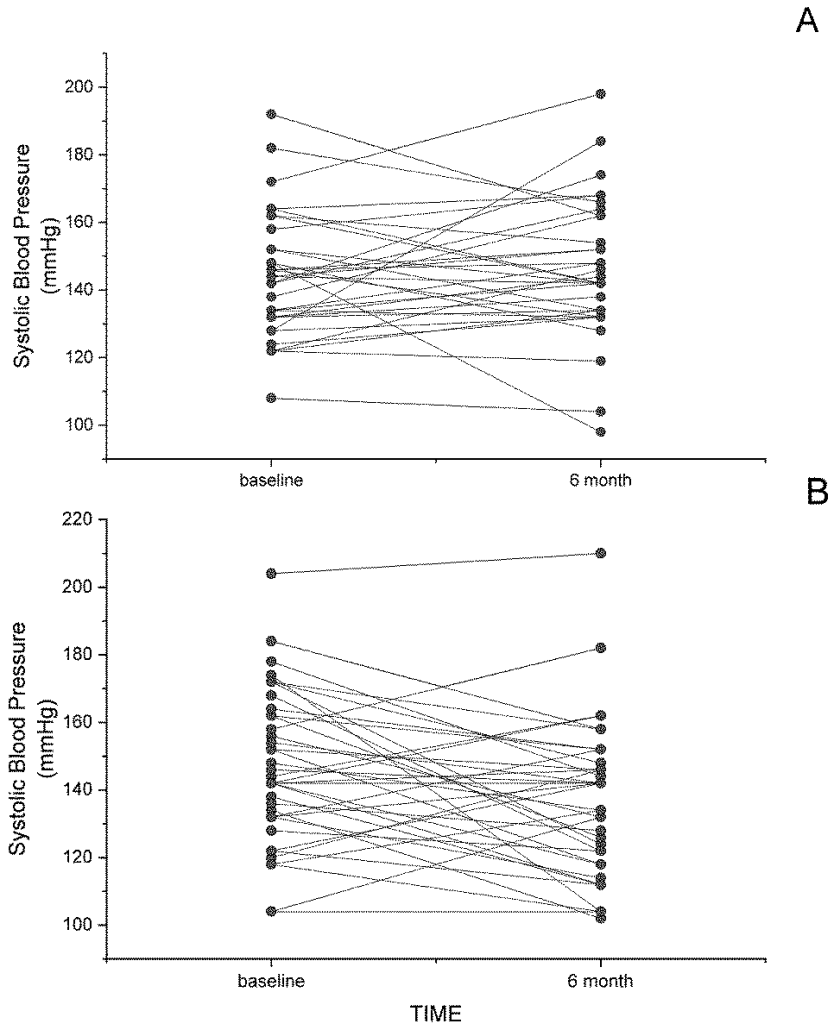


Figure 18. Individual values of systolic blood pressure at baseline or after 6 months of dietary supplementation with a placebo (A) or 30 g of milled flaxseed per day (B). Baseline systolic blood pressures in the placebo group were 143.5 ± 2.88 at baseline and 144.8 ± 3.33 after 6 months (n= 36). Baseline systolic blood pressures in the flax group were 146.6 ± 3.37 at baseline and 136.5 ± 3.53 after 6 months (n= 41). Results are obtained from Rodriguez-Leyva et al, 2013 [5].

Dietary Flaxseed and Diabetes

Flaxseed also impacts another major disease that is growing in incidence across the globe: diabetes. Flaxseed supplementation reduced blood glucose in subjects with type 2 diabetes (30,31) and lowered blood glucose in subjects with prediabetes (32). Flaxseed derived gum and lignan supplement also decreased blood glucose in subjects with Type 2 diabetes (33,34). Pre-clinical studies have reported anti-hyperglycemic effects of the flaxseed lignan SDG in animals with Type I diabetes (35). Whether SDG or flaxseed supplementation improves glycemic control in human subjects with Type 1 diabetes remains unknown and could be a topic of experimentation in the future.

Dietary Flaxseed and Cancers

Flaxseed is already used extensively in animal studies to treat a variety of cancers (36). Perhaps the most studied cancer with respect to the impact of dietary flaxseed is breast cancer. In both experimental animal studies (36,37) and in human trials (36-38), dietary flaxseed has significant protective effects against breast cancer. A systematic review of 10 human trials led to the conclusion that flaxseed reduced tumour growth in women with breast cancer (36,38). They also found evidence in support of flax-associated protection against primary breast cancer as well as a reduced risk of mortality in women living with breast cancer (38). Beneficial effects were observed with 25 g doses of milled flaxseed (38).

Flaxseed lignan are nonsteroidal phytoestrogens that have a chemical structure that resembles mammalian estrogens and hence produce estrogen-like effects in mammals. Flax lignans are metabolized by intestinal bacteria to become bioavailable in the plasma (39). Lignans derived from food occur mainly as glucosides and undergo deglycosylation by β-glucosidases from gut

microbiota before their intestinal absorption. Secoisolariciresinol diglucoside (SDG) is hydrolyzed in this way to release secoisolariciresinol (SECO). Deglycosylation of SECO is followed by demethylation by microbiota to produce dihydroxyenterodiol, which further results in the formation of enterodiol through dehydroxylation. Finally, dehydrogenation of enterodiol produces enterolactone. Enterodiol and enterolactone are absorbed from large intestine, and ultimately appear as glucuronide and sulfate conjugates in body fluid and are excreted in urine (40). The metabolism of SDG to enterolactone occurs in all subjects irrespective of gender differences (39). A pharmacokinetic study in humans demonstrated the appearance of enterolignans in the plasma 8-10 h after ingestion of SDG, with the mean maximum plasma concentration of enterodiol and enterolactone attaining ~14.8 h and ~19.7 h, respectively, after consumption (40). The mean elimination half-life reported for enterodiol is ~4.4 h, and enterolactone is ~12.6 h (40). Epidemiological studies in postmenopausal breast cancer patients have reported an association of higher blood concentration of enterolactone with a reduced risk of breast cancer, decreased mortality rate and better survival. Clinical evidence also exists supporting the protective effect of enterolactone in the breast, colon, prostate, gut and lung (41).

Breast cancer is not the only cancer that has shown sensitivity to dietary flaxseed or its components. Cancer of the prostate gland (42), lung (43,44), colon (45), ovary (41), endometrium (41), hepatocellular (41) and cervix have been inhibited by flaxseed.

The mechanisms responsible for the beneficial anti-oncotic action of milled flaxseed have been studied in cell culture, in animal experiments and in human trials. The rich compositions of both SDG and ALA have been postulated to play a role in the beneficial action of flaxseed on cancer (37,38,45,46). Lignans like SDG reduce the breast cancer mortality by 33-70% and reduce all-cause mortality by 40-53% (36). Doses of 50 mg of SDG will reduce tumours (38). However,

it is unlikely that SDG is itself responsible for the anti-tumour activity. SDG is restricted to the gastro-intestinal space and does not enter the blood stream. It is metabolized within the gastrointestinal tract to the enterolignans, enterolactone and enterodiol (47). These compounds have estrogenic and antioxidative properties (36). Enterolactone and enterodiol can bind to estrogen receptors and alter cell growth (37). It is likely that these metabolites of SDG are ultimately responsible for the anti-tumour capacity of SDG (41). The anti-inflammatory action of ALA may also play a role (41). Flaxseed oil (enriched in ALA) also potentiates the anti-tumour action of drugs used in oncotic therapy. Flax oil administered with trastuzumab for epidermal growth factor receptor 2 positive breast cancer enhanced the anti-tumour effects of trastuzumab and significantly lowered the concentrations of trastuzumab needed to kill tumours in athymic mice (48). Similar results have been observed using flaxseed with tamoxifen, another anti-tumour drug (37).

The cellular oncotic pathways affected by flaxseed and its bioactive components include a modulation of estrogen metabolism (36), an inhibition of cellular proliferation, angiogenesis, metastasis and inflammation, as well as a stimulation of apoptosis within the tumour (36,38,41-43,45,46). The molecular targets for the anti-tumour actions of flaxseed and its bioactive components includes a suppression of the phosphorylation of p-AKT, p-ERK and p-JNK kinases (resulting in a general slowing of the MAPK pathways) (43,46), inhibition of CDK4 (45), down regulation of multiple miRNAs (44), decreased expression of mRNAs for Bcl2, cell cycle proteins, ERalpha and beta, and epidermal and insulin-like growth factor receptors (46).

It would appear that increasing our understanding of the value of dietary flaxseed, flax oil and SDG in this field and its efficacy in human trials may be a profitable avenue of future study. As in the case of the effects of flaxseed and its bioactive constituents in cardiovascular disease, it would

appear that sufficient data already exists for the institution of these dietary therapies in specific cases of cancer prevention and treatment.

Dietary Flaxseed and The Brain

The critical role that omega-3 fatty acids obtained from marine sources play in brain development is well known (49). This work has focused in the past primarily on the omega-3 fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Whereas research has established an essential role for DHA in pre- and post-natal brain development (49,50), EPA appears to modulate behaviour and mood (50). A loss of brain DHA content has been associated with poorer performance in spatial and learning tasks (51).

These data suggest that another omega-3 fatty acid, ALA, which is enriched in flaxseed, may have similar functional significance for the brain. When mothers of rats were fed flaxseed during pregnancy, the brains of newborn pups were heavier and contained significantly greater amounts of both ALA and DHA (52). In pups given milled flaxseed or flaxseed oil soon after birth, these pups showed higher brain mass demonstrating the value of milled flaxseed particularly in contributing to early postnatal brain development (53). However, although dietary flaxseed may improve brain development and spatial memory, research has cautioned that the diet may depress body growth due to an imbalance between omega-3 and omega-6 fatty acid levels (54).

Dietary flaxseed may also improve aspects of brain function during conditions of neural disease. Maternal intake of flaxseed prevented depressive symptoms in offspring and demonstrated neuroprotection during experimental neonatal hypoxic-ischemic encephalopathy by lessening brain mass loss together with improvements in motor hyperactivity and spatial memory (55). In mice exposed to chronic mild stress, dietary flaxseed reduced all parameters of chronic stress (56).

Supplementation to the diet with flaxseed lignans like SDG have shown anti-depressant-like effects in mice subjected to chronic stress (57) and demonstrated protective effects in cortical neurons against NMDA-induced neurotoxicity (58).

Dietary Flaxseed and Female Hormonal Status

Dietary flaxseed may also exhibit a protective effect against menopausal symptoms (59). Several studies have examined the effects of flaxseed or its bioactive ingredients on quality of life and hot flashes frequency and severity in post-menopausal women. The estrogenic action of certain metabolites of flaxseed suggested a potentially positive effect on these post-menopausal symptoms. In a study of 140 postmenopausal women, menopausal symptoms decreased and the quality of life increased in women who ingested a flaxseed supplemented diet (60). A particularly large trial (199 women) of an unusually long duration (1 year) on a high dose of flaxseed (40 g per day) reported a significant decrease in menopausal symptoms but this effect did not differ from the control group that ingested a wheat germ placebo (8). Another study reported similar inconclusive evidence of a positive effect of flaxseed on menopausal symptoms with up to 90 g of flaxseed per day (61). Further randomized controlled trials and systematic reviews of clinical trials found no significant effect of flaxseed or on quality of life or hot flashes during menopause (62-65). It is concluded that randomized, placebo-controlled trials are necessary to determine conclusive effects on menopausal symptoms. When carefully controlled trials are carried out in this manner, it would appear that flaxseed does not induce a significant effect on quality of life or the incidence and severity of hot flashes in menopausal women.

Caution has been advised for flaxseed consumption during pregnancy and lactation. SDG and flaxseed oil given to pregnant rats have shown opposite effects on total fat mass of the body. SDG

administration resulted in higher fat mass while flaxseed oil lowered the fat mass. Furthermore, flax oil also reduced serum and milk triglycerides and cholesterol levels in the female rats during lactation (66,67). Similarly, male and female pups of mothers on flaxseed oil exhibited lower body fat mass and triglycerides levels (66,67). With such changes in maternal and offspring biochemical parameters, it may appear prudent to exercise caution when using flaxseed or its components in pregnancy. However, as no other studies have reported similar changes, randomized, placebo-controlled trials in pregnant subjects are warranted to provide any conclusive evidence.

Postmenopausal women are at increased risk of osteoporosis. In a short 6 weeks study by Arjmandi et al (68), a slightly lower bone resorption marker (tartrate-resistant acid phosphatase) was observed in postmenopausal women receiving flaxseed. However, no influence was reported when using anabolic bone agents. Conversely, most studies have shown no effect of flaxseed consumption on bone mineral density, bone mineral content, or bone turnover in postmenopausal women (69). In animal model, flaxseed supplementation resulted in an additional benefit when combined with estrogen therapy. Flax oil rich in ALA showed a positive effect on bone health, particularly in pathological conditions such as obesity and kidney disease. ALA may be responsible for this improvement in osteoporotic bone conditions than the estrogenic lignan content of flaxseed (69).

Dietary Flaxseed and Skin Health

A 12 week, randomized, double-blinded study on healthy female volunteers with sensitive skin reported a positive improvement in skin properties with the ingestion of flaxseed oil. A significant decrease was noted in skin sensitivity, transepidermal water loss, skin roughness and scaling, with an increase in skin hydration and smoothness (70). ALA was identified as the main

bioactive responsible for these effects on skin and aging. Compared to a younger population, older individuals have higher concentrations of proinflammatory oxylipins including 5-HETE, 9,10,13-TriHOME, and 9,12-13-TriHOME, which could explain higher levels of inflammation in this older demographic (71). Dietary supplementation of flaxseed has been shown to correct the balance of pro- and anti-inflammatory oxylipins and thus may exert a healthy effect on aging (71).

Dietary Flaxseed and Gastro-Intestinal Health

The influence of the microbiome on human and animal health is receiving increasing attention from the research community (72). However, our understanding of the impact of dietary flaxseed on the gut microbiome in either healthy or diseased populations is limited. It was known that the lignans that are unusually high in content within flaxseed need to be metabolized by intestinal bacteria in order to gain access to the systemic circulation in humans. The production of enterolignans like enterodiol and enterolactone from SDG is best generated from milled flaxseed and defatted flaxseed meal in comparison to a variety of other foods that contain SDG (73). This intestinal metabolism is accomplished via the biotransformative action of specific intestinal bacteria (74) including *Ruminococcus bromii* and *Ruminococcus lactaris* (75). Others have found *L. casei* and *L. acidophilus* were important for the digestion of whole flaxseed in order to increase enterodiol bioaccessibility (76).

Early studies have shown the capacity of flaxseed supplementation in the diet to alter the bacterial flora in the intestines of animals. For example, flaxseed supplemented to the diet of mice will alter *Enterobacteriaceae* diversity and prevalence in the feces and cecum (77). Power and colleagues (78) showed that a flaxseed supplemented diet will alter the microbial microenvironment in the colon with a 20-fold increases in *Prevotella spp*. and a 30-fold decrease

in *Akkermansia muciniphila* abundance. Others have shown that a combined high fat, flaxseed and fish oil supplemented diet increased the abundance of intestinal *Bifidobacterium* (79).

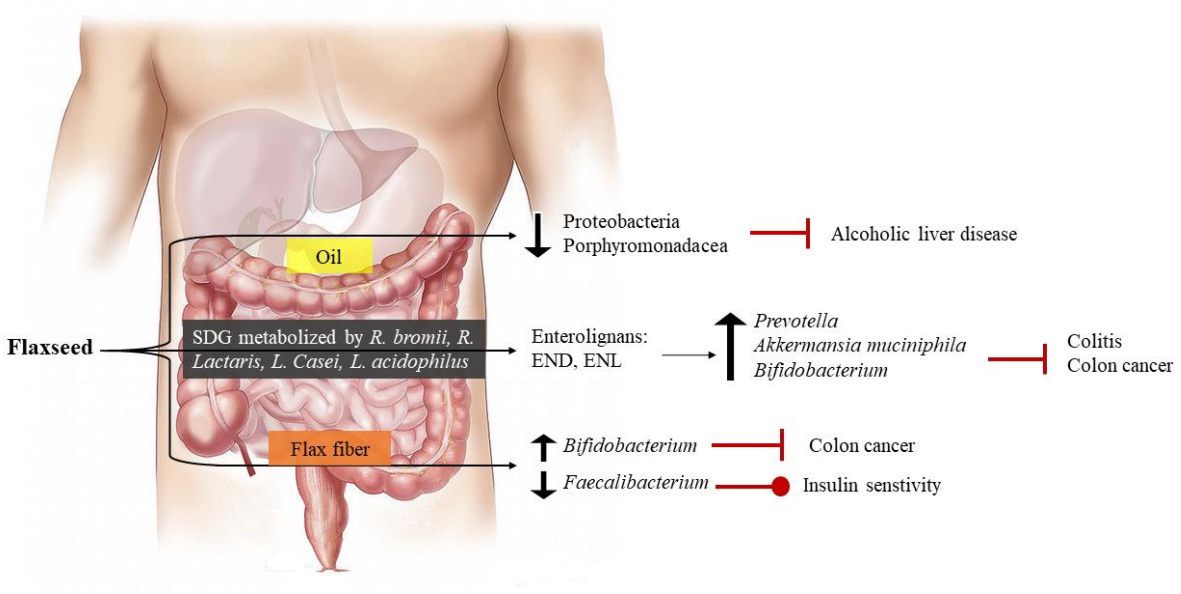


Figure 19. The effect of dietary flaxseed on the gastrointestinal health.

Changes in specific bacteria in the microbiome may have implications on disease progression (Figure 19). Dietary flax oil consumption can, under some conditions, reduce the presence of *Proteobacteria* and *Porphyromonadaceae* in the gut microbiome, which may have a positive impact on alcoholic liver disease (80). Defatted flaxseed meal (which is a partially delipidated form of flaxseed) reduced the appearance of aberrant crypt foci in the colon of mice through an increase in the expression of *Bifidobacterium* (81). Daily intake of flaxseed mucilage resulted in changes in the abundance of 33 different metagenomics species in the gut microbiota including eight different species of *Faecalibacterium*. This altered insulin sensitivity in these women (82). These data contrast with those of Lagkouvardos et al (75) who found flaxseed did not significantly

change the fecal microbiome nor the abundance of the dominant bacterial species present. Others have also challenged the efficacy of dietary flaxseed to alter the appearance of disease based upon a change in the gut microbiome. For example, a 5% flaxseed supplemented diet had no protective effect against the development of intestinal tumours (83). Caution in the use of flaxseed in the diet has also been introduced by the results of Maatanen and co-workers who showed that whereas a reduced fat diet could protect against colitis, the high levels of PUFAs provided by flaxseed supplementation to the diet diminished these beneficial effects (84).

The potential for dietary flaxseed to replace or reduce the use of constipation medication in different populations at risk (the elderly, the ill, in personal health care homes, etc) and the reduction in side-effects associated with conventional constipation medication is worthy of study. Two recent randomized trials have generated results of great interest for the introduction of flaxseed into diets for relief from constipation. In the first trial (30), flaxseed baked into cookies was ingested in constipated patients with Type 2 diabetes. They found the flaxseed reduced constipation symptoms, weight, fasting plasma glucose, triglycerides, and LDL and HDL cholesterol levels. In a subsequent trial using the same type of patient population, flaxseed affected all of these parameters once again and, importantly, was superior in its capacity to reduce constipation symptoms to psyllium (85). Flaxseed may also be of use in reducing the symptoms of irritable bowel syndrome, however, further research is required (86). Flaxseed oil was also beneficial in reducing experimental diarrhea (87).

The mechanism of action of flaxseed in stimulating fecal output during constipation clearly lies largely with its high fiber content. It is not surprising, therefore, that even partially defatted flaxseed meal was a valuable laxative in both normal and constipated condition (78). However,

flaxseed oil has been shown to act on intestinal muscarinic receptors and blocks K+ channels (77), actions that would both lead to modifications of intestinal motility.

Toxicity of Flaxseed

Although no toxicity has ever been reported in clinical studies with dietary supplementation of flaxseed, some compounds such as cyanogenic glycosides and linatine have been identified as potential toxic compounds. Cyanogenic glycosides such as linamarin, linustatin, neolinustatin, lotaustralin, and amygdalin are nitrogenous secondary plant metabolites (3). These compounds are not exclusively found in flaxseed but are also present in other food items including apples, spinach and cassava (88-90). The glycoside is converted by intestinal β-glycosidase to cyanohydrin, which then decomposes to hydrogen cyanide (90). In flaxseed, this process is catalyzed by two distinct enzymes: linustatinase and linamarase β-glucosidase (90). Hydrogen cyanide could potentially cause acute cyanide poisoning, which may place the respiratory and nervous system at risk (3). However, no increase in plasma cyanide levels above baseline have been observed with the consumption of 15-100 g flaxseed (90). The linustatin and neolinustatin found in flaxseed are thought to be the slowest cyanide producers compared to other cyanogenic glycosides. This is because flaxseed glycosides have gentiobiose moiety that needs to be hydrolyzed to glucose. The cyanohydrin formed by metabolism is highly stable (90), which, therefore, resists any spontaneous decomposition to hydrogen cyanide. Theoretically, 1-2 tablespoons of flaxseed will produce approximately 5-10 mg of hydrogen cyanide after ingestion. This is highly unlikely to cause toxicity for three reasons: a) 50-60 mg dose of cyanide is required to cause acute toxicity, b) human body can routinely detoxify up to 100 mg/day of cyanide (3,89), and c) cooking destroys cyanide (cyanide is heat labile) (91). Human studies with 50 g/day flaxseed did not increase urinary thiocyanate levels (3). Based on these data, humans would need to

consume the unrealistic amount of 1 kg of flaxseed daily for cyanide toxicity to ever manifest itself.

Another potentially toxic compound is linatine (antipyridoxin factor) which has been identified as vitamin B6 antagonist in chicks (89). However, flaxseed has never been shown to induce vitamin B6 deficiency in clinical studies (91). Other compounds such as phytic acid and trypsin inhibitor have also been suggested to induce negative effects on the nutritional status after flaxseed ingestion. However, once again, no studies have reported any alterations in zinc status due to phytic acid, or any difference in trypsin inhibitor activity in flaxseed compared with canola or soybean seeds (89,91). In conclusion, it is important to recognize that no definite scientific data have been produced to support the concept of toxicity from dietary flaxseed because of any of these compounds.

Conclusions

Supplementation of the diet with milled flaxseed has many healthy benefits to the body. Although cardiovascular disease and cancer are probably the best researched areas that have shown convincing evidence of a beneficial action for dietary flaxseed, other areas like gastro-intestinal health and diabetes have also been receptive to the beneficial effects of dietary flaxseed. Other areas in human health require further research to make definitive conclusions but the preliminary data is encouraging. With little or no evidence of toxicity for dietary supplementation with flaxseed, there appears to be a clear argument to support its inclusion in the daily diet and little reason to oppose it.

References

1. Brown, L.; Caligiuri, S.P.B.; Brown, D.; Pierce, G.N. Clinical trials using functional foods provide unique challenges. J. Funct. Foods. 2018, 45, 233–238.
2. Caligiuri, S.P.B.; Pierce, G.N. A review of the relative efficacy of dietary, nutritional supplements, lifestyle and drug therapies in the management of hypertension. Crit. Rev. Food Sci. Nutr. 2017, 57, 3508–3527.
3. Parikh, M.; Netticadan, T.; Pierce, G.N. Flaxseed: Its bioactive components and their cardiovascular benefits. Am. J. Physiol. Heart Circ. Physiol. 2018, 314, H146–H159.
4. Ramicharitrar, A.; Badrie, N.; Mattfeldt-Beman, M.; Matsuo, H.; Ridley, C. Consumer acceptability of muffins with flaxseed (Linum usitatissimum). J. Food Sci. 2005, 70, 5504–5507.
5. Rodriguez-Leyva, D.; Weighell, W.; Edel, A.L.; La Vallee, R.; Dibrov, E.; Pinneker, R.; Maddaford, T.G.; Ramjiawan, B.; Aliani, M.; Guzman, R.; et al. Potent anti-hypertensive action of dietary flaxseed in hypertensive patients. Hypertension 2013, 62, 1081–1089.
6. Pohjanheimo, T.A.; Hakala, M.A.; Tahvonen, R.L.; Salminen, S.J.; Kallio, H.P. Flaxseed in breadmaking: Effects on sensory quality, aging, and composition of bakery products. J. Food Sci. 2006, 71, S343–S348.
7. Aliani, M.; Ryland, D.; Pierce, G.N. Effect of flax addition on the flavor profile of muffins and snack bars. Food Res. Int. 2011, 44, 2489–2496.
8. Dodin, S.; Lemay, A.; Jacques, H.; Légaré, F.; Forest, J.C.; Mâsse, B. The effects of flaxseed dietary supplement on lipid profile, bone mineral density, and symptoms in menopausal women; a randomized, double-blind, wheat germ placebo-controlled clinical trial. J. Clin. Endocrinol. Metab. 2005, 90, 1390–1397.

9. Chen, Z.Y.; Ratnayake,W.M.N.; Cunnane, S.C. Oxidative stability of flaxseed lipids during baking. J. Am. Oil Chem. Soc. 1994, 71, 629–632.
10. Aliani, M.; Ryland, D.; Pierce, G.N. Effect of flax addition on the flavor profile and acceptability of bagels. J. Food Sci. 2012, 71, S62–S79.
11. Austria, J.A.; Aliani, M.; Malcolmson, L.J.; Dibrov, E.; Blackwood, E.P.; Maddaford, T.G.; Guzman, R.; Pierce, G.N. Daily food choices over one year when patient diets are supplemented with milled flaxseed. J. Funct. Foods 2016, 26, 772–780.
12. Edel, A.E.; Aliani, M.; Pierce, G.N. Stability of bioactives in flaxseed and flaxseed-fortified foods. Food Res. Int. 2015, 77, 140–155.
13. Austria, J.A.; Richard, M.N.; Chahine, M.N.; Edel, A.L.; Malcolmson, L.J.; Dupasquier, C.M.C.; Pierce, G.N. Bioavailability of alpha linolenic acid in subjects after ingestion of three different forms of flaxseed. J. Am. Coll. Nutr. 2008, 27, 214–221.
14. Dzuvor, C.K.O.; Taylor, J.T.; Acquah, C.; Pan, S.; Agyei, D. Bioprocessing of functional ingredients from flaxseed. Molecules 2018, 23, 2444.
15. Dupasquier, C.M.C.; Weber, A.-M.; Ander, B.P.; Rampersad, P.P.; Steigerwald, S.; Wigle, J.T.; Mitchell, R.W.; Kroeger, E.A.; Gilchrist, J.S.C.; Moghadasian, M.M.; et al. The effects of dietary flaxseed on vascular contractile function and atherosclerosis in rabbits during prolonged hypercholesterolemia. Am. J. Physiol. 2006, 291, H2987–H2996.
16. Dupasquier, C.M.C.; Dibrov, E.; Kneesh, A.L.; Cheung, P.K.M.; Lee, K.G.Y.; Alexander, H.K.; Yeganeh, B.; Moghadasian, M.H.; Pierce, G.N. Dietary flaxseed inhibits atherosclerosis in the LDL receptor deficient mouse in part through anti-proliferative and anti-inflammatory actions. Am. J. Physiol. 2007, 293, H2394–H2402.

17. Bassett, C.M.C.; McCullough, R.S.; Edel, A.L.; Patenaude, A.; La Vallee, R.; Pierce, G.N. The alpha linolenic acid content of flaxseed can prevent the atherogenic effects of dietary trans-fat. Am. J. Physiol. 2011, 301, H2220–H2226.
18. Parikh, M.; Raj, P.; Austria, J.A.; Yu, L.; Garg, B.; Netticadan, T.; Pierce, G.N. Dietary flaxseed protects against ventricular arrhythmias and left ventricular dilation after a myocardial infarction. J. Nutr. Biochem. 2019, 71, 63-71.
19. Francis, A.A.; Austria, J.A.; La Vallee, R.K.; Deniset, J.F.; Maddaford, G.G.; Dibrov, E.; Pierce, G.N. The effects of dietary flaxseed on atherosclerotic plaque regression. Am. J. Physiol. Heart Circ. Physiol. 2013, 304, H1743–H1751.
20. Ander, B.P.;Weber, A.R.; Rampersad, P.; Gilchrist, J.S.C.; Pierce, G.N.; Lukas, A. Dietary flaxseed protects against ventricular fibrillation induced by ischemia- reperfusion in normal and hypercholesterolemic rabbits. J. Nutr. 2004, 134, 3250–3256.
21. Ander, B.P.; Hurtado, C.; Raposo, C.S.; Maddaford, T.G.; Deniset, J.F.; Hryshko, L.V.; Pierce, G.N.; Lukas, A. Di_erential sensitivities of the NCX 1.1 and NCX 1.3 isoforms of the Na+-Ca2+ exchanger to alpha-linolenic acid. Cardiovasc. Res. 2007, 73, 395–403.
22. Prasad, K. Flax lignan complex slows down the progression of atherosclerosis in hyperlipidemic rabbits. J. Cardiovasc. Pharmacol. Ther. 2009, 14, 38–48.
23. Caligiuri, S.P.B.; Rodriguez-Leyva, D.; Aukema, H.; Ravandi, A.; Weighell, W.; Guzman, R.; Pierce, G.N. Dietary flaxseed reduces central aortic blood pressure without cardiac involvement but through changes in plasma oxylipins. Hypertension 2016, 68, 1031–1038.
24. Rodriguez-Leyva, D.; Zahradka, P.; Ramjiawan, B.; Guzman, R.; Aliani, M.; Pierce, G.N. The effect of dietary flaxseed on improving symptoms of cardiovascular disease in patients with

peripheral arterial disease: The rationale and design of the FlaxPAD randomized controlled trial. Contemp. Clin. Trials 2011, 32, 724–730.

25. Caligiuri, S.P.B.; Aukema, H.M.; Ravandi, A.; Guzman, R.; Dibrov, E.; Pierce, G.N. Flaxseed consumption reduces blood pressure in hypertensive patients by altering circulating oxylipins via an alpha linolenic acid-induced inhibition of soluble epoxide hydrolase. Hypertension 2014, 64, 53–59.
26. Ogawa, A.; Suzuki, Y.; Aoyama, T.; Takeuchi, H. Dietary _-linolenic acid inhibits angiotensin-converting enzyme activity and mRNA expression levels in the aorta of spontaneously hypertensive rats. J. Oleo Sci. 2009, 58, 355–360.
27. Udenigwe, C.C.; Adebiyi, A.P.; Doyen, A.; Li, H.; Bazinet, L.; Aluko, R.E. Low molecular weight flaxseed protein-derived arginine-containing peptides reduced blood pressure of spontaneously hypertensive rats faster than amino acid form of arginine and native flaxseed protein. Food Chem. 2012, 132, 468–475.
28. Sawant, S.H.; Bodhankar, S.L. Flax lignan concentrate reverses alterations in blood pressure, left ventricular functions, lipid profile and antioxidant status in DOCA-salt induced renal hypertension in rats. Renal Fail. 2016, 38, 411–423.
29. Park, J.B.; Velasquez, M.T. Potential effects of lignan-enriched flaxseed powder on bodyweight, visceral fat, lipid profile, and blood pressure in rats. Fitoterapia. 2012, 83, 941–946.
30. Soltanian, N.; Janghorbani, M. A randomized trial of the effects of flaxseed to manage constipation, weight, glycemia, and lipids in constipated patients with type 2 diabetes. Nutr. Metab. 2018, 15, 36.

31. Mani, U.V.; Mani, I.; Biswas, M.; Kumar, S.N. An open-label study on the effect of flax seed powder (Linum usitatissimum) supplementation in the management of diabetes mellitus. J. Diet. Suppl. 2011, 8, 257–265.
32. Hutchins, A.M.; Brown, B.D.; Cunnane, S.C.; Domitrovich, S.G.; Adams, E.R.; Bobowiec, C.E. Daily flaxseed consumption improves glycemic control in obese men and women with pre-diabetes: A randomized study. Nutr. Res. 2013, 33, 367–375.
33. Thakur, G.; Mitra, A.; Pal, K.; Rousseau, D. E_ect of flaxseed gum on reduction of blood glucose and cholesterol in type 2 diabetic patients. Int. J. Food Sci. Nutr. 2009, 60 (Suppl. 6), 126–136.
34. Pan, A.; Sun, J.; Chen, Y.; Ye, X.; Li, H.; Yu, Z.; Wang, Y.; Gu, W.; Zhang, X.; Chen, X.; et al. Effects of a flaxseed-derived lignan supplement in type 2 diabetic patients: A randomized, double-blind, cross-over trial. PLoS ONE 2007, 2, e1148.
35. Prasad, K.; Mantha, S.V.; Muir, A.D.;Westcott, N.D. Protective e_ect of secoisolariciresinol diglucoside against streptozotocin-induced diabetes and its mechanism. Mol. Cell. Biochem. 2000, 206, 141–149.
36. Mason, J.K.; Thompson, L.U. Flaxseed and its lignan and oil components: Can they play a role in reducing the risk of and improving the treatment of breast cancer? Appl. Physiol. Nutr. Metab. 2014, 39, 663–678.
37. Calado, A.; Neves, P.M.; Santos, T.; Ravasco, P. The e_ect of flaxseed in breast cancer: A Literature Review. Front. Nutr. 2018, 5, 4.
38. Flower, G.; Fritz, H.; Balneaves, L.G.; Verma, S.; Skidmore, B.; Fernandes, R.; Kennedy, D.; Cooley, K.; Wong, R.; Sagar, S.; et al. Flax and Breast Cancer: A systematic review. Integr. Cancer Ther. 2014, 13, 181–192.

39. Gaya, P.; Medina, M.; Sánchez-Jiménez, A.; Landete, J.M. Phytoestrogen metabolism by adult human gut microbiota. Molecules 2016, 21, 1034.

40. Kuijsten, A.; Arts, I.C.W.; Vree, T.B.; Hollman, P.C.H. Pharmacokinetics of enterolignans in healthy men and women consuming a single dose of secoisolariciresinol diglucoside. J. Nutr. 2005, 135, 795–801.

41. Mali, A.V.; Padhye, S.B.; Anant, S.; Hegde, M.V.; Kadam, S.S. Anticancer and antimetastatic potential of enterolactone: Clinical, preclinical and mechanistic perspectives. Eur. J. Pharmacol. 2019, 852, 107–124.

42. Lin, X.; Gingrich, J.R.; Bao,W.; Li, J.; Haroon, Z.A.; Demark-Wahnefried,W. E_ect of flaxseed supplementation on prostatic carcinoma in transgenic mice. Urology 2002, 60, 919–924.

43. Chikara, S.; Mamidi, S.; Sreedasyam, A.; Chittem, K.; Pietrofesa, R.; Zuppa, A.; Moorthy, G.; Dyer, N.; Christofidou-Solomidou, M.; Reindl, K.M. Flaxseed consumption inhibits chemically induced lung tumorigenesis and modulates expression of Phase II enzymes and inflammatory cytokines in A/J mice. Cancer Prev. Res. 2018, 11, 27–37.

44. Christofidou-Solomidou, M.; Pietrofesa, R.; Arguiri, E.; McAlexander, M.A.;Witwer, K.W. Dietary flaxseed modulates the miRNA profile in irradiated and non-irradiated murine lungs: a novel mechanism of tissue radioprotection by flaxseed. Cancer Biol. Ther. 2014, 15, 930–937.

45. Shah, N.R.; Patel, B.M. Secoisolariciresinol diglucoside rich extract of L. usitatissimum prevents diabetic colon cancer through inhibition of CDK4. Biomed. Pharmacother. 2016, 83, 733–739.

46. Chen, J.; Saggar, J.K.; Corey, P.; Thompson, L.U. Flaxseed and pure secoisolariciresinol diglucoside, but not flaxseed hull, reduce human breast tumor growth (MCF-7) in athymic mice. J. Nutr. 2009, 139, 2061–2066.
47. Edel, A.L.; Patenaude, A.F.; Richard, M.N.; Dibrov, E.; Austria, J.A.; Aukema, H.M.; Pierce, G.N.; Aliani, M. The effect of flaxseed dose on circulating concentrations of alpha-linolenic acid and secoisolariciresinol diglucoside derived enterolignans in young healthy adults. Eur. J. Nutr. 2016, 55, 651–663.
48. Mason, J.K.; Chen, J.; Thompson, L.U. Flaxseed oil-trastuzumab interaction in breast cancer. Food Chem. Toxicol. 2010, 48, 2223–2226.
49. Lauritzen, L.; Brambilla, P.; Mazzocchi, A.; Harsløf, L.B.; Ciappolino, V.; Agostoni, C. DHA effects in brain development and function. Nutrients 2016, 8, 6.
50. Kidd, P.M. Omega-3 DHA and EPA for cognition, behavior, and mood: clinical findings and structural-functional synergies with cell membrane phospholipids. Altern. Med. Rev. 2007, 12, 207–227.
51. Salem, N., Jr.; Moriguchi, T.; Greiner, R.S.; McBride, K.; Ahmad, A.; Catalan, J.N.; Slotnick, B. Alterations in brain function after loss of docosahexaenoate due to dietary restriction of n-fatty acids. J. Mol. Neurosci. 2001, 16, 299–307.
52. Lenzi Almeida, K.C.; Teles Boaventura, G.; Guzman Silva, M.A. Influence of omega-3 fatty acids from the flaxseed (Linum usitatissimum) on the brain development of newborn rats. Nutr. Hosp. 2011, 26, 991–996.
53. Pessanha, C.R.; da Camara Boueri, B.F.; da Costa, L.R.; Ferreira, M.R.; Melo, H.S.; de Abreu, M.D.; Pessoa, L.R.; da Silva, P.C.; Pereira, A.D.; Ribeiro, D.C.; et al. Brain development in

male rats subjected to early weaning and treated with diet containing flour or flaxseed oil after 21 days until 60 days. J. Dev. Orig. Health Dis. 2015, 6, 268–271.

54. Fernandes, F.S.; de Souza, A.S.; do Carmo, M.; Boaventura, G.T. Maternal intake of flaxseed-based diet (Linum usitatissimum) on hippocampus fatty acid profile: implications for growth, locomotor activity and spatial memory. Nutrition 2011, 27, 1040–1047.
55. Mucci Dde, B.; Fernandes, F.S.; Souza Ados, S.; Sardinha, F.L.; Soares-Mota, M.; Tavares do Carmo, M. Flaxseed mitigates brain mass loss, improving motor hyperactivity and spatial memory, in a rodent model of neonatal hypoxic-ischemic encephalopathy. Prostaglandins Leukot. Essent. Fatty Acids 2015, 97, 13–19.
56. Naveen, S.; Siddalingaswamy, M.; Singsit, D.; Khanum, F. Anti-depressive effect of polyphenols and omega-3 fatty acid from pomegranate peel and flax seed in mice exposed to chronic mild stress. Psychiatry Clin. Neurosci. 2013, 67, 501–508.
57. Ma, X.; Wang, R.; Zhao, X.; Zhang, C.; Sun, J.; Li, J.; Zhang, L.; Shao, T.; Ruan, L.; Chen, L.; et al. Antidepressant-like effect of flaxseed secoisolariciresinol diglycoside in ovariectomized mice subjected to unpredictable chronic stress. Metab. Brain Dis. 2013, 28, 77–84.
58. Li, X.B.; Yang, Z.X.; Yang, L.; Chen, X.L.; Zhang, K.; Yang, Q.; Wu, Y.M.; Liu, S.B.; Tao, K.S.; Zhao, M.G. Neuroprotective effects of flax lignan against NMDA-induced neurotoxicity in vitro. CNS Neurosci. Ther. 2012, 18, 927–933.
59. Landete, J.M.; Arqués, J.; Medina, M.; Gaya, P.; de Las Rivas, B.; Munoz, R. Bioactivation of phytoestrogens: Intestinal bacteria and health. Crit. Rev. Food Sci. Nutr. 2016, 56, 1826–1843.

60. Cetisli, N.E.; Saruhan, A.; Kivcak, B. The e_ects of flaxseed on menopausal symptoms and quality of life. Holist. Nurs. Pract. 2015, 29, 151–157.
61. Colli, M.C.; Bracht, A.; Soares, A.A.; de Oliveira, A.L.; Bôer, C.G.; de Souza, C.G.; Peralta, R.M. Evaluation of the efficacy of flaxseed meal and flaxseed extract in reducing menopausal symptoms. J. Med. Food. 2012, 15, 840–845.
62. Lewis, J.E.; Nickell, L.A.; Thompson, L.U.; Szalai, J.P.; Kiss, A.; Hilditch, J.R. A randomized controlled trial of the effect of dietary soy and flaxseed muffins on quality of life and hot flashes during menopause. Menopause 2006, 13, 631–642.
63. Dew, T.P.; Williamson, G. Controlled flax interventions for the improvement of menopausal symptoms and postmenopausal bone health: A systematic review. Menopause 2013, 20, 1207–1215.
64. Lethaby, A.; Marjoribanks, J.; Kronenberg, F.; Roberts, H.; Eden, J.; Brown, J. Phytoestrogens for menopausal vasomotor symptoms. Cochrane Database Syst. Rev. 2013, 12, CD001395.
65. Pruthi, S.; Qin, R.; Terstreip, S.A.; Liu, H.; Loprinzi, C.L.; Shah, T.R.; Tucker, K.F.; Dakhil, S.R.; Bury, M.J.; Carolla, R.L.; et al. A phase III, randomized, placebo-controlled, double-blind trial of flaxseed for the treatment of hot flashes: North Central Cancer Treatment Group N08C7. Menopause 2012, 1, 48–53.
66. Guarda, D.S.; Lisboa, P.C.; de Oliveira, E.; Nogueira-Neto, J.F.; de Moura, E.G.; Figueiredo, M.S. Flaxseed oil during lactation changes milk and body composition in male and female suckling pups rats. Food Chem. Toxicol. 2014, 69, 69–75.

67. Troina, A.A.; Figueiredo, M.S.; Passos, M.C.F.; Reis, A.M.; Oliveira, E.; Lisboa, P.C.; Moura, E.G. Flaxseed bioactive compounds change milk, hormonal and biochemical parameters of dams and offspring during lactation. Food Chem. Toxicol. 2012, 50, 2388–2396.
68. Arjmandi, B.; Juma, S.; Lucas, E.; Wei, L.; Venkatesh, S.; Khan, D. Flaxseed supplementation positively influences bone metabolism in postmenopausal women. JANA 1998, 1, 27–32.
69. Kim, Y.; Ilich, J.Z. Implications of dietary _-linolenic acid in bone health. Nutrition 2011, 27, 1101–1107.
70. Neukam, K.; De Spirt, S.; Stahl, W.; Bejot, M.; Maurette, J.M.; Tronnier, H.; Heinrich, U. Supplementation of flaxseed oil diminishes skin sensitivity and improves skin barrier function and condition. Skin Pharmacol. Physiol. 2011, 24, 67–74.
71. Caligiuri, S.P.B.; Aukema, H.M.; Ravandi, A.; Pierce, G.N. Elevated levels of pro-inflammatory oxylipins in older subjects are normalized by flaxseed consumption. Exp. Gerontol. 2014, 59, 51–57.
72. Ettinger, G.; MacDonald, K.; Reid, G.; Burton, J.P. The influence of the human microbiome and probiotics on cardiovascular health. Gut Microbes 2014, 5, 719–728.
73. Thompson, L.U.; Robb, P.; Serraino, M.; Cheung, F. Mammalian lignan production from various foods. Nutr. Cancer 1991, 16, 43–52.
74. Wang, C.Z.; Ma, X.Q.; Yang, D.H.; Guo, Z.R.; Liu, G.R.; Zhao, G.X.; Tang, J.; Zhang, Y.N.; Ma, M.; Cai, S.Q.; et al. Production of enterodiol from defatted flaxseeds through biotransformation by human intestinal bacteria. BMC Microbiol. 2010, 10, 115.
75. Lagkouvardos, I.; Kläring, K.; Heinzmann, S.S.; Platz, S.; Scholz, B.; Engel, K.H.; Schmitt-Kopplin, P.; Haller, D.; Rohn, S.; Skurk, T. Gut metabolites and bacterial community

networks during a pilot intervention study with flaxseeds in healthy adult men. Mol. Nutr. Food Res. 2015, 59, 1614–1628.

76. Munoz, O.; Fuentealba, C.; Ampuero, D.; Figuerola, F.; Estévez, A.M. The e_ect of Lactobacillus acidophilus and Lactobacillus casei on the in vitro bioaccessibility of flaxseed lignans (Linum usitatissimum L.). Food Funct. 2018, 9, 2426–2432.
77. Pulkrabek, M.; Rhee, T.; Gibbs, P.; Hall, C. Flaxseed- and Buckwheat-supplemented diets altered enterobacteriaceae diversity and prevalence in the cecum and feces of obese mice. J. Diet. Suppl. 2017, 14, 667–678.
78. Power, K.A.; Lepp, D.; Zarepoor, L.; Monk, J.M.; Wu, W.; Tsao, R.; Liu, R. Dietary flaxseed modulates the colonic microenvironment in healthy C57BI/6 male mice which may alter susceptibility to gut-associated diseases. J. Nutr. Biochem. 2016, 28, 61–69.
79. Patterson, E.; O'Doherty, R.M.; Murphy, E.F.; Wall, R.; O'Sullivan, O.; Nilaweera, K.; Fitzgerald, G.F.; Cotter, P.D.; Ross, R.P.; Stanton, C. Impact of dietary fatty acids on metabolic activity and host intestinal microbiota composition on C57BL/6J mice. Br. J. Nutr. 2014, 111, 1905–1917.
80. Zhang, X.; Wang, H.; Yin, P.; Fan, H.; Sun, L.; Liu, Y. Flaxseed oil ameliorates alcoholic liver disease via anti-inflammation and modulating gut microbiota in mice. Lipids Health Dis. 2017, 16, 44.
81. Gomides, A.F.; Concalves, R.V.; de Paula, S.O.; Ferreira, C.L.; Comastri, D.S.; Peluzio Mdo, C. Defatted flaxseed meal prevents the appearance of aberrant crypt foci in the colon of mice increasing the gene expression of p53. Nutr. Hosp. 2015, 31, 1675–1681.
82. Brahe, L.K.; Le Chatelier, E.; Prifti, E.; Pons, N.; Kennedy, S.; Blaedel, T.; Håkansson, J.; Dalsgaard, T.K.; Hansen, T.; Pedersen, O.; et al. Dietary modulation of the gut microbiota—

A randomized controlled trial in obese postmenopausal women. Br. J. Nutr. 2015, 114, 406–417.

83. van Kranen, H.J.; Mortensen, A.; Sørensen, I.K.; van den Berg-Wijnands, J.; Beems, R.; Nurmi, T.; Adlercreutz, H.; van Kreijl, C.F. Lignan precursors from flaxseed or rye bran do not protect against the development of intestinal neoplasia in ApcMin mice. Nutr.Cancer 2003, 45, 203–210.
84. Määttänen, P.; Lurz, E.; Botts, S.R.; Wu, R.Y.; Yeung, C.W.; Li, B.; Abi_, S.; Johnson-Henry, K.C.; Lepp, D.; Power, K.A.; et al. Ground flaxseed reverses protection of a reduced-fat diet against Citrobacter rodentium-induced colitis. Am. J. Physiol. Gastrointest. Liver Physiol. 2018, 315, G788–G798.
85. Soltanian, N.; Janghorbani, M. E_ect of flaxseed or psyllium vs. placebo on management of constipation, weight, glycemia, and lipids: A randomized trial in constipated patients with type 2 diabetes. Clin. Nutr. ESPEN 2019, 29, 41–48.
86. Cockerell, K.M.;Watkins, A.S.; Reeves, L.B.; Goddard, L.; Lomer, M.C. Effects of linseeds on the symptoms of irritable bowel syndrome: A pilot randomized controlled trial. J. Hum. Nutr. Diet. 2012, 5, 435–443.
87. Hanif, P.A.; Gilani, A.H. Dual effectiveness of flaxseed in constipation and diarrhea: possible mechanism. J. Ethnopharmacol. 2015, 169, 60–68.
88. Bolarinwa, I.F.; Orfila, C.; Morgan, M.R. Determination of amygdalin in apple seeds, fresh apples and processed apple juices. Food Chem. 2015, 170, 437–442.
89. Touré, A.; Xueming, X. Flaxseed lignans: Source, biosynthesis, metabolism, antioxidant activity, bio-active components, and health benefits. Comp. Rev. Food Sci. Food Saf. 2010, 9, 261–269.

90. Cressey, P.; Reeve, J. Metabolism of cyanogenic glycosides: A review. Food Chem. Toxicol. 2019, 125, 225–232.

91. Kajla, P.; Sharma, A.; Sood, D.R. Flaxseed-a potential functional food source. J. Food Sci. Technol. 2015, 52, 1857–1871.

CHAPTER 2: METHODS AND RESULTS

2.1. Dietary flaxseed and acute myocardial infarction

An anti-atherosclerotic, a cholesterol-lowering and an antihypertensive effect of flaxseed has been observed previously. However, it's *in vivo* effects on cardiac structure, function and electrical activity needs to be elucidated. Due to the positive effects of dietary flaxseed on cardiovascular disease and its risk factors, we investigated the effects of flaxseed and its bioactive components ALA and SDG in an experimental model of MI.

It was hypothesized that in an MI induced by coronary artery ligation in rats, pretreatment with dietary flaxseed and its components will reduce the incidence of cardiac arrhythmias, and treatment after the surgery will a) prevent extensive fibrosis after infarction, and, b) improve cardiac structure and function post-MI.

Dietary Flaxseed Protects Against Ventricular Arrhythmias And Left Ventricular Dilation After A Myocardial Infarction

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Running Title: Effects of dietary flaxseed on myocardial infarction

ABSTRACT

Dietary omega-3 polyunsaturated fatty acids (n-3 PUFAs) have been associated with a lower incidence of cardiovascular events and sudden cardiac death. Flaxseed is one of the richest plant sources of n-3 PUFAs and can retard the progression and accelerate the regression of atherosclerotic plaques. The aim of the study was to examine the preventive effects of dietary flaxseed on arrhythmias and curative effects on heart dysfunction that develops after a myocardial infarction (MI). The left anterior descending coronary artery was ligated in rats to induce the MI. Rats were randomized into five groups: sham MI with normal chow, MI with normal chow, MI with 10% milled flaxseed supplementation (flax), MI with 4.4% supplemented flax oil enriched in

alpha-linolenic acid (ALA), and MI with flax lignan secoisolariciresinol diglucoside (SDG) supplementation (0.44%). Animals were fed with their respective diets for two weeks before and for 8 weeks after the surgery. Echocardiography and continuous ECG recordings were obtained after ligation to confirm the induction of the MI, to check for arrhythmias and to assess cardiac function. Histological examination was also performed to evaluate cardiac fibrosis. Pretreatment with dietary flax, ALA, and SDG significantly reduced the incidence of arrhythmias. Supplementation of flax and component diets after the MI resulted in significantly small infarct size, less left ventricle dilation, decreased myocardial fibrosis and tumor necrosis factor-α levels compared to the MI group. Together, this study generates evidence for a beneficial effect of flaxseed in patients post-MI for the prevention of arrhythmias and ventricular remodeling.

Key words: Flaxseed, alpha linolenic acid, arrhythmias, echocardiography, myocardial infarction, diet

INTRODUCTION

Cardiovascular diseases (CVD) are the leading cause of death [1], with 43.8% mortality attributed to coronary artery disease (CAD) alone. The latest updates estimate that by 2035, more than 130 million adults will have CVD in the US with a concomitant rise in health expenditure to $1.1 trillion [2]. Omega-3 polyunsaturated fatty acids (n-3 PUFAs) were recently recommended by the American Heart Association (AHA) for the secondary prevention of CAD and sudden cardiac death (SCD), and also for heart failure patients with reduced ejection fraction [3]. n-3 PUFAs are essential fatty acids that have a role in cellular functions including membrane structure and function, cell signaling and gene expression [4]. Several studies have shown an inverse association between CVD risk with fish consumption [5]. Recently, a large clinical trial, REDUCE-IT, demonstrated a 25% lower cardiovascular risk including myocardial infarction (MI)

and stroke in patients that received n-3 PUFA [6]. Fish are an important source of n-3 PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [4]. However, despite the recommendation by AHA to have fatty fish at least 1-2 servings weekly [3], fish consumption is lower because of physical and physiological concerns such as smell, taste, and potential toxins [7]. In addition to this, vegetarians and vegans cannot have this source at all. A solution to this is the availability of an alternate source of n-3 PUFAs obtained from plants. Flaxseed, *Linum usitatissium*, has one of the highest amounts of an essential n-3 PUFA obtained from plants, alpha linolenic acid (ALA) [7]. Other than ALA, flax is also rich in the lignan phytoestrogen secoisolariciresinol diglucoside (SDG) and dietary fiber [7].

Flaxseed has been found to have beneficial cardiovascular effects. The FLAX-PAD trial, a one year, randomized controlled trial, demonstrated a significant reduction of 10 mm Hg systolic blood pressure and 7 mm Hg diastolic blood pressure in peripheral artery disease patients with elevated blood pressure [8]. In LDL receptor knockout mice, flaxseed suppressed atherosclerotic plaque formation [9, 10] and was found to be effective in regression of established plaques [11]. Flaxseed supplementation has also reduced the incidence of ventricular arrhythmias in hearts challenged by ischemia-reperfusion (I/R) *ex-vivo* [12]. In addition to its effect on cardiac electrical conduction, the ALA found within flaxseed reduces apoptosis in cardiomyocytes during I/R insult [13]. Flaxseed also regulates glucose levels, thereby exerting a hypoglycemic effect in diabetes [14].

Although much has been studied about the vascular actions of flaxseed, it's *in vivo* effects on cardiac structure, function and electrical activity needs to be elucidated. In light of the positive effects of dietary flaxseed on CVD and risk factors, we investigated the effects of flaxseed and its bioactive components ALA and SDG in an experimental model of MI. We hypothesized that in an

animal model of MI induced by coronary artery ligation (CAL), pretreatment with dietary flaxseed and its components will reduce the incidence of cardiac arrhythmias, and treatment after the surgery will a) prevent extensive fibrosis after infarction, and, b) improve cardiac structure and function post-MI.

MATERIALS AND METHODS

Nutritional composition of the experimental diets

The core proximate and chemical composition of the diet sample for crude protein, carbohydrate, fat, fibre, ash, mineral, and moisture was carried out by the Central Testing Laboratory (Winnipeg, MB, Canada).

Animal care

This study protocol (17-005) was approved by the University of Manitoba Office of Research Ethics & Compliance and Animal Care Committee and was done in accordance with the guidelines by the Canadian Council for Animal Care. Male Sprague Dawley rats (100-140 g; Charles River Laboratories, Quebec, Canada) were housed in a temperature and humidity-controlled room with a 12 h light/dark cycle. Animals were randomized into five groups: sham MI fed normal chow, MI fed normal chow, MI fed chow supplemented with 10% milled flaxseed (flax), MI fed chow supplemented with 4.4% flax oil enriched in alpha-linolenic acid (ALA), and MI fed chow supplemented with the flax lignan secoisolariciresinol diglucoside (SDG) (0.44%). These dietary interventions and the concentrations used are similar to those used successfully in previous studies [10].

The four diets included a Prolab® RMH 3000 rodent regular chow (TestDiet, Richmond, IN), a 10% ground flaxseed (BakePur Milled Golden Flaxseed, Pizzey Ingredients, Russell, Manitoba, Canada) supplemented chow diet, or a 4.4.% flax oil (Cold Pressed Flax Oil, Royal Harvest, Shape Foods, Brandon, Manitoba, Canada) supplemented chow diet, or a 0.44% SDG (Archer Daniels Midland, Chicago, IL, USA) supplemented diet.

Animals were anesthetized with 1–5% isoflurane and oxygen at a flow rate of 2 L min^{-1} and kept in a surgical plane of anesthesia with 2% isoflurane during surgery. A left thoracotomy was carried out, and the heart was gently exposed from the pericardial sac through the incision. The left anterior descending coronary artery (LAD) was located and occluded with 6-0 polypropylene silk suture at about 2 mm from aortic root. The suture was tied and the ligation was estimated to be successful when the anterior wall of the left ventricle turned pale. The heart was repositioned, then the chest compressed to remove any air from the cavity and the incision was closed using a purse string suture. Sham-operated animals that served as controls were subjected to similar surgical procedures except that the LAD artery was not ligated. Buprenorphine 0.05 mg kg^{-1} was administered pre- and post-surgery (2 times a day for 2 days) subcutaneously as an analgesic agent to all animals.

Animals were fed with their respective diets for two weeks before and for 8 weeks after surgery. Thus, animals were sacrificed after a total of 10 weeks on the study protocol.

Ventricular arrythmias assessment

An electrocardiogram was recorded during the surgical period for: a) 2 min before and b) 10 min after the coronary artery ligation in stabilized animals under isoflurane anesthesia. ECG tracings were obtained and analyzed using a BIOPAC data acquisition system. The Lambeth

convention [15] was used to classify and interpret ventricular arrhythmias, as follows: Ventricular premature beat (VPB): defined as a ventricular electrical complex that occurs prematurely and is different in shape (voltage and/or duration) from the preceding ventricular complex; Bigeminy: defined as repeated sequence of VPB, normal sinus beat, and VPB; Salvos: defined as series of 2-3 consecutive VPBs; Ventricular tachycardia (VT): defined as a sequence of a minimum of 4 consecutive VPBs; Ventricular fibrillation (VF): defined as a minimum of 4 consecutive VPBs that varies from beat to beat in shape and rate. The total arrhythmia incidence as well as the types of arrhythmias was defined as the percentage of animals affected relative to all of the animals in a group.

Transthoracic echocardiography

All experimental rats were weighed and anesthetized with 3% isoflurane in a chamber, and then kept under 1.5–2% isoflurane throughout the procedure. An echocardiogram was obtained at 4 and 8 weeks post-surgery by 2D guided M-mode and Doppler modalities with a 13 MHz probe (Vivid E9; GE Medical Systems, Milwaukee, WI, USA) as described earlier [16]. 2D M-mode parasternal short-axis view images were obtained to determine systolic functional parameters such as the percentage of left ventricle (LV) ejection fraction (EF), fractional shortening (FS), end-systolic volume (ESV) and end-diastolic volume (EDV). Doppler measurements included isovolumic relaxation time (IVRT), mitral valve (MV) E wave, A wave, and E wave deceleration time. The cardiac structural parameters such as interventricular septal thickness (IVS), LV posterior wall thickness (LVPW) and LV internal diameter (LVID) at diastole and systole were determined from parasternal short-axis view images. All echocardiographic images were analyzed to calculate the listed parameters using EchoPAC software (GE Medical Systems, Milwaukee,

WI). All measurements were performed blinded and averaged from three cardiac cycles to account for inter-beat variability.

Biological sample collection and analysis

All animals were anesthetized with ketamine/xylazine (9.0 mg per 100 g and 0.9 mg per 100 g IM). The depth of anesthesia was assessed by a pedal withdrawal reflex. The blood sample was collected from the inferior vena cava by opening the thoracic cavity and the heart was immediately excised. Whole heart was rinsed in PBS, LV's, septum, and fibrotic scar tissues were separated, weighed and flash frozen in liquid nitrogen.

Percentage of infarct (scarred/fibrotic) LV tissue was calculated by dividing the weight of scarred LV tissue by whole weight of LV tissue as described previously [17]. Evidence of overt heart failure was assessed by determining the presence of ascites and by calculating the lung and liver wet-to-dry weight ratio in all rats.

To determine MI-associated inflammation, the levels of tumor necrosis factor-α (TNF α) were assessed in heart tissue using commercial Elisa kits purchased from Abcam (ab100785).

Total fatty acids were extracted from the plasma samples and derivatized as previously described [18]. The fatty acid methyl esters were separated on a DB225MS column (30 m X 0.25 mm diameter and 0.25 μm film thickness; Agilent Technologies Canada Inc., Mississauga, Ontario) using a Varian 450 GC with FID. The temperature program was 70°C for 2 minutes, the temperature was raised to 180°C at 30°C/minute, held for 1 minute, raised to 200°C at 10°C/minute, held for 2 minutes, then raised to 220°C at 2°C/minute and held for 10 minutes before finally raising to240°C at 20°C/minute and holding for 15 minutes. Total run time was

46.67 minutes, and samples were run with a 20:1 split ratio and a 1.3ml/min column flow. Hydrogen was used as the carrier gas. Components were identified by comparison with authentic standards (Nu-Chek Prep, Elysian, MN)

Collagen volume fraction

To examine the degree of myocardial fibrosis, heart tissue slices were stained with Masson's trichrome. Each heart tissue was cut into three thick slices from apex to base along the long axis using a tissue slicer (Rodent heart slicer matrix HSRS002:1, Zivic Instruments). After OCT embedding, 7 µm thin sections were obtained using a cryostat (Microm HM 550) and then sections were stained with Trichrome stains (Masson HT15) from Sigma Aldrich with the following modification in the protocol. Briefly, tissue slides were fixed in preheated Bouin's solution at 56º for 1 hour. After this, samples on slides were washed and stained with Weigert's Iron Hematoxylin solution (10 minutes), rinsed in deionized water, stained with Biebrich Scarlet-Acid Fucshin (10 minutes), rinsed again and placed in phosphotungstic/phosphomolybdic acid solution (10 minutes), stained with Aniline Blue solution (10 minutes), placed in 1% acetic acid (3 minutes), rinsed and dehydrated through 70%, and 90% ethanol, cleared in xylene and mounted. Images were recorded using a Cytation 5 BioTek Instruments, Vermont, USA. The collagen volume fraction in the infarcted and non-infarcted areas of LV was calculated by using NIS-Elements AR Analysis 4.60, Nikon Instruments. Myocardial fibrosis was expressed as a percentage of fibrotic area to left ventricle area (% of LV).

Statistical analysis

The incidence of ventricular arrhythmias was treated as categorical data, presented as percentages (%), and a chi-squared test followed by Fisher's exact test for *p* values was used for

comparison between treatment groups. For normally distributed data, a one-way ANOVA with a Dunnett post hoc test for multiple comparisons between groups was performed. Data distribution was assessed by Kolmogorov-Smirnov test, D'Agostino-Pearson omnibus normality test, and Shapiro-Wik normality test. All data are expressed as mean ± S.E.M and analyzed using GraphPad Prism v 7. Statistical significance was accepted when $p<0.05$.

RESULTS

Experimental diets

The nutritional composition of the four diets used in this study is listed in Table 3. The ash, protein, fiber, and carbohydrate content of all the diets were controlled and similar among the groups. Although the SDG supplemented diet had a lower fat content, the digestible energy levels provided by the diets were all in a similar range (within 3.6 and 3.9 kcal/g).

Table 3. Nutritional composition of the experimental diets

Diets	Ash, %	Protein, %	Fibre, %	Fat, %	CHO, %	Protein, kcal/100g	Fat, kcal/100g	CHO, kcal/100g	Energy, kcal/g
Control	6.7	25.07	4.07	7.47	55.79	100.28	67.23	223.16	3.9
10% ground flaxseed	6.67	26.58	4.17	6.3	55.37	106.32	56.7	221.48	3.8
4.4% flax oil	6.67	25.38	3.7	7.43	55.92	101.52	66.87	223.68	3.9

0.44% SDG	7.1	26.75	4	2.93	58.32	107	26.37	233.28	3.6

Ash, protein, fiber, carbohydrates, and fat (g/100 g diet) are represented as a percentage of total nutrients, measured by proximate analysis (as fed). Caloric content of fat, protein, and CHO constituents in the diet were calculated as 9, 4, and 4 kcal/g. Fiber content of the CHO component was subtracted to yield the total caloric content of CHO. Energy was calculated as the sum of energies of protein, carbohydrate, and fat. CHO, carbohydrates; SDG, the lignan secoisolariciresinol diglucoside; protein, crude protein; fiber, crude fiber; fat, crude fat.

Body and organ weights as a function of diet and MI

At the start of the dietary regimen, all of the animals had similar body weights. As expected, over the course of 10 weeks, there was a significant within-group (p <0.0001) rise in body weight amongst all of the groups. No significant differences were observed in the body weight of MI groups when compared to the sham control. However, body weight in the SDG group at the end of the protocol was significantly lower than the body weights in the flax and flax oil group but not with respect to MI group (respectively p<0.05 and p<0.001) (Table 4). Morphological examinations were performed on heart, liver and lung weight as well as heart weight to tibia length ratio. No statistically significant alterations were observed as a function of dietary intervention in any of these parameters, as shown in Table 4.

Table 4. Body and organ characteristics of animals at 8 weeks post-MI

Parameters	Sham	MI	Flax	Oil	SDG
Body weight (g)	411±10	415±6	421±7	429±5	388±7*#
Liver weight (g)	10.90±0.36	11.13±0.18	11.31±0.25	12.27±0.22	11.59±0.20
Liver wet/dry weight	3.15±0.04	3.10±0.05	3.09±0.05	3.08±0.04	3.09±0.04
Lung weight (g)	1.328±0.02	1.317±0.03	1.271±0.02	1.327±0.02	1.302±0.04
Lung wet/dry weight	5.20±0.09	5.20±0.10	5.08±0.07	5.14±0.06	5.12±0.08
Heart weight (g)	1.06±0.02	1.10±0.02	1.08±0.01	1.09±0.01	1.05±0.02
Heart weight/Tibia length (g/cm)	0.25±0.01	0.26±0.01	0.26±0.01	0.26±0.00	0.25±0.01
LV weight (g)	0.56±0.02	0.52±0.02	0.54±0.01	0.55±0.01	0.53±0.02
RV weight (g)	0.19±0.01	0.18±0.01	0.18±0.00	0.18±0.01	0.19±0.01
SP weight (g)	0.23±0.01	0.22±0.01	0.24±0.01	0.23±0.01	0.23±0.01

Values represented are mean ± SEM. The body weight values are final values at 10 weeks before termination. LV, left ventricle; RV, right ventricle; SP, septum. *significantly different from Flax, $p<0.05$; #significantly different from Oil, $p<0.01$.

Dietary changes in the plasma levels of fatty acids

A comprehensive list of plasma fatty acids is compiled in the Table 5. Notable difference were observed in the levels of palmitic acid (C16:0), stearic acid (C18:0), arachidonic acid (C20:0),

lignoceric acid (C24:0), eicosenoic acid (C20:1), linoleic acid (LA; C18:2 n-6), gamma-linoleic acid (GLA; C18:3 n-6), eicosadienoic acid (C20:2 n-6), dihomo-gamma-linoleic acid (DGLA; C20:3 n-6), arachidonic acid (AA; C20:4 n-6), adrenic acid (C22:4 n-6), and docosapentaenoic acid (DPA; C22:5 n-3). The total amount of n-6 PUFAs was significantly (p<0.0001) decreased with diets supplemented with flaxseed and its components. As shown in Figure 20, the supplementation with ground flaxseed, and flax oil produced a significant (p<0.0001) increase of 17-fold and 24-fold respectively in the plasma levels of alpha linolenic acid (ALA; C18:3 n-3). A relatively modest but significant (p<0.0001) increase was also observed in the levels of another long chain omega-3 fatty acid - eicosapentaenoic acid (EPA; C20:5 n-3), with a 7-fold increase with ground flaxseed, 8-fold with flax oil, and 2-fold with SDG supplementation. However, no impact of dietary intervention was observed on the levels of docosahexaenoic acid (DHA; C22:6 n-3). The total amount of n-3 PUFAs was significantly (p<0.0001) increased with diets supplemented with flaxseed and its components. Consequently, the ratios of n-6 to n-3 were significantly lowered in the flaxseed and its components supplemented groups (~ 6-fold, p<0.0001) compared to the control groups, as shown in Figure 20.

Table 5. Circulating plasma fatty acid analysis

Fatty acids	**SHAM**	**MI**	**FLAX**	**OIL**	**SDG**
C14:0	0.09±0.00	0.09±0.00	0.09±0.00	0.10±0.00	0.13±0.00*
C16:0	9.32±0.12	9.31±0.19	7.86±0.11*	8.20±0.11*	8.84±0.15
C18:0	6.58±0.16	6.15±0.15	5.94±0.09*	6.36±0.08	4.95±0.10*

C20:0	0.06±0.00	0.06±0.00	0.05±0.00*	0.04±0.00*	0.04±0.00*
C22:0	0.09±0.00	0.07±0.00	0.07±0.00	0.08±0.00	0.07±0.00*
C24:0	0.32±0.01	0.26±0.01	0.25±0.01*	0.24±0.01*	0.23±0.01*
C16:1	0.14±0.01	0.17±0.01	0.18±0.01	0.20±0.01	0.42±0.04*
C18:1	3.21±0.06	3.22±0.14	3.09±0.10	3.53±0.11	2.46±0.11*
C20:1	0.06±0.00	0.06±0.00	0.04±0.00*	0.05±0.00*	0.04±0.00*
C22:1	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00
C18:2 (n-6)	12.76±0.21	11.63±0.58	11.23±0.12*	12.27±0.15	10.04±0.18*
C18:3 (n-6)	0.04±0.00	0.05±0.00	0.02±0.00*	0.03±0.00*	0.04±0.00
C20:2 (n-6)	0.15±0.00	0.12±0.00	0.07±0.00*	0.07±0.00*	0.06±0.00*
C20:3 (n-6)	0.26±0.01	0.22±0.01	0.33±0.01*	0.33±0.00*	0.32±0.01*
C20:4 (n-6)	8.23±0.21	7.33±0.15	3.56±0.09*	3.35±0.09*	4.32±0.11*
C20:3 (n-3)	t	t	0.04±0.00	0.06±0.00	t
C22:4 (n-6)	0.11±0.00	0.10±0.00	0.02±0.00*	0.02±0.00*	0.05±0.00*
C22:5 (n-3)	0.11±0.00	0.09±0.00	0.48±0.02*	0.51±0.02*	0.13±0.00
ΣPUFA	23.11±0.36	20.28±1.01	20.67±0.37	22.98±0.38	16.71±0.27*

Σn-6 PUFA	21.59±0.33	20.22±0.37	15.25±0.22*	16.08±0.17*	14.87±0.23*

Values represented are mean ± S.E.M. *significantly different from control diet groups - Sham and MI, $p<0.0001$.

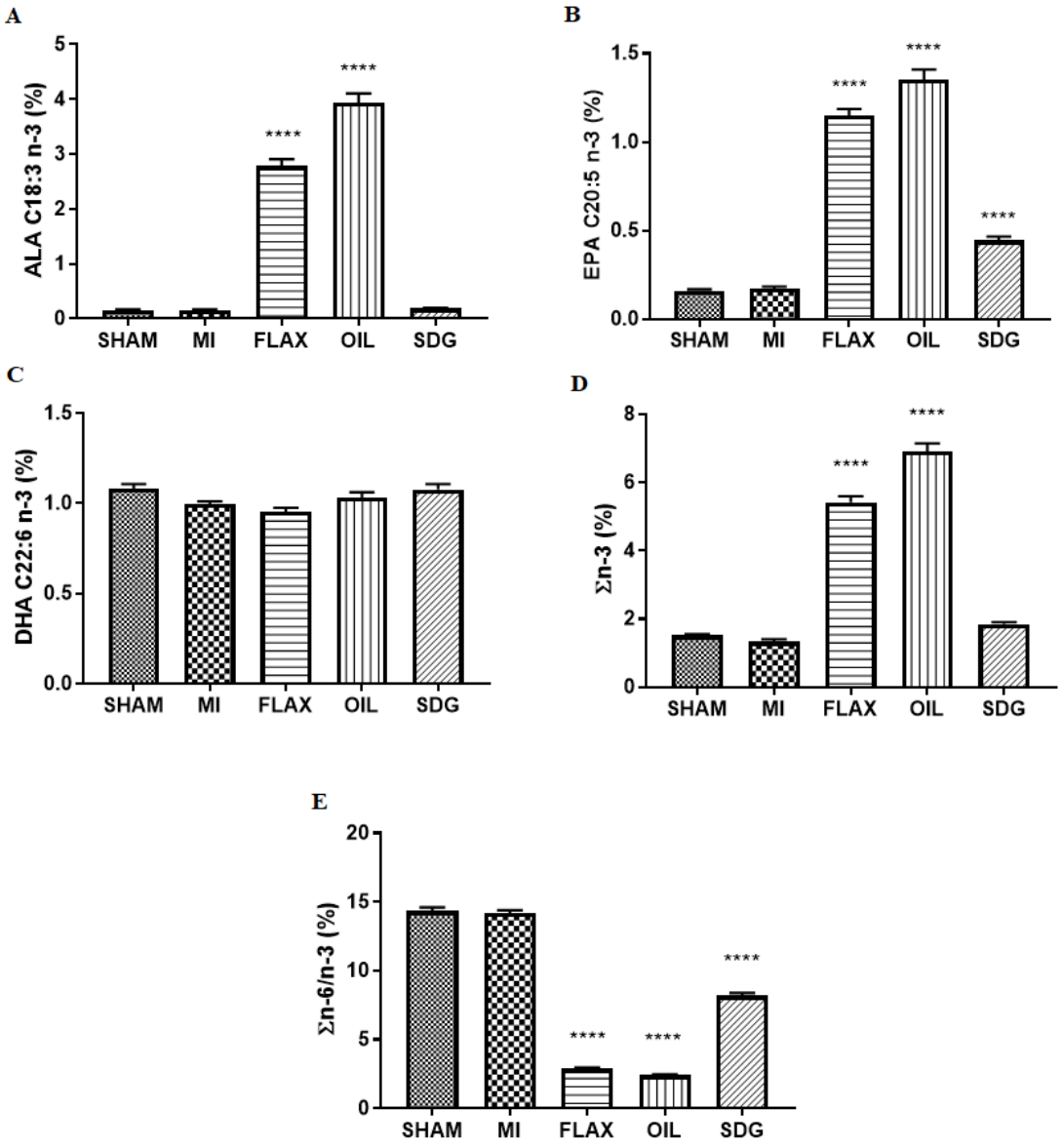


Figure 20. Effect of flaxseed on plasma fatty acids. (**A**) Alpha-linolenic acid **(**ALA); (**B**) eicosapentaenoic acid (EPA); (**C**) docosahexaenoic acid (DHA); (**D**) sum of omega-3 fatty acids

(∑n-3); (**E**) ratio of omega-6 and omega-3 fatty acids (∑n-6/n-3). Values represented are mean ± SEM. **** significantly different from the control diet groups – Sham and MI, $p<0.0001$ (one-way ANOVA one-way ANOVA with a Dunnett's *post hoc* test).

Scar size as a function of diet

Scar size in the heart after ligation of the coronary artery was assessed in the animals 8 weeks post-surgery (Figure 21). The scar size was measured as a % of total left ventricle weight. The percentage of scar weight was significantly lower ($p<0.05$) in the flax-supplemented MI group compared to the control-diet MI.

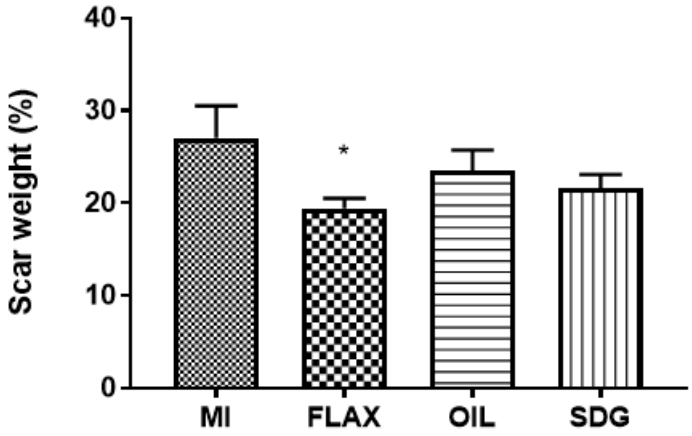


Figure 21. Effect of flaxseed on myocardial infarct size. Values represented are mean ± SEM. * significantly different from MI, $p<0.05$ (one-way ANOVA one-way ANOVA with a Dunnett's *post hoc* test).

Occurrence of ventricular arrhythmias after MI

As shown in Figure 22, sinus rhythm was observed on the electrocardiogram (ECG) of all the animals before the surgery. Within 10 min after the coronary artery ligation, several changes

appeared indicating the impact of the coronary artery occlusion on the cardiac electrical conduction. The MI created by the coronary artery ligation was confirmed by the appearance of ST segment elevation on ECG tracings during the surgery procedure.

Various arrhythmic events such as premature ventricular contractions (PVCs), bigeminy, salvos of three PVCs, ventricular tachycardia (VT), and ventricular fibrillation (VF) were observed for 10 min after the coronary artery ligation (Figure 22). While no alterations occurred in the sinus rhythm in the sham rats on the control diet, in the MI group on a normal chow diet, 73% of the animals exhibited arrhythmic events within 10 min of the coronary artery ligation. In contrast, a significantly lower incidence of arrhythmias was noted in the ground flaxseed ($p<0.05$), flax oil ($p<0.0001$), and SDG ($p<0.0001$) treated groups compared to the MI group. Furthermore, a significant decrease was also observed in specific types of arrhythmias including PVCs, bigeminy and salvo events, VT, and VF as shown in Figure 22.

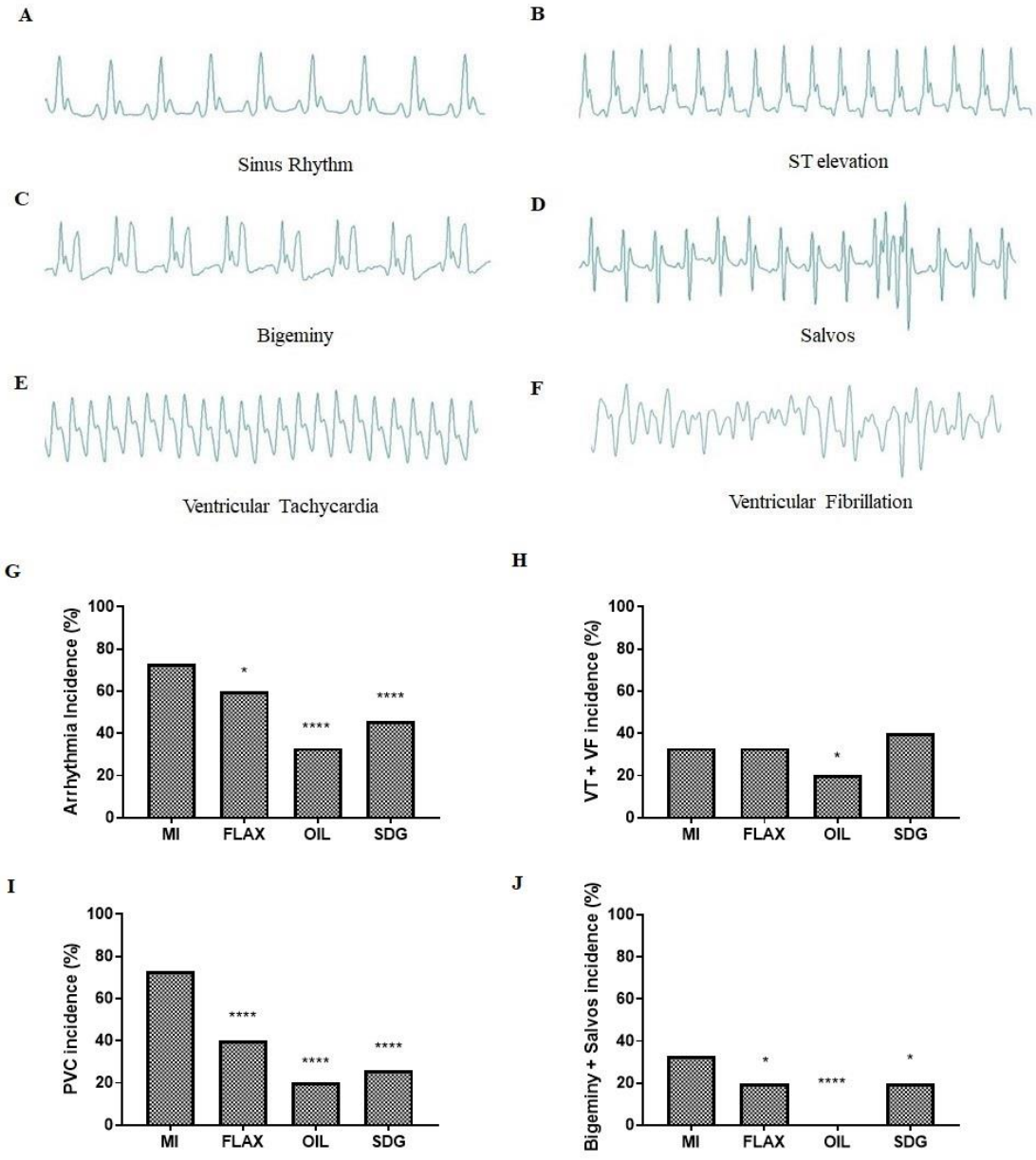


Figure 22. Effect of dietary interventions on ventricular arrhythmias. Representative images of ECG tracings A-F. (**A**) Sinus rhythm; (**B**) ST elevation; (**C**) bigeminy; (**D**) salvos; (**E**) ventricular tachycardia (VT); (**F**) ventricular fibrillation (VF). Antiarrhythmic effect of dietary interventions G-J. (**G**) Arrhythmia incidence; (**H**) VT and VF incidence; (**I**) PVC incidence; (**J**) bigeminy and salvos incidence. Values represented are mean ± SEM. * significantly different from

MI, $p<0.05$; **** significantly different from MI, $p<0.0001$ (a chi-squared test followed by Fisher's exact test for p values). There were no arrhythmias observed in the sham control group.

Echocardiographic results

Echocardiographic outcomes were obtained to examine the effect of flaxseed on ventricular remodeling at 4- and 8-weeks post-MI. Figure 23 shows representative images of the M-mode view of cardiac performance of all the groups. At 4 and 8 weeks, left ventricle internal diameter in diastole (LVIDd) and in systole (LVIDs) values were significantly increased (respectively $p<0.01$ and $p<0.0001$) in MI animals compared to the sham group implying LV dilatation, as shown in Figure 23. Animals that received diets supplemented with ground flaxseed and SDG showed significantly ($p<0.05$) decreased LVIDs at 8 weeks compared to the MI group on control diet.

As presented in Table 6, LV end-diastolic volume (EDV) was found to be altered at 4 and 8 weeks, as the MI group demonstrated significantly (respectively $p<0.01$ and $p<0.0001$) higher values compared to the sham animals. A similar trend was observed with end-systolic volume ($p<0.0001$) at both the time points. EDV and ESV values in the dietary treatment groups and in the untreated-MI group were similar.

Values for left ventricle posterior wall thickness in diastole (LVPWd) and in systole (LVPWs) were comparable between all the groups at 4- and 8-weeks post-surgery (Table 6). Interventricular septum thickness in diastole (IVSd) and in systole (IVSs) was significantly decreased at both the time points ($p<0.01$ and $p<0.0001$, respectively) in the MI group when compared to the IVS in sham animals. No impact of dietary treatment was observed on LVPW and IVS.

Systolic function was reduced in the MI group as evidenced by significantly decreased values of LV ejection fraction (EF) and fractional shortening (FS) at 4 and 8 weeks ($p<0.0001$) when compared to the sham control (Table 6). Doppler echocardiography performed to assess the effect on cardiac relaxation revealed altered diastolic function in the MI groups compared to the sham animals. Although all the groups had similar values for early [E] and late atrial [A] filling velocity ratio, isovolumetric relaxation time (IVRT) was significantly ($p<0.01$) increased in the MI group at 8 weeks compared to the sham control. However, no statistically significant differences were observed for systolic and diastolic functions in the dietary treatment groups compared to the untreated MI group. The effect on heart rate (HR), stroke volume (SV), and cardiac output (CO) are presented in Table 6.

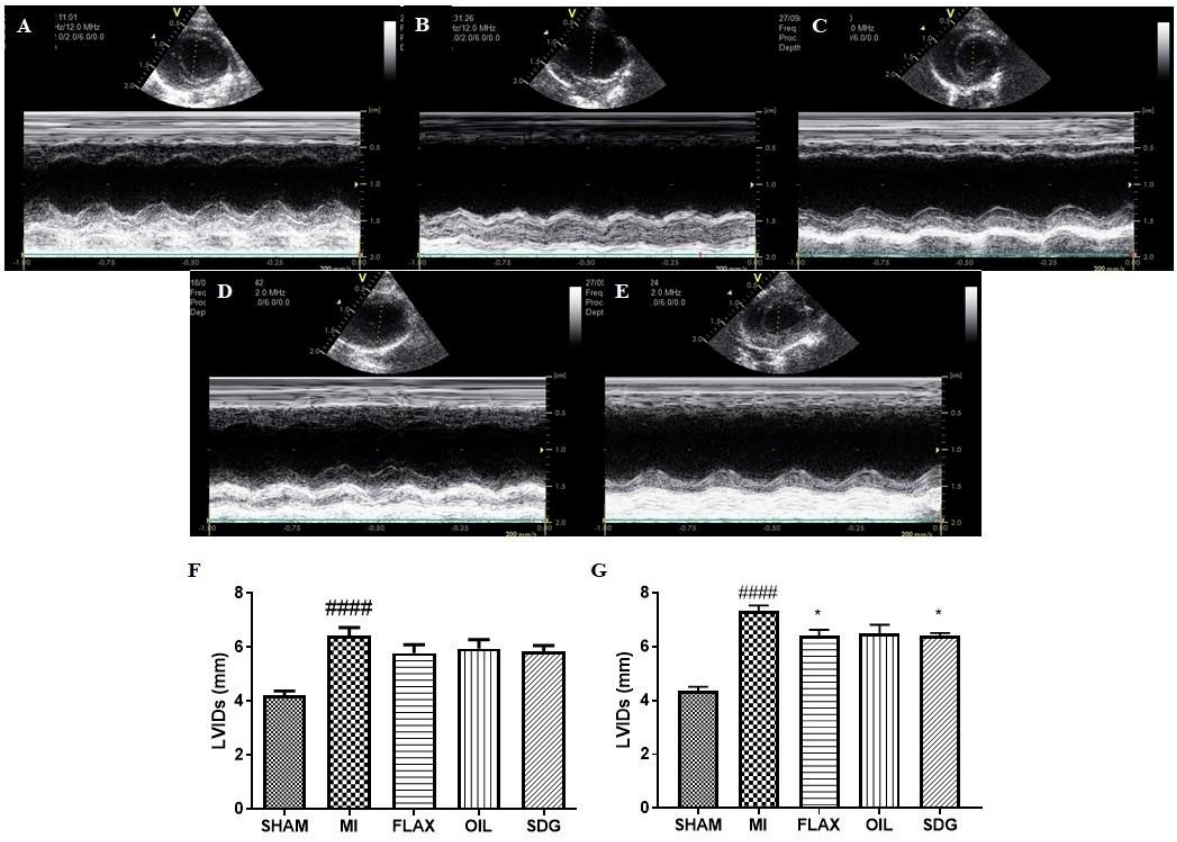


Figure 23. Effect of flaxseed on ventricular dilatation. Representative images of M-mode echocardiograms. (**A**) Sham; (**B**) MI; (**C**) Flax; (**D**) Oil; (**E**) SDG; (**F**) left ventricle internal diameter in systole (LVIDs) at 4 weeks; (**G**) LVIDs at 8 weeks. Values represented are mean ± S.E.M. #### significantly different from SHAM, $p<0.0001$; * significantly different from MI, $p<0.05$ (one-way ANOVA one-way ANOVA with a Dunnett's *post hoc* test).

Myocardial fibrosis and inflammation

Estimates of myocardial fibrosis demonstrated profound collagen accumulation in the MI groups compared to the sham control. As shown in Figure 24, dietary treatment with ground flaxseed ($p<0.0001$), flax oil ($p<0.0001$), and SDG ($p<0.01$) significantly decreased the fibrosis compared to the MI animals on a regular chow diet. Increased levels of proinflammatory cytokine tumor necrosis factor-alpha (TNF-α) were found in the control MI group compared to the sham group ($p<0.05$). All of the dietary treatment groups showed a significant decrease ($p<0.05$; $p<0.01$; $p<0.01$) in the cardiac levels of TNF-α compared to the animals which underwent an MI and were maintained on a regular chow diet.

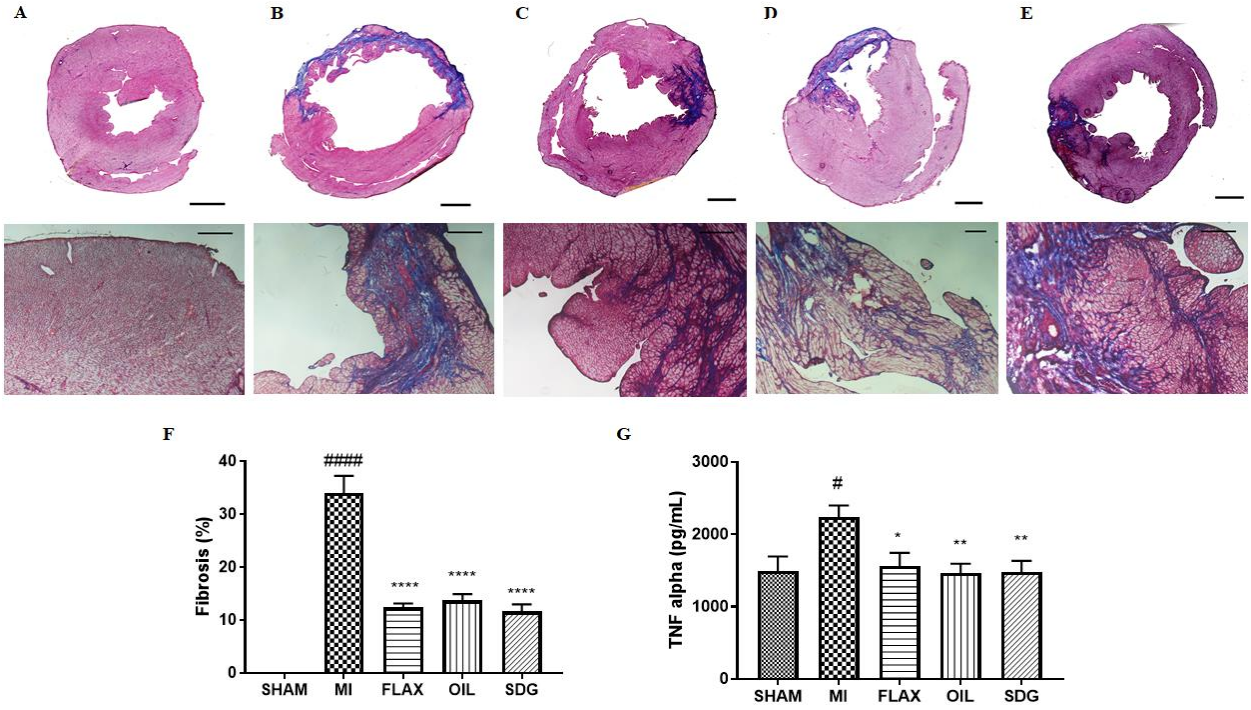


Figure 24. Effect of dietary interventions on myocardial fibrosis and inflammation. Representative images of the LV cross-sections showing myocardial fibrosis; upper and lower panel scale bar = 2000 μm and 250 μm, respectively. Myocardial tissue is stained in red and collagen fibers are stained in blue by Masson staining. (**A**) Sham; (**B**) MI; (**C**) Flax; (**D**) Oil; (**E**) SDG; (**F**) degree of fibrosis (%); (**G**) tumor necrosis factor-α (TNF-α). Values represented are mean ± S.E.M. #### significantly different from SHAM, $p<0.0001$; # significantly different from SHAM, $p<0.05$; * significantly different from MI, $p<0.05$; ** significantly different from MI, $p<0.01$; **** significantly different from MI, $p<0.0001$ (one-way ANOVA one-way ANOVA with a Dunnett's *post hoc* test).

Table 6. Echocardiographic measurements at 4- and 8-weeks post-MI

4 weeks					
	SHAM	MI	FLAX	OIL	SDG
LVPWd, mm	1.85±0.08	1.87±0.06	1.80±0.08	1.95±0.07	2.02±0.08
LVPWs, mm	2.52±0.09	2.63±0.08	2.61±0.10	2.70±0.09	2.88±0.13
EDV, mL	0.76±0.04	1.51±0.13†	1.26±0.10	1.31±0.11	1.30±0.11
ESV, mL	0.19±0.01	0.65±0.07†	0.49±0.07	0.54±0.07	0.48±0.05
LVIDd, mm	6.92±0.14	8.82±0.29†	8.29±0.24	8.36±0.27	8.35±0.27
IVSd	1.75±0.04	1.47±0.07*	1.65±0.07	1.71±0.06†	1.53±0.04
IVSs	2.26±0.06	1.79±0.07*	1.81±0.09	1.99±0.10	1.80±0.05
EF, %	74.33±1.93	58.67±2.20†	63.14±2.99	61.07±2.94	62.54±1.70
FS, %	39.07±1.74	27.67±1.42†	30.86±2.00	29.47±2.13	30.00±1.14
E/A	1.36±0.08	1.4±0.08	1.29±0.07	1.36±0.04	1.27±0.06
IVRT, ms	19.02±0.69	19.4±0.769	20.06±0.79	20.31±0.37	21.95±0.72
HR, BPM	340±7	337±5	328±5	341±6	320±9
SV, mL	0.56±0.03	0.86±0.06	0.76±0.03	0.76±0.04	0.81±0.06
CO, mL/min	192.8±0.01	289.1±0.01*	252.8±0.01	262.0±0.01	261.2±0.02
8 weeks					
	SHAM	SHAM	SHAM	SHAM	SHAM
LVPWd, mm	1.90±0.09	1.94±0.09	2.02±0.07	2.03±0.11	1.97±0.11
LVPWs, mm	2.59±0.10	2.44±0.11	2.61±0.05	2.59±0.12	2.64±0.10

EDV, mL	0.86±0.07	1.27±0.11*	1.22±0.09	1.36±0.10	1.23±0.05
ESV, mL	0.21±0.02	0.70±0.08†	0.63±0.06	0.68±0.08	0.61±0.02
LVIDd, mm	7.21±0.21	8.26±0.28*	8.21±0.21	8.50±0.24	8.25±0.13
IVSd	1.62±0.04	1.22±0.05†	1.23±0.06	1.30±0.08	1.29±0.04
IVSs	2.59±0.06	1.58±0.11†	1.58±0.06	1.69±0.13	1.55±0.05
EF, %	75.13±1.76	47.2±3.14†	49.36±2.30	52±3.17	49.85±1.95
FS, %	39.60±1.61	21.07±1.77†	22.07±1.27	24.07±2.11	22.38±1.11
E/A	1.30±0.06	1.48±0.17	1.25±0.04	1.32±0.07	1.26±0.07
IVRT, ms	19.73±0.51	23.11±0.74*	21.76±0.68	21.05±0.59	22.77±0.63
HR, BPM	335±7	335±6	329±7	342±4	320±7
SV, mL	0.65±0.06	0.56±0.04	0.59±0.03	0.67±0.04	0.62±0.04
CO, mL/min	218.9±0.01	190.3±0.01	195.5±0.01	233.5±0.01	199.5±0.01

Values represented are mean ± SEM. LV, left ventricle; LVPWd, LV posterior wall thickness in diastole; LVPWs, LV posterior wall thickness in systole; EDV, LV end-diastolic volume; ESV, LV end-systolic volume; LVIDd, LV internal diameter in diastole; LVIDs, LV internal diameter in systole; IVSd, LV interventricular septum thickness in diastole; IVSs, LV interventricular septum thickness in systole; EF, LV ejection fraction; FS, LV fractional shortening; E/A, ratio of early [E] and late atrial [A] ventricular filling velocity; IVRT, isovolumetric relaxation time; HR, heart rate; SV, stroke volume; CO, cardiac output. * significantly different from Sham group, $p<0.01$; † significantly different from Sham group, $p<0.0001$; † significantly different from MI group, $p<0.05$.

DISCUSSION

This is the first study to investigate dietary flaxseed in the animal model of an acute MI induced by coronary artery ligation. The data demonstrate that dietary flaxseed given to animals before and after an MI: a) reduced the incidence of arrhythmias; b) limited the size of the infarct; c) prevented excessive dilation of the left ventricular chamber; d) exerted an anti-inflammatory effect, and e) reduced cardiac fibrosis. The dosage of flaxseed and its bioactive components employed in this study is similar to that used previously in animal studies [9, 12, 19] and is applicable and relevant to that used in human dietary interventions with flaxseed [8, 20, 21].

In this study, dietary flaxseed and, in particular, flax oil demonstrated a significant antiarrhythmic action *in vivo*. This anti-arrhythmic result is consistent with a previous *ex-vivo* study from our lab that exhibited a significant reduction in the incidence of ventricular fibrillation during ischemia in isolated hearts from rabbits fed a flaxseed-supplemented diet [12]. It is possible from our study to determine the bioactive components within the diet that may be responsible for this anti-arrhythmic action. Flaxseed, and particularly flax oil, is enriched in the omega-3 fatty acid, ALA. Flaxseed is the richest plant source of ALA [22]. The mechanism responsible for the effects of dietary flaxseed on the heart post-MI, therefore, likely involves, directly or indirectly, this rich content of ALA. Isolated hearts have shown a decreased capacity for arrhythmias when perfused with ALA [23-25]. However, it is not possible to determine if ALA or a metabolic product of ALA was responsible for the anti-arrhythmic effect of the flaxseed diet. A significant reduction in ventricular fibrillation was observed in dogs intravenously administered ALA, EPA, and DHA [23]. Dietary supplementation of flaxseed and flax oil enriched in ALA in this study increased the plasma levels of both ALA and EPA, clear evidence that ALA is metabolized in rats to the longer chain polyunsaturated fatty acid EPA but not to DHA. This effect in rats aligns with a previous

report from our lab that showed similar results in mice [9] but contrasts with rabbits [19] and humans [20, 26] which show limited if any metabolism of ALA to the longer chain fatty acids EPA and DHA. Thus, the anti-arrhythmic action of dietary flaxseed may be primarily due to ALA and/or EPA. In a large prospective cohort study, every 0.1% increase in energy content from ALA intake was associated with a 12% reduction in the risk of sudden cardiac death in women. ALA intake had a neutral effect on fatal CHD and nonfatal MI [25].

The ionic mechanism responsible for the anti-arrhythmic action of the dietary flaxseed is more difficult to identify based upon data obtained from the present study. However, previous work in the literature is relevant and may shed some insight into the electrophysiological changes induced by the dietary intervention. Ventricular arrhythmias occur in two phases in an animal model of acute myocardial infarction. The phase I (immediate ventricular arrhythmias) usually occur within 2 to 15 min of coronary artery ligation (CAL). This period represents reversible myocardial injury [27]. Phase II (delayed ventricular arrhythmias) occurs 15 to 30 min post-CAL and is associated with the early inflammatory period of myocardial necrosis and infarct evolution [27]. Phase I arrhythmias occur as a result of slow conduction and delayed activation of subepicardial muscle. The dispersion in refractoriness that increases after CAL causes unidirectional flow facilitating re-entry circuit development in Phase I [28]. Increased automaticity contributes to arrhythmia occurrence during the delayed phase [29]. Dietary flaxseed has been shown to reduce the QT interval and thus shorten the action potential duration [12]. Several ion channels are targeted by omega-3 fatty acids. Voltage-dependent sodium currents are suppressed by omega-3 fatty acids, thus prolonging and shifting the inactivation state of the Na^+ channels to more negative potentials. Omega-3 fatty acids also decrease L-type calcium currents, preventing the cytosolic Ca^{2+} fluctuations which are responsible for delayed afterdepolarizations which

produce triggered arrhythmias [23]. ALA can also inhibit the activity of the Na^+/Ca^{2+} exchanger which is responsible for the delayed afterdepolarizations [30]. In total, these molecular sites of action represent plausible mechanisms through which dietary flaxseed (via its content of ALA) exerts its effects on cardiac electrical activity.

The process by which the dietary flaxseed decreased the size of the infarct in the present study may involve a multi-factorial mechanism. Following coronary artery occlusion, a wavefront of necrosis gradually progresses from subendocardium to subepicardium [31]. Dead cardiomyocytes are removed through an inflammatory process and are replaced by collagenous scar tissue that originates from fibroblasts. After infarction, collagen content rises over the next 4-7 days and reaches a plateau by 3-6 weeks [32]. The collagen volume fraction increases from 3% to 30% in rats after coronary artery ligation [33]. As the most important determinant, infarct size correlates with the post-infarction remodeling and the development of heart failure [33]. In this study, dietary treatment with flaxseed significantly limited the infarct size 8 weeks after the coronary artery ligation and prevented excessive left ventricle dilation. Powerful anti-inflammatory and anti-fibrotic effects were also observed with dietary flaxseed. This is in accord with a previous report where the ALA component of flaxseed reduced the MI size after ischemia/reperfusion insult [34]. Part of the cardioprotective mechanism may involve the capacity of ALA to block apoptosis in cardiomyocytes after ischemic reperfusion challenge [13]. ALA has been shown to decrease the production of reactive oxygen species, upregulate the expression of anti-apoptotic proteins in the myocardium, and inhibit the activity of angiotensin converting enzyme [34-36]. SDG has also been reported to reduce infarct size and improve cardiac contractility [37]. SDG increases the expression of VEGF, angiopoietin-1, and endothelial NO synthase. These angiogenic proteins induce neovascularization and may reduce the imbalance of

myocardial O_2 supply and demand [37]. This is also consistent with the anti-inflammatory action of the diet shown in the present investigation. In summary, the synergistic action of ALA and SDG may be important for the beneficial effects exhibited here on infarct size and fibrosis observed as a function of the dietary flaxseed.

The ventricular dilatation exhibited after an MI was expected. After an MI, the pathological process of left ventricular remodeling impacts ventricular cavity shape and impairs contractile function. A progressive ventricular dilation that is due to the myocyte side-to-side slippage [38] increases the myocardial wall stress. The degree of left ventricular dilation determines the risk of major cardiovascular events including ventricular arrhythmias and heart failure [39]. Inflammation is a critical factor influencing ventricular remodeling and serves as an important target for intervention. The inflammatory response is characterized by infiltrating neutrophils, monocytes/macrophages, and lymphocytes [40]. Macrophages produce a variety of proinflammatory cytokines and growth factors in the myocardium, this in turn causes amplification of inflammatory response and cardiac fibrosis. An inflammatory chemokine, MCP-1, has been shown to stimulate the fibroblast collagen expression through the production of several profibrotic cytokines [41]. The anti-inflammatory action shown in the present study through the inhibition of TNF-α expression is consistent with previous work that has shown dietary flaxseed can reduce the expression of anti-inflammatory markers like mac-3, IL-6 and VCAM-1 during cardiovascular disease [9]. Thus, the inhibition of cardiac dilatation and fibrosis may, in part, be mediated by the reduction in inflammation induced by the ALA and SDG components of flaxseed. The lack of improvement in cardiac function despite the positive effect of dietary flaxseed supplementation on cardiac structure and electrical activity warrants further investigations with longer duration of flaxseed supplementation with or without a higher dose.

Conclusion

Taken together, the present results support dietary flaxseed as a prophylaxis for cardiovascular disease. Dietary flaxseed and/or its bioactive ingredients reduce the occurrence of arrhythmias, limit infarct size, attenuate left ventricular dilation, and decrease cardiac fibrosis after an MI. These data further support the consumption of omega-3 fatty acids from marine and plant sources as an approach to reduce the risk of coronary artery disease and sudden cardiac death. It is important to recognize that the present results demonstrate the impact of this dietary intervention which was carried out before and after the induction of the MI. The effects of a dietary treatment induced solely after an MI will need further investigation. The results presented here represent a template to further investigate the efficacy of using flaxseed as a dietary supplement in patients who have already had an MI as well as to encourage the use of flaxseed as a preventative measure before an MI occurs.

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REFERENCES

[1] Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980-2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet. 2018;392:1736-88.

[2] Benjamin EJ, Virani SS, Callaway CW, Chamberlain AM, Chang AR, Cheng S, et al. Heart Disease and Stroke Statistics - 2018 Update: A Report From the American Heart Association. Circulation. 2018;137:e67-e492.

[3] Siscovick DS, Barringer TA, Fretts AM, Wu JH, Lichtenstein AH, Costello RB, et al. Omega-3 Polyunsaturated Fatty Acid (Fish Oil) Supplementation and the Prevention of Clinical Cardiovascular Disease: A Science Advisory From the American Heart Association. Circulation. 2017;135:e867-e84.

[4] Jia X, Kohli P, Virani SS. Omega-3 Fatty Acid and Cardiovascular Outcomes: Insights From Recent Clinical Trials. Curr Atheroscler Rep. 2019;21:1.

[5] Mozaffarian D, Wu JH. Omega-3 fatty acids and cardiovascular disease: effects on risk factors, molecular pathways, and clinical events. J Am Coll Cardiol. 2011;58:2047-67.

[6] Bhatt DL, Steg PG, Miller M, Brinton EA, Jacobson TA, Ketchum SB, et al. Cardiovascular Risk Reduction with Icosapent Ethyl for Hypertriglyceridemia. N Engl J Med. 2019;380:11-22.

[7] Parikh M, Netticadan T, Pierce GN. Flaxseed: its bioactive components and their cardiovascular benefits. Am J Physiol Heart Circ Physiol. 2018;314:H146-h59.

[8] Rodriguez-Leyva D, Weighell W, Edel AL, LaVallee R, Dibrov E, Pinneker R, et al. Potent antihypertensive action of dietary flaxseed in hypertensive patients. Hypertension. 2013;62:1081-9.

[9] Dupasquier CM, Dibrov E, Kneesh AL, Cheung PK, Lee KG, Alexander HK, et al. Dietary flaxseed inhibits atherosclerosis in the LDL receptor-deficient mouse in part through antiproliferative and anti-inflammatory actions. Am J Physiol Heart Circ Physiol. 2007;293:H2394-402.

[10] Bassett CM, McCullough RS, Edel AL, Patenaude A, LaVallee RK, Pierce GN. The alpha-linolenic acid content of flaxseed can prevent the atherogenic effects of dietary trans fat. Am J Physiol Heart Circ Physiol. 2011;301:H2220-6.

[11] Francis AA, Deniset JF, Austria JA, LaVallee RK, Maddaford GG, Hedley TE, et al. Effects of dietary flaxseed on atherosclerotic plaque regression. Am J Physiol Heart Circ Physiol. 2013;304:H1743-51.

[12] Ander BP, Weber AR, Rampersad PP, Gilchrist JS, Pierce GN, Lukas A. Dietary flaxseed protects against ventricular fibrillation induced by ischemia-reperfusion in normal and hypercholesterolemic Rabbits. J Nutr. 2004;134:3250-6.

[13] Ganguly R, Hasanally D, Stamenkovic A, Maddaford TG, Chaudhary R, Pierce GN, et al. Alpha linolenic acid decreases apoptosis and oxidized phospholipids in cardiomyocytes during ischemia/reperfusion. Mol Cell Biochem. 2018;437:163-75.

[14] Prasad K, Dhar A. Flaxseed and Diabetes. Curr Pharm Des. 2016;22:141-4.

[15] Curtis MJ, Hancox JC, Farkas A, Wainwright CL, Stables CL, Saint DA, et al. The Lambeth Conventions (II): guidelines for the study of animal and human ventricular and supraventricular arrhythmias. Pharmacol Ther. 2013;139:213-48.

[16] Parikh M, Raj P, Yu L, Stebbing J-A, Prashar S, Petkau JC, et al. Ginseng Berry Extract Rich in Phenolic Compounds Attenuates Oxidative Stress but not Cardiac Remodeling post Myocardial Infarction. International Journal of Molecular Sciences. 2019;20:983.

[17] Ju H, Zhao S, Jassal DS, Dixon IM. Effect of AT1 receptor blockade on cardiac collagen remodeling after myocardial infarction. Cardiovasc Res. 1997;35:223-32.

[18] Bassett CMC, Edel AL, Patenaude AF, McCullough RS, Blackwood DP, Chouinard PY, et al. Dietary Vaccenic Acid Has Antiatherogenic Effects in LDLr−/− Mice. The Journal of Nutrition. 2009;140:18-24.

[19] Dupasquier CM, Weber AM, Ander BP, Rampersad PP, Steigerwald S, Wigle JT, et al. Effects of dietary flaxseed on vascular contractile function and atherosclerosis during prolonged hypercholesterolemia in rabbits. Am J Physiol Heart Circ Physiol. 2006;291:H2987-96.

[20] Austria JA, Richard MN, Chahine MN, Edel AL, Malcolmson LJ, Dupasquier CM, et al. Bioavailability of alpha-linolenic acid in subjects after ingestion of three different forms of flaxseed. J Am Coll Nutr. 2008;27:214-21.

[21] Kaul N, Kreml R, Austria JA, Richard MN, Edel AL, Dibrov E, et al. A comparison of fish oil, flaxseed oil and hempseed oil supplementation on selected parameters of cardiovascular health in healthy volunteers. J Am Coll Nutr. 2008;27:51-8.

[22] Parikh M, Pierce GN. Dietary flaxseed: what we know and don't know about its effects on cardiovascular disease. Can J Physiol Pharmacol. 2019;97:75-81.

[23] Billman GE, Kang JX, Leaf A. Prevention of sudden cardiac death by dietary pure omega-3 polyunsaturated fatty acids in dogs. Circulation. 1999;99:2452-7.

[24] Kromhout D, Giltay EJ, Geleijnse JM. n-3 fatty acids and cardiovascular events after myocardial infarction. N Engl J Med. 2010;363:2015-26.

[25] Albert CM, Oh K, Whang W, Manson JE, Chae CU, Stampfer MJ, et al. Dietary alpha-linolenic acid intake and risk of sudden cardiac death and coronary heart disease. Circulation. 2005;112:3232-8.

[26] Patenaude A, Rodriguez-Leyva D, Edel AL, Dibrov E, Dupasquier CM, Austria JA, et al. Bioavailability of alpha-linolenic acid from flaxseed diets as a function of the age of the subject. Eur J Clin Nutr. 2009;63:1123-9.

[27] Weiss SM, Saint DA. The persistent sodium current blocker riluzole is antiarrhythmic and anti-ischaemic in a pig model of acute myocardial infarction. PLoS One. 2010;5:e14103-e.

[28] Ben-Haim SA, Becker B, Gutterman DD, Edoute Y, Kaplinsky E, Palti Y. Prediction of immediate ventricular arrhythmias after coronary artery ligation. J Am Coll Cardiol. 1992;20:1270-6.

[29] Hope RR, Williams DO, El-Sherif N, Lazzara R, Scherlag BJ. The Efficacy of Antiarrhythmic Agents During Acute Myocardial Ischemia and the Role of Heart Rate. Circulation. 1974;50:507-14.

[30] Lukas A, Ander BP, Pierce GN, Raposo CS, Hurtado C, Deniset JF, et al. Differential sensitivities of the NCX1.1 and NCX1.3 isoforms of the Na+–Ca2+ exchanger to α-linolenic acid. Cardiovasc Res. 2007;73:395-403.

[31] Kloner RA, Jennings RB. Consequences of Brief Ischemia: Stunning, Preconditioning, and Their Clinical Implications. Circulation. 2001;104:2981-9.

[32] Clarke SA, Richardson WJ, Holmes JW. Modifying the mechanics of healing infarcts: Is better the enemy of good? J Mol Cell Cardiol. 2016;93:115-24.

[33] Fomovsky GM, Holmes JW. Evolution of scar structure, mechanics, and ventricular function after myocardial infarction in the rat. Am J Physiol Heart Circ Physiol. 2010;298:H221-8.

[34] Xie N, Zhang W, Li J, Liang H, Zhou H, Duan W, et al. alpha-Linolenic acid intake attenuates myocardial ischemia/reperfusion injury through anti-inflammatory and anti-oxidative stress effects in diabetic but not normal rats. Arch Med Res. 2011;42:171-81.

[35] Carotenuto F, Minieri M, Monego G, Fiaccavento R, Bertoni A, Sinigaglia F, et al. A diet supplemented with ALA-rich flaxseed prevents cardiomyocyte apoptosis by regulating caveolin-3 expression. Cardiovasc Res. 2013;100:422-31.

[36] Derbali A, Mnafgui K, Affes M, Derbali F, Hajji R, Gharsallah N, et al. Cardioprotective effect of linseed oil against isoproterenol-induced myocardial infarction in Wistar rats: a biochemical and electrocardiographic study. J Physiol Biochem. 2015;71:281-8.

[37] Penumathsa SV, Koneru S, Thirunavukkarasu M, Zhan L, Prasad K, Maulik N. Secoisolariciresinol diglucoside: relevance to angiogenesis and cardioprotection against ischemia-reperfusion injury. J Pharmacol Exp Ther. 2007;320:951-9.

[38] Pfeffer MA, Braunwald E, Moye LA, Basta L, Brown EJ, Jr., Cuddy TE, et al. Effect of captopril on mortality and morbidity in patients with left ventricular dysfunction after myocardial infarction. Results of the survival and ventricular enlargement trial. The SAVE Investigators. N Engl J Med. 1992;327:669-77.

[39] Sutton MSJ, Lee D, Rouleau JL, Goldman S, Plappert T, Braunwald E, et al. Left Ventricular Remodeling and Ventricular Arrhythmias After Myocardial Infarction. Circulation. 2003;107:2577-82.

[40] Seropian IM, Toldo S, Van Tassell BW, Abbate A. Anti-inflammatory strategies for ventricular remodeling following ST-segment elevation acute myocardial infarction. J Am Coll Cardiol. 2014;63:1593-603.

[41] Yamazaki T, Izumi Y, Nakamura Y, Yamashita N, Fujiki H, Osada-Oka M, et al. Tolvaptan improves left ventricular dysfunction after myocardial infarction in rats. Circ Heart Fail. 2012;5:794-802.

2.2. Dietary flaxseed and microRNA

Endogenous microRNAs are being intensively investigated for their effect on cardiovascular health and disease. Specific microRNAs have been implicated in the pathogenesis of left ventricular remodeling after an MI. Due to the observed beneficial effects of dietary flaxseed in the first study, we explored the molecular mechanisms especially the regulation of microRNA expression by dietary flaxseed in an MI.

It was hypothesized that in an MI induced by coronary artery ligation in rats, pretreatment with dietary flaxseed and its components will modulate the expression of specific microRNAs and proteins involved in adverse cardiac remodeling after an MI.

Supplementation of the Diet with Flaxseed Oil Regulates the Expression of MicroRNAs after a Myocardial Infarction

(To be Submitted)

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Abstract

MicroRNAs (miRs) are a novel class of molecular mediators known to be involved in the cardiac remodeling process after a myocardial infarction (MI). miR-1, miR-133a, miR-133b, miR-135a, and miR-29b play a key role in many cardiac pathological processes including apoptosis, fibrosis and arrhythmias. Dietary flaxseed previously reduced the incidence of arrhythmias and decreased cardiac infarct size and tissue fibrosis in a rat coronary artery ligation-induced model of MI. The present study was carried out to test the hypothesis that dietary flaxseed supplementation before and after an MI regulates the expression of specific miRs to produce its cardioprotective effect. The MI was induced in rats by ligating the left anterior descending coronary artery. Animals were randomized into: a) sham MI with normal chow, b) MI with normal chow, c) MI with 10% milled

flaxseed supplementation, d) MI with 4.4% supplemented flax oil enriched in alpha-linolenic acid (ALA), and e) MI with flax lignan secoisolariciresinol diglucoside (SDG) supplementation (0.44%). The feeding protocol consisted of 2 weeks before and 8 weeks after the surgery. Dietary flaxseed oil supplementation upregulated the expression of miR133a, miR 135a, and miR 29b. The levels of collagen I expression were reduced in the flaxseed oil group. We conclude that miR-133a, miR-135a, and miR-29b are sensitive to dietary flaxseed oil likely due to its rich ALA content.

Introduction

Cardiovascular disease (CVD) is a leading cause of morbidity and mortality, and according to the latest estimates by the Heart Disease and Stroke Statistics – 2019 update, approximately 1 of every 3 deaths occur due to CVD. Ischemic heart disease is the major cause of mortality as 43.2% of cardiovascular deaths are attributed to it alone (1). Ischemia results in acute cardiac damage through myocardial infarction (MI). Insufficient blood flow to the myocardium results in an MI which in turn leads to inflammatory and fibrotic changes in both viable and infarcted myocardium and consequently leads to adverse cardiac remodeling and ventricular dysfunction (2). Recent advancements in the understanding of molecular mechanisms of heart function have shone a light on the impact of endogenously encoded microRNAs (miRs) on cardiovascular health and disease (3). In contrast to standard pharmacological drugs acting on single targets, miRs are capable of modulating several downstream pathways in parallel (4).

MiRs are endogenous, noncoding, single-stranded RNAs of 22-26 nucleotides that regulate messenger RNA (mRNA) expression through posttranslational inhibition or degradation (5). Several miRs have been shown to be involved in cardiac pathological processes such as acute MI, cardiac arrhythmias, and hypertrophy (6). miRs can either promote or inhibit apoptosis of

cardiomyocytes, they can modulate angiogenesis, alter cardiac regeneration and reprogram cardiac fibroblasts into cardiomyocytes (7). These effects clearly support a relationship between altered miR expression and ischemic heart disease. Recent studies have highlighted the importance of miRs in the regulation of cardiac rhythm (7). miRs such as miR-1, miR-133, miR-29, *and* miR-135 play important roles in cardiac remodeling and arrhythmias.

A recent meta-analysis of 13 randomized controlled trials with a total of 127,477 participants demonstrated a significant reduction in the risk of MI, total CAD, and total CVD through supplementation of the diet with marine omega-3 polyunsaturated fatty acids (n-3 PUFAs) (8). An alternative source of n-3 PUFAs is being investigated due to limiting physical and physiological concerns regarding n-3 PUFAs obtained from marine sources (9). Flaxseed is a rich source of alpha-linolenic acid (ALA), which is a plant-based n-3 PUFAs. In addition to the high content of ALA, flaxseed is also a good source of lipid-lowering dietary fiber and the antioxidant secoisolariciresinol diglucoside (SDG), which is a plant lignan (9). Dietary flaxseed recently demonstrated several positive effects in a coronary artery ligation induced model of MI in rats. Flaxseed supplementation reduced the incidence of ventricular arrhythmias as well as prevented an excessive increase in infarct size, myocardial fibrosis, inflammation, and left ventricular dilation (10). However, the mechanism responsible for this cardioprotective effect of flaxseed was unclear. In view of the above discussion of the impact of miRs on cardiac performance under pathological challenge, it is possible that dietary flaxseed may alter miR expression and, ultimately, affect myocardial adaptive processes. In support of this hypothesis, diet can induce significant cardioprotective effects via a direct action on the epigenome (11). The current investigation was thus performed to test the hypothesis that flaxseed supplementation modulates

the expression of specific miRs, and protein molecules involved in the adverse cardiac remodeling process after an MI.

Methods

Animals

This study protocol (17-005) was approved by the University of Manitoba Office of Research Ethics & Compliance and Animal Care Committee and was done in accordance with the guidelines by the Canadian Council for Animal Care. Male Sprague Dawley rats (100-140 g; Charles River Laboratories, Quebec, Canada) were housed in a temperature and humidity-controlled room with a 12 h light/dark cycle. Animals were randomized into five groups: sham MI fed normal chow, MI fed normal chow, MI fed chow supplemented with 10% milled flaxseed (flax), MI fed chow supplemented with 4.4% flax oil enriched in alpha-linolenic acid (ALA), and MI fed chow supplemented with the flax lignan secoisolariciresinol diglucoside (SDG) (0.44%). These dietary interventions and the concentrations used are similar to those used successfully in previous studies (12). The four diets included a Prolab® RMH 3000 rodent regular chow (TestDiet, Richmond, IN), a 10% ground flaxseed (BakePur Milled Golden Flaxseed, Pizzey Ingredients, Russell, Manitoba, Canada) supplemented chow diet, or a 4.4.% flax oil (Cold Pressed Flax Oil, Royal Harvest, Shape Foods, Brandon, Manitoba, Canada) supplemented chow diet, or a 0.44% SDG (Archer Daniels Midland, Chicago, IL, USA) supplemented diet. Animals were fed with their respective diets for two weeks before and for 8 weeks after surgery. Thus, animals were sacrificed after a total of 10 weeks on the study protocol.

Induction of MI

Animals were anesthetized with 1–5% isoflurane and oxygen at a flow rate of 2 L min^{-1} and kept in a surgical plane of anesthesia with 2% isoflurane during surgery. A left thoracotomy was carried out, and the heart was gently exposed from the pericardial sac through the incision. The left anterior descending coronary artery (LAD) was located and occluded with 6-0 polypropylene silk suture at about 2 mm from aortic root. The suture was tied, and the ligation was estimated to be successful when the anterior wall of the left ventricle turned pale. The heart was repositioned, then the chest compressed to remove any air from the cavity and the incision was closed using a purse string suture. Sham-operated animals that served as controls were subjected to similar surgical procedures except that the LAD artery was not ligated. Buprenorphine 0.05 mg kg^{-1} was administered pre- and post-surgery (2 times a day for 2 days) subcutaneously as an analgesic agent to all animals.

Biological sample collection and analysis

All animals were anesthetized with ketamine/xylazine (9.0 mg per 100 g and 0.9 mg per 100 g IM). The depth of anesthesia was assessed by a pedal withdrawal reflex. The blood sample was collected from the inferior vena cava by opening the thoracic cavity and the heart was immediately excised. Whole heart was rinsed in PBS, LV's, septum, and fibrotic scar tissues were separated, weighed and flash frozen in liquid nitrogen.

MicroRNA detection in cardiac tissue

Total RNA was isolated from the left ventricle tissue using *mir*Vana™ miRNA isolation kit (Ambion, USA). Isolation was performed as per manufacturer's instructions. The concentration of the total isolated RNA from the heart homogenate was measured immediately with NanoDrop™

Lite Spectrophotometer (Thermo Scientific, USA). Total RNA was reverse transcribed to cDNA with TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, USA) using T100™ Thermal Cycler (Bio-Rad, USA) as per the manufacturer's instructions. cDNA was amplified using the TaqMan® Universal Master Mix II (Applied Biosystems, USA) with specific miRNA assays for miRNA-1 (Assay ID 002064), miRNA-133a (Assay ID 002246), miRNA-133b (Assay ID 002247), miRNA-135a (Assay ID 000460), and miRNA-29b (Assay ID 000413) (Applied Biosystems, USA). qPCR analysis was performed on CFX96™ Real-Time System (Bio-Rad, USA), as per the manufacturer's instructions. Amplification data were normalized to U6 snRNA (Assay ID 001973) (Applied Biosystems, USA).

Western blot analysis for Collagen 1A1

The viable (non-infarcted) left ventricle tissue samples were homogenized in RIPA buffer (Teknova, USA). The extracted protein was quantified using Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA). Equal amounts of protein samples (20 μg) were separated on 7.5% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE) using TGX Stain-Free™ FastCast™ Acrylamide Kit (Bio-Rad, USA). After electrophoresis, the separate proteins were transferred using the Trans-Blot Turbo Mini Nitrocellulose Transfer Packs and the Trans-Blot Transfer System (Bio-Rad, USA). The membranes were blocked with 5% dry milk in Tris-buffered saline (TBS)/0.1% Tween 20 for 1 h at room temperature, and then incubated overnight at 4ºC with a primary anti-rat collagen type I (1:1000, CL50141AP, Cedarlane, Canada). The membrane was washed with TBS and then incubated with secondary antibody IgG (1:10,000, Abcam, USA) conjugated with horseradish peroxidase. The membrane blots were developed using Luminata™ Forte Western HRP Substrate (MilliporeSigma, USA) and imaged on a Gel Doc™ XR+ imager (Bio-Rad, USA).

Statistical analysis

All data are expressed as mean ± S.E.M and analyzed by one-way ANOVA with a Tukey's multiple comparisons tests using GraphPad Prism v 7. Data distribution was assessed by Kolmogorov-Smirnov test, D'Agostino-Pearson omnibus normality test, and Shapiro-Wik normality test. Statistical significance was accepted when $p<0.05$.

Results

Dietary fatty acid analysis

The macronutrient composition of the experimental diets has been reported previously (10). The fatty acid profile of the diets is presented in Table **7**. Notable differences were observed in the levels of saturated and monounsaturated fatty acids. As shown in Figure 25, due to the supplementation of the diets with flaxseed and flax oil, a significant increase of 14-fold and 15-fold, respectively, was observed in the levels of ALA (C18:3n3) as compared to the control diet. Similarly, total n-3 fatty acid levels were also significantly higher in the flaxseed supplemented diets when compared with the control diet. Consequently, the ratios of n-6 to n-3 fatty acid levels were significantly lowered in the flaxseed and the flax oil supplemented diets compared to the control diet, Figure 25.

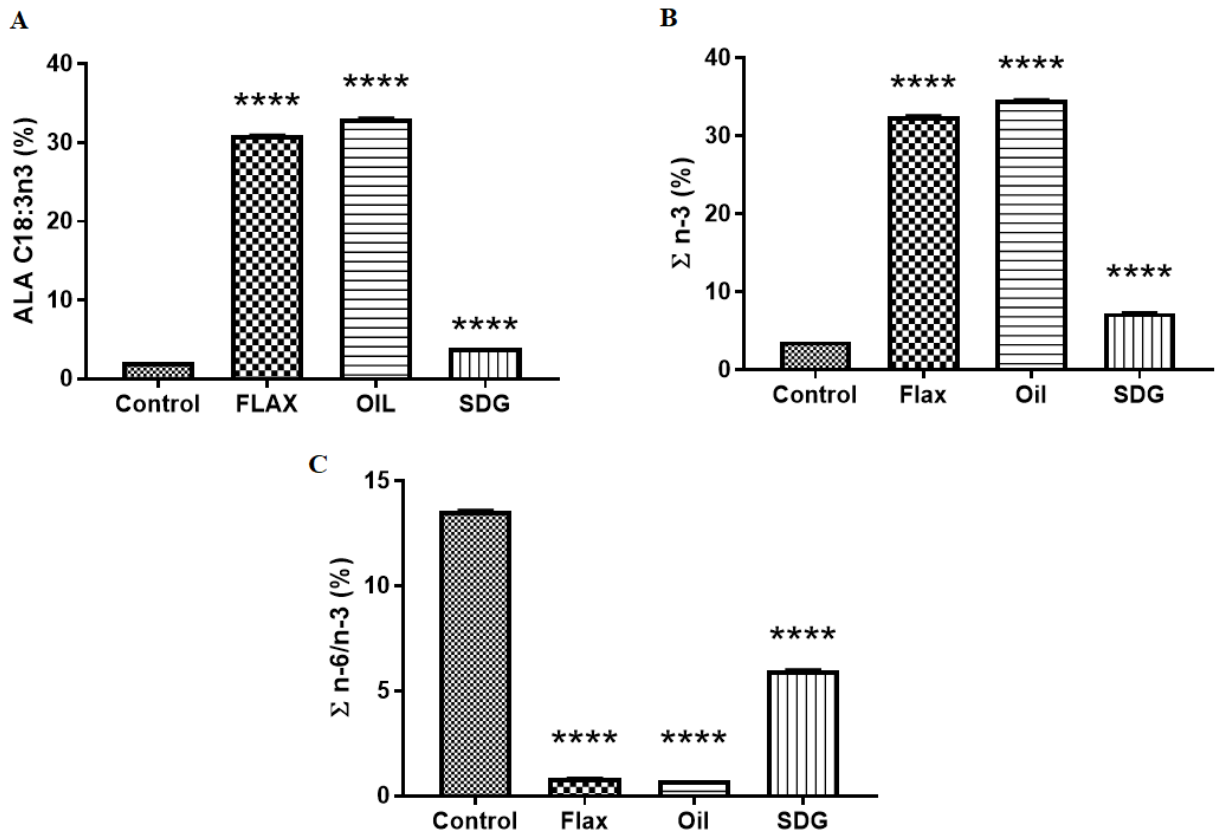


Figure 25. Effect of flaxseed supplementation in the diets. **A**, ALA (C18:3n3) levels. **B**, sum of n-3 fatty acids (∑n-3) levels. **C**, ratio of n-6 and n-3 fatty acids (∑n-6/n-3) levels. Data are presented as mean±S.E.M. **** significantly different from the control diet, $p<0.0001$.

Table 7. Dietary fatty acid analysis

Fatty acid	CONTROL	FLAX	OIL	SDG
C10:0	t	t	t	t
C12:0	0.01±0.00	0.01±0.00	0.01±0.00	0.02±0.00*
C14:0	0.49±0.00	0.51±0.01	0.50±0.00	1.06±0.00*

C15:0	0.07±0.00*	0.08±0.00*	0.08±0.00*	0.16±0.00*
C16:0	13.67±0.02*	10.17±0.02*	10.16±0.02*	16.37±0.01*
C17:0	0.12±0.00	0.12±0.00ǂ	0.12±0.00*	0.21±0.00*
C18:0	1.92±0.00	3.00±0.00*	4.10±0.00*	2.37±0.00*
C20:0	0.32±0.00	0.19±0.00*	0.21±0.00*	0.22±0.00*
C22:0	0.17±0.00	0.18±0.00*	0.19±0.00*	0.22±0.00*
C24:0	0.19±0.00	0.17±0.00ǂ	0.15±0.00*	0.21±0.00
Total SFA	16.98±0.02	14.43±0.02*	15.51±0.02*	20.82±0.01*
C14:1	t	t	t	t
C16:1t	0.05±0.00	0.05±0.00ǂ	0.04±0.00*	0.07±0.00*
C16:1	0.68±0.01	0.66±0.01	0.60±0.01ǂ	1.33±0.02*
C17:1	4.19±0.03	4.16±0.01	3.93±0.02*	9.57±0.06*
C18:1	23.16±0.01	19.53±0.01*	18.43±0.01*	15.15±0.01*
C18:1n7c	0.81±0.00	0.87±0.00*	0.83±0.00ǂ	1.19±0.01*
C20:1	0.39±0.00	0.33±0.00*	0.25±0.01*	0.50±0.01*
C22:1	0.03±0.00	0.03±0.00	0.04±0.00*	0.05±0.00*
C24:1	0.05±0.00	0.04±0.00	0.04±0.00	0.10±0.00*

Total MUFA	29.36±0.03	25.66±0.01*	24.16±0.01*	27.95±0.05*
C18:2	49.82±0.04	27.13±0.03*	25.55±0.06*	43.53±0.03*
C18:3n6	0.03±0.00	0.03±0.00	0.03±0.00	0.06±0.00*
C20:2	0.04±0.00	0.05±0.00ǂ	0.04±0.00	0.08±0.00*
C20:3n6	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
C20:4	0.06±0.00	0.08±0.00*	0.07±0.00*	0.16±0.00*
C20:3n3	0.00±0.00	0.01±0.00*	0.01±0.00*	0.00±0.00
C20:5	0.75±0.00	0.77±0.00	0.72±0.00	1.65±0.01*
C22:2	t	t	t	t
C22:4	0.01±0.00	0.01±0.00	0.01±0.00	0.02±0.00*
C22:5n6	0.01±0.00	0.02±0.00*	0.01±0.00	0.04±0.00*
C22:5n3	0.09±0.00	0.10±0.00*	0.09±0.00	0.21±0.00*
C22:6n3	0.70±0.00	0.75±0.00*	0.70±0.00	1.58±0.01*
Total PUFA	53.66±0.04	59.91±0.02*	60.33±0.02*	51.23±0.04*

Data are presented as mean±S.E.M, n=5-6. ǂ Significantly different from the control diet, $p<0.05$; * significantly different from the control diet, $p<0.001$. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; t, trace.

The expression of miR 133a is sensitive to the flax oil treatment

Downregulation of miR 133 expression after an MI is related to the death of cardiomyocytes as well as the development of ventricular fibrillation. In this study, the expression of miR 133a and -133b was analyzed in the postinfarct cardiac tissue after 8 weeks of CAL surgery. No significant differences in the expression patterns of both the isoforms of miR 133 were observed in the untreated MI group when compared to the sham control (Figure 26). However, flax oil treatment significantly upregulated the expression of miR 133a compared to the control tissue. Interestingly, miR 133b expression was not sensitive to the flax oil treatment.

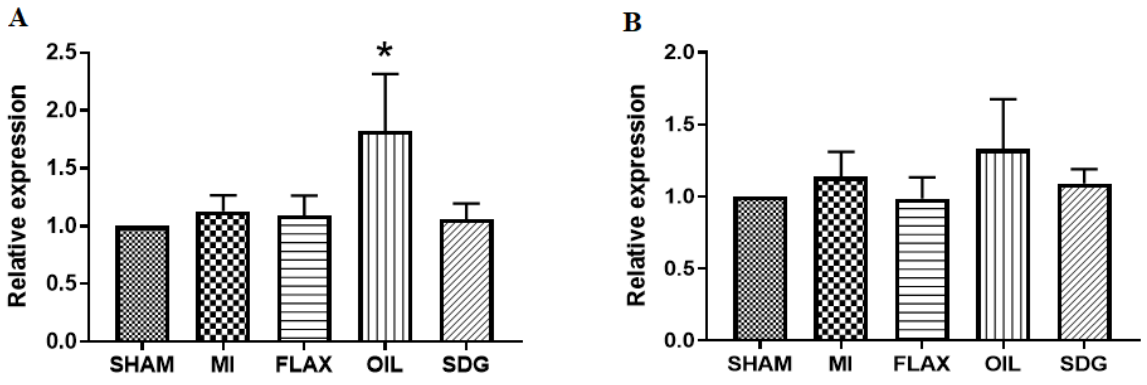


Figure 26. Effect of dietary interventions on miR 133 expression levels. **A**, miR133a. **B**, miR133b. Data are presented as mean±S.E.M, n=5-6. * Significantly different from sham, $p<0.05$.

Upregulation of miR 135a and -29b by the flax oil supplementation

Increased expression of miR 1 is arrhythmogenic in the ischemic hearts, whereas the downregulation of miR 135a and 29b is associated with apoptosis of cardiomyocytes and extensive cardiac fibrosis, respectively. The flax oil supplementation significantly upregulated the expression of miR 135a and -29b in the postinfarct cardiac tissue 8 weeks after CAL surgery (Figure 27). An approximately 3.5-fold and 3.3-fold increase in the expression levels of miR 135a

and -29b, respectively, was observed with respect to the control group, as shown in Figure 27. No such changes were observed in the expression levels of miR 1 amongst experimental groups.

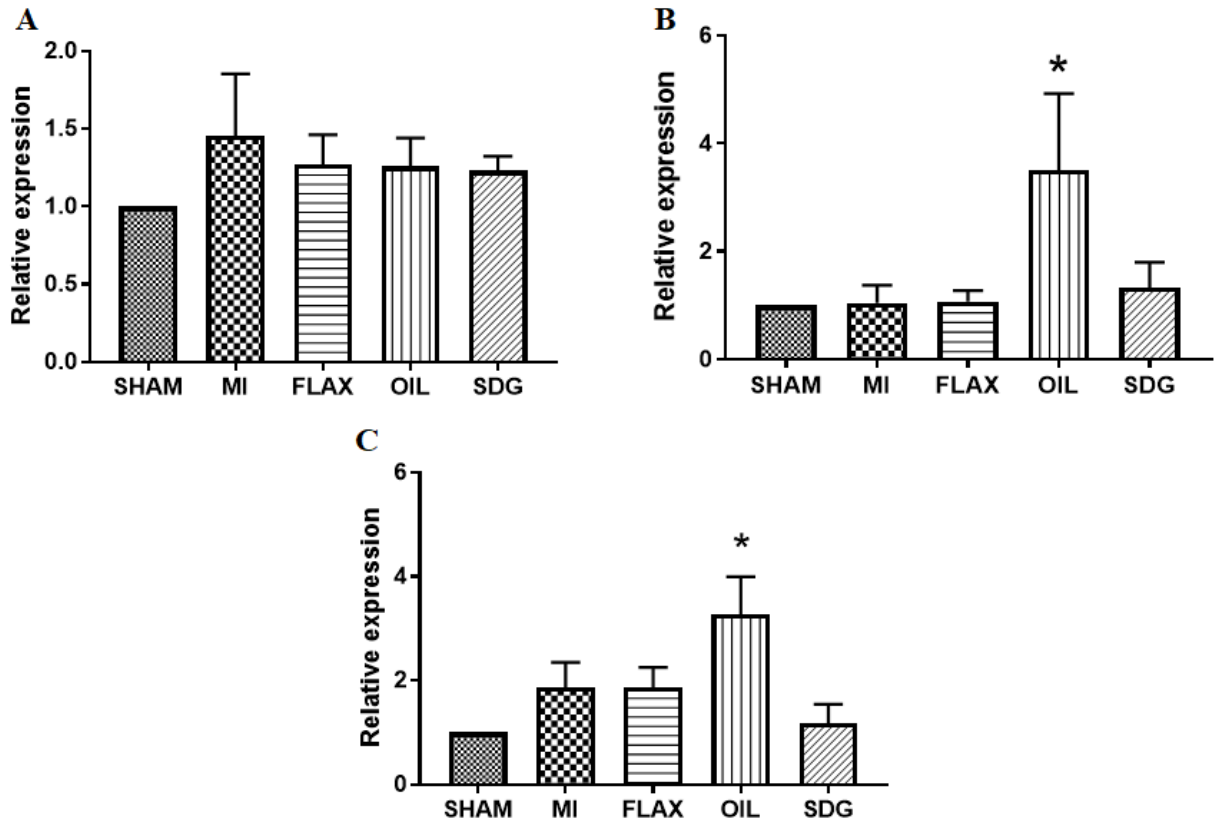


Figure 27. Effect of dietary interventions on miR 1 (A), miR 135a (B) and miR 29b (C) expression levels. Data are presented as mean±S.E.M, n=5-6. * Significantly different from sham, $p<0.05$.

The effect of dietary flaxseed and its components on the expression of collagen I

After observing the effect of flax oil on upregulating the expression of miR 29b, a western blot analysis was performed to evaluate the expression levels of collagen protein. Previous morphometric staining data demonstrated a decrease in fibrosis and mir29b has been associated with a modulation of tissue fibrosis as well (10). A significant 2-fold increase in the levels of

collagen type I and alpha 1 (Col 1α1) protein was found in the untreated MI group compared to the sham control (Figure 28). Dietary supplementation with flax, flax oil, and SDG decreased the protein levels of Col 1α1 by 2.36, 2.69, and 2.13-fold, respectively, compared to the untreated MI group as shown in Figure 28.

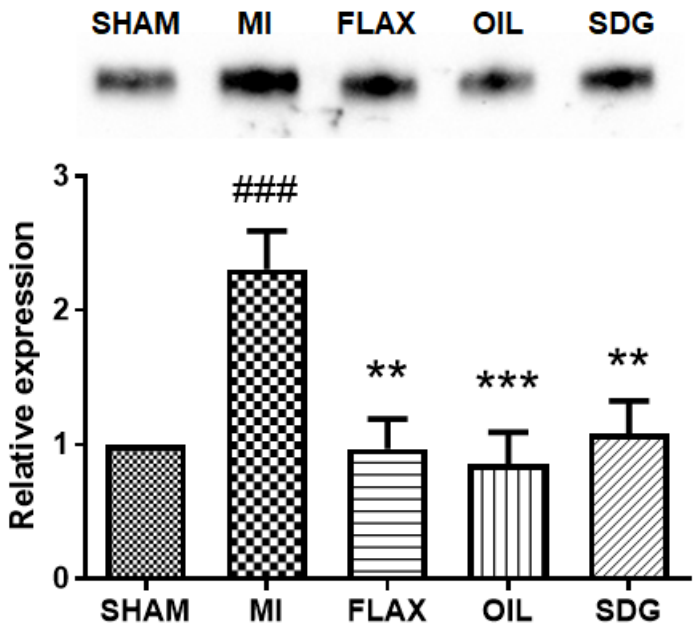


Figure 28. Effect of the dietary interventions on collagen I levels. Data are presented as mean±S.E.M, n=5-6. ### Significantly different from sham, $p<0.001$; *** significantly different from MI, $p<0.001$; and ** significantly different from MI, $p<0.05$.

Discussion

Dietary supplementation with flaxseed before and after an MI has been reported to decrease the incidence of arrhythmias, reduce the myocardial infarct size, decrease cardiac fibrosis, exert an anti-inflammatory effect, and prevent excessive left ventricular dilatation (10). The present study was thus undertaken to: a) elucidate the molecular mechanisms involved in the cardioprotective effect of flaxseed in an MI, and, b) identify the potential bioactive within flaxseed

that may be responsible for the observed effects. In the current study, the data demonstrate that in an animal model of acute MI induced by coronary artery ligation, dietary supplementation with flaxseed regulates the expression of miR 133a, miR 135a, miR 29b, and collagen I.

MiRs are short, non-coding nucleotides that negatively regulate the expression of target genes by mRNA degradation or translational repression. Altered expression profiles of miRs have been associated with various cardiovascular diseases including arrhythmias, MI and heart failure (13, 14). Notably, an essential role of miRs has been reported in several pathological processes of ischemia-induced cardiac damage including cardiac fibrosis and even the death of cardiomyocytes (15, 16). Various nutritional components can modulate the expression of diverse miRs (17). Omega-3 PUFAs, for example, protect cardiomyocytes after an MI by upregulating the anti-apoptotic miRs and downregulating the pro-apoptotic miRs (18, 19). Flaxseed is the richest source of the plant-based omega-3 PUFA, alpha-linolenic acid (ALA). Dietary supplementation with flaxseed has demonstrated antiarrhythmic, antiatherogenic, antihypertensive and cholesterol-lowering effects in animal and human studies (9). Flaxseed and its components modulate the expression of miRs in breast cancer cells (20, 21) and irradiated lung tissue (22). However, no study has examined the effect of flaxseed or ALA on miRs in the setting of an MI.

MiR 133 is expressed in cardiomyocytes and cardiac fibroblasts (23). miR 133, which is frequently associated with an MI, has two isoforms –133a and –133b (24). Our data showed that the expression of miR 133a but not 133b was upregulated by dietary flaxseed oil supplementation in rat hearts 8 weeks after coronary artery ligation. The location of miR -133a and -133b on separate chromosomes may explain why only one of the isoforms was upregulated (25). Overexpression of miR 133 reduces the apoptosis of cardiomyocytes (26). Pro-apoptotic genes such as DAPK2, APAF1, caspase-9, Bcl-2, and BMF have been identified as the target of miR 133

(24). Interestingly, miR 133a also improved cardiac reprogramming to produce beating cardiomyocytes from murine and human fibroblasts by repressing Snai1 which regulates epithelial to mesenchymal transition (27). This is important because cardiomyocytes are unable to replicate and regenerate any lost contractile tissue after an MI (28). By upregulating the expression of cardiogenic miR 133, dietary flaxseed may be a viable strategy to promote cardiac repair.

Our results also demonstrated that flaxseed oil treatment upregulated the expression of miR 135a and -29b after an MI. The upregulation of miR 135a has previously reduced MI size and exerted an anti-apoptotic and antifibrotic effect in a model of ischemia-reperfusion injury (29). miR 29b is highly expressed in cardiofibroblasts and is reported to target numerous genes involved in extracellular matrix production and fibrosis such as collagen isoforms, elastin and matrix metalloproteinases (30-32). An upregulation of miR 135a and miR 29b expression results in decreased myocardial fibrosis (29, 33, 34). Increased accumulation of fibrillar collagen in the cardiac matrix is a hallmark of myocardial fibrosis (35). Type I collagen is the most abundant as it accounts for 85% of the collagen content within the myocardium (36). Previous work from our lab has shown increased histochemical staining after an MI induced by coronary artery ligation as evidence of increased myocardial fibrosis (10). The tissue fibrosis was prevented when flaxseed and flaxseed oil was included in the diet before and after the MI was induced (10). Consistent with these findings, an increased expression of collagen I was detected in the present study after the MI which was normalized when dietary flaxseed oil was included in the diet of the rats. These effects on myocardial fibrosis by miRs 29b and 135a are complemented by the actions of miR 133. miR 133 inhibits the expression of collagen 1A1 and connective tissue growth factor (CTGT) to exert its anti-fibrotic effect (37). During overexpression of miR 133, inflammatory cell infiltration in the myocardium is reduced after an MI (38). Taken together, upregulation of miRs 29b, 135b and 133a

and the subsequent decrease in collagen I levels by flaxseed oil may represent the primary mechanisms through which the ALA content of flaxseed exhibits its anti-fibrotic effect.

The anti-arrhythmic action of dietary flaxseed oil may also be associated with its capacity to alter miR expression. The downregulation of miR 133 has been related to the incidences of ventricular fibrillation in MI patients (39). miR 133 can increase the expression of hyperpolarization-activated cyclic nucleotide-gated ion channel 2 (HCN2) which determines cardiac automaticity (39). Alternatively, miR 135a can modulate the expression of the thioredoxin-interacting protein (TXNIP), protein tyrosine phosphatase 1B (PTP1B) protein, and the transient receptor potential melastatin 7 (TRPM7) channel (29, 40). miR 135a also targets the sodium-calcium exchanger type 1 (NCX1) (41). NCX1 activity can be arrhythmogenic causing early- and delayed-after depolarizations (41). In addition, increased expression of miR 135a can reduce the spontaneous beating frequency of rat neonatal cardiomyocytes (41). Previous work from our lab has shown dietary flaxseed and flaxseed oil is antiarrhythmic in models of ischemia-reperfusion injury to the heart (42) and coronary artery ligation-induced MI (10). Flaxseed was particularly effective against VF (42) and decreased expression of miR 133 increases the incidence of VF (39). The capacity of dietary flaxseed oil to induce the expression of miRs 133 and 135a may, therefore, represent the anti-arrhythmic mechanism of dietary flaxseed oil, likely through an action on NCX1. Consistent with this line of reasoning, we have previously demonstrated that ALA can inhibit NCX activity (43). Overall, these studies suggest that the reduced MI size, fibrosis, inflammation and incidence of arrhythmias shown previously (10) may have been associated with the ALA content of dietary flaxseed.

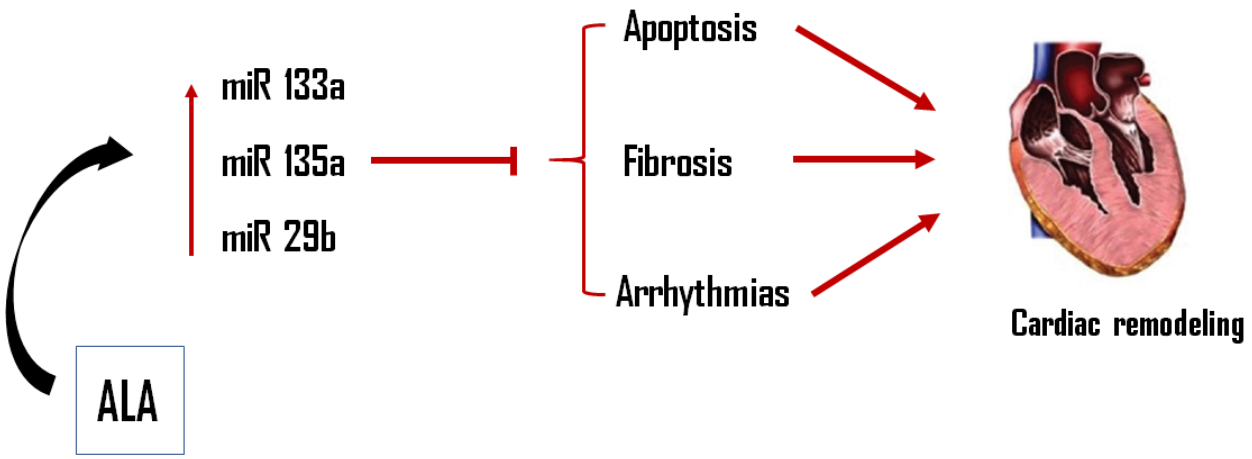


Figure 29. ALA-rich flaxseed oil upregulates the expression of miR 133a, miR 135a, and miR 29b and consequently exerts antiapoptotic, antifibrotic and antiarrhythmic effects.

In summary, this study has provided the first evidence that miR 133a, 135a, and 29b are sensitive to dietary flaxseed oil and associated with significant cardioprotection in a model of MI. The present study also suggests the cardioprotective effect of flaxseed in the setting of an acute MI is mediated, at least in part, via its ALA content. These data complement previous studies on hypertension and atherosclerosis that have also attributed the health benefits of dietary flaxseed to its content of ALA (44-47). Overall, the experimental evidence that has accumulated on the beneficial actions of dietary flaxseed and ALA on cardiovascular disease may warrant serious consideration of the use of these dietary interventions as a viable strategy to reduce cardiovascular disease globally.

References

1. Benjamin EJ, Muntner P, Alonso A, Bittencourt MS, Callaway CW, Carson AP, et al. Heart disease and stroke statistics-2019 update: A report from the american heart association. Circulation. 2019;139(10):e56-e528.

2. Sun Y. Myocardial repair/remodelling following infarction: Roles of local factors. Cardiovascular research. 2009;81(3):482-90.

3. Kura B, Parikh M, Slezak J, Pierce GN. The influence of diet on micrornas that impact cardiovascular disease. Molecules (Basel, Switzerland). 2019;24(8).

4. Fiedler J, Thum T. Micrornas in myocardial infarction. Arteriosclerosis, thrombosis, and vascular biology. 2013;33(2):201-5.

5. Catalanotto C, Cogoni C, Zardo G. Microrna in control of gene expression: An overview of nuclear functions. Int J Mol Sci. 2016;17(10):1712.

6. Liu X, Zhang Y, Du W, Liang H, He H, Zhang L, et al. Mir-223-3p as a novel microrna regulator of expression of voltage-gated k+ channel kv4.2 in acute myocardial infarction. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology. 2016;39(1):102-14.

7. Kim GH. Microrna regulation of cardiac conduction and arrhythmias. Translational research : the journal of laboratory and clinical medicine. 2013;161(5):381-92.

8. Hu Y, Hu FB, Manson JE. Marine omega-3 supplementation and cardiovascular disease: An updated meta-analysis of 13 randomized controlled trials involving 127 477 participants. Journal of the American Heart Association. 2019;8(19):e013543.

9. Parikh M, Netticadan T, Pierce GN. Flaxseed: Its bioactive components and their cardiovascular benefits. American journal of physiology Heart and circulatory physiology. 2018;314(2):H146-h59.

10. Parikh M, Raj P, Austria JA, Yu L, Garg B, Netticadan T, et al. Dietary flaxseed protects against ventricular arrhythmias and left ventricular dilation after a myocardial infarction. The Journal of nutritional biochemistry. 2019;71:63-71.

11. Lionetti V, Tuana BS, Casieri V, Parikh M, Pierce GN. Importance of functional food compounds in cardioprotection through action on the epigenome. European heart journal. 2019;40(7):575-82.

12. Bassett CMC, McCullough RS, Edel AL, Patenaude A, LaVallee RK, Pierce GN. The α-linolenic acid content of flaxseed can prevent the atherogenic effects of dietary trans fat. American Journal of Physiology-Heart and Circulatory Physiology. 2011;301(6):H2220-H6.

13. Thum T, Galuppo P, Wolf C, Fiedler J, Kneitz S, van Laake LW, et al. Micrornas in the human heart: A clue to fetal gene reprogramming in heart failure. Circulation. 2007;116(3):258-67.

14. Thum T, Gross C, Fiedler J, Fischer T, Kissler S, Bussen M, et al. Microrna-21 contributes to myocardial disease by stimulating map kinase signalling in fibroblasts. Nature. 2008;456(7224):980-4.

15. Wang JX, Zhang XJ, Li Q, Wang K, Wang Y, Jiao JQ, et al. Microrna-103/107 regulate programmed necrosis and myocardial ischemia/reperfusion injury through targeting fadd. Circulation research. 2015;117(4):352-63.

16. van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS, et al. Dysregulation of microrna after myocardial infarction reveals a role of mir-29 in cardiac fibrosis.

Proceedings of the National Academy of Sciences of the United States of America. 2008;105(35):13027-32.

17. Kura B, Parikh M, Slezak J, Pierce GN. The influence of diet on micrornas that impact cardiovascular disease. Molecules (Basel, Switzerland). 2019;24(8):1509.

18. Zheng Z, Ge Y, Zhang J, Xue M, Li Q, Lin D, et al. Pufa diets alter the microrna expression profiles in an inflammation rat model. Molecular medicine reports. 2015;11(6):4149-57.

19. Ma H, Chen P, Sang C, Huang D, Geng Q, Wang L. Modulation of apoptosis-related micrornas following myocardial infarction in fat-1 transgenic mice vs wild-type mice. Journal of cellular and molecular medicine. 2018;22(11):5698-707.

20. LeMay-Nedjelski L, Mason-Ennis JK, Taibi A, Comelli EM, Thompson LU. Omega-3 polyunsaturated fatty acids time-dependently reduce cell viability and oncogenic microrna-21 expression in estrogen receptor-positive breast cancer cells (mcf-7). Int J Mol Sci. 2018;19(1).

21. Taibi A, Lin Z, Tsao R, Thompson LU, Comelli EM. Effects of flaxseed and its components on mammary gland mirnome: Identification of potential biomarkers to prevent breast cancer development. Nutrients. 2019;11(11).

22. Christofidou-Solomidou M, Pietrofesa R, Arguiri E, McAlexander MA, Witwer KW. Dietary flaxseed modulates the mirna profile in irradiated and non-irradiated murine lungs: A novel mechanism of tissue radioprotection by flaxseed. Cancer biology & therapy. 2014;15(7):930-7.

23. Li N, Zhou H, Tang Q. Mir-133: A suppressor of cardiac remodeling? Frontiers in Pharmacology. 2018;9(903).

24. Chistiakov DA, Orekhov AN, Bobryshev YV. Cardiac-specific mirna in cardiogenesis, heart function, and cardiac pathology (with focus on myocardial infarction). Journal of molecular and cellular cardiology. 2016;94:107-21.

25. Liu Y, Liang Y, Zhang JF, Fu WM. Microrna-133 mediates cardiac diseases: Mechanisms and clinical implications. Experimental cell research. 2017;354(2):65-70.

26. Bostjancic E, Zidar N, Stajner D, Glavac D. Microrna mir-1 is up-regulated in remote myocardium in patients with myocardial infarction. Folia biologica. 2010;56(1):27-31.

27. Muraoka N, Yamakawa H, Miyamoto K, Sadahiro T, Umei T, Isomi M, et al. Mir-133 promotes cardiac reprogramming by directly repressing snai1 and silencing fibroblast signatures. EMBO J. 2014;33(14):1565-81.

28. Eschenhagen T, Bolli R, Braun T, Field LJ, Fleischmann BK, Frisen J, et al. Cardiomyocyte regeneration: A consensus statement. Circulation. 2017;136(7):680-6.

29. Wu Y, Liu Y, Pan Y, Lu C, Xu H, Wang X, et al. Microrna-135a inhibits cardiac fibrosis induced by isoproterenol via trpm7 channel. Biomedicine & Pharmacotherapy. 2018;104:252-60.

30. Fiedler J, Thum T. Micrornas in myocardial infarction. Arteriosclerosis, thrombosis, and vascular biology. 2013;33(2):201-5.

31. van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS, et al. Dysregulation of micrornas after myocardial infarction reveals a role of mir-29 in cardiac fibrosis. Proceedings of the National Academy of Sciences. 2008;105(35):13027-32.

32. Kriegel AJ, Liu Y, Fang Y, Ding X, Liang M. The mir-29 family: Genomics, cell biology, and relevance to renal and cardiovascular injury. Physiological Genomics. 2012;44(4):237-44.

33. Zhang Y, Huang XR, Wei LH, Chung AC, Yu CM, Lan HY. Mir-29b as a therapeutic agent for angiotensin ii-induced cardiac fibrosis by targeting tgf-beta/smad3 signaling. Molecular therapy : the journal of the American Society of Gene Therapy. 2014;22(5):974-85.

34. Zhu J-N, Chen R, Fu Y-H, Lin Q-X, Huang S, Guo L-L, et al. Smad3 inactivation and mir-29b upregulation mediate the effect of carvedilol on attenuating the acute myocardium infarction-induced myocardial fibrosis in rat. PLOS ONE. 2013;8(9):e75557.

35. Kong P, Christia P, Frangogiannis NG. The pathogenesis of cardiac fibrosis. Cell Mol Life Sci. 2014;71(4):549-74.

36. Worke LJ, Barthold JE, Seelbinder B, Novak T, Main RP, Harbin SL, et al. Densification of type i collagen matrices as a model for cardiac fibrosis. Advanced Healthcare Materials. 2017;6(22):1700114.

37. Castoldi G, Di Gioia CR, Bombardi C, Catalucci D, Corradi B, Gualazzi MG, et al. Mir-133a regulates collagen 1a1: Potential role of mir-133a in myocardial fibrosis in angiotensin ii-dependent hypertension. Journal of cellular physiology. 2012;227(2):850-6.

38. Xiao Y, Zhao J, Tuazon JP, Borlongan CV, Yu G. Microrna-133a and myocardial infarction. Cell Transplant. 2019;28(7):831-8.

39. Bostjancic E, Brandner T, Zidar N, Glavac D, Stajer D. Down-regulation of mir-133a/b in patients with myocardial infarction correlates with the presence of ventricular fibrillation. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie. 2018;99:65-71.

40. Wang S, Cheng Z, Chen X, Xue H. Microrna-135a protects against myocardial ischemia-reperfusion injury in rats by targeting protein tyrosine phosphatase 1b. Journal of Cellular Biochemistry. 2019;120(6):10421-33.

41. Duong E, Xiao J, Qi XY, Nattel S. Microrna-135a regulates sodium-calcium exchanger gene expression and cardiac electrical activity. Heart rhythm. 2017;14(5):739-48.

42. Ander BP, Weber AR, Rampersad PP, Gilchrist JSC, Pierce GN, Lukas A. Dietary flaxseed protects against ventricular fibrillation induced by ischemia-reperfusion in normal and hypercholesterolemic rabbits. The Journal of Nutrition. 2004;134(12):3250-6.

43. Ander BP, Hurtado C, Raposo CS, Maddaford TG, Deniset JF, Hryshko LV, et al. Differential sensitivities of the ncx1.1 and ncx1.3 isoforms of the na+-ca2+ exchanger to alpha-linolenic acid. Cardiovascular research. 2007;73(2):395-403.

44. Rodriguez-Leyva D, Weighell W, Edel AL, LaVallee R, Dibrov E, Pinneker R, et al. Potent antihypertensive action of dietary flaxseed in hypertensive patients. Hypertension (Dallas, Tex : 1979). 2013;62(6):1081-9.

45. Bassett CM, McCullough RS, Edel AL, Patenaude A, LaVallee RK, Pierce GN. The alpha-linolenic acid content of flaxseed can prevent the atherogenic effects of dietary trans fat. American journal of physiology Heart and circulatory physiology. 2011;301(6):H2220-6.

46. Dupasquier CM, Dibrov E, Kneesh AL, Cheung PK, Lee KG, Alexander HK, et al. Dietary flaxseed inhibits atherosclerosis in the ldl receptor-deficient mouse in part through antiproliferative and anti-inflammatory actions. American journal of physiology Heart and circulatory physiology. 2007;293(4):H2394-402.

47. Francis AA, Deniset JF, Austria JA, LaVallee RK, Maddaford GG, Hedley TE, et al. Effects of dietary flaxseed on atherosclerotic plaque regression. American journal of physiology Heart and circulatory physiology. 2013;304(12):H1743-51.

2.3. Dietary flaxseed and myocardial injury as part of metabolic syndrome

After investigating the effect of flaxseed in a surgically induced model of myocardial infarction, further examinations were carried out in the JCR:LA-*cp* genetic rat model of metabolic syndrome. Unlike the experimental model used in the first study, JCR:LA-*cp* rats are reported to develop spontaneous myocardial ischemia. In addition, this rat strain is also claimed to exhibit atherosclerosis and hypertension.

It was hypothesized that dietary flaxseed supplementation in the presence or absence of a high-fat, high sucrose diet will alter the incidence of myocardial lesions, BP hemodynamics, atherosclerosis, and cardiac function in male and female JCR:LA-*cp* rats.

Dietary Flaxseed Reduces Myocardial Ischemic Lesions, Improves Cardiac Function and Lowers Cholesterol Levels Despite the Presence of Severe Obesity in JCR:LA-c*p* Rats

(To be Submitted)

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Abstract

The purpose of this study was to determine the effect of dietary flaxseed supplementation on myocardial ischemic lesions as well as the impact of a high-fat, high sucrose (HFHS) diet on the development of hypertension, atherosclerosis, and cardiac dysfunction in JCR:LA-*cp* rats, a model of genetic obesity. Twelve-week-old male and female obese rats were randomized into four groups (n=8 each) to receive, for 12 weeks, either a) control diet (Con), b) control diet supplemented with 10% ground flaxseed (CFlax), c) HFHS diet, and d) HFHS supplemented with 10% ground flaxseed (HFlax). Male and female lean rats served as control animals and received similar dietary

interventions. 24-week old male and female obese JCR rats had higher plasma levels of total cholesterol, LDL-C, HDL-C, and TGs compared to the lean control animals. However, no such changes were observed in the plasma glucose levels between the two genotypes. Dietary flaxseed supplementation in the CFlax diet lowered the levels of total cholesterol and LDL-C significantly compared to the Con diet in male obese rats. Compared to the Con, the HFHS diet administration in male obese rats resulted in increased liver weights and liver enzyme levels which were significantly reduced in the HFlax group. Although lean female rats showed less marked metabolic changes, the HFHS diet resulted in reduced fractional shortening and increased left ventricular dilatation (LVID) as well as end-systolic volume (ESV). Flaxseed supplemented HFlax diet improved cardiac structure and function in lean female rats compared to the HFHS. Obese rats on the HFHS diet exhibited increased myocardial ischemic lesions and diastolic dysfunction regardless of sex. The HFlax diet significantly lowered the frequency of cardiac lesions and improved diastolic function in male and female obese rats compared to the HFHS diet. After 12 weeks on the Con diet, the blood pressure values were similar in obese and lean rats and en face aortic analysis for atherosclerotic lesions revealed no detectable aortic lesions. Surprisingly, the HFHS diet did not elevate blood pressure or induce detectable atherosclerotic lesions. Collectively, this trial shows: i) a HFHS diet can increase myocardial ischemic lesion frequency and can exacerbate cardiac dysfunction in lean and obese animals; ii) a HFHS diet abolished the protective effect of female sex on cardiac function; iii) a lack of hypertension and atherosclerosis in JCR:LA-*cp* rats despite severe obesity even after the administration of an atherogenic diet; iv) dietary flaxseed reduced myocardial ischemic lesions, exerted a cholesterol-lowering effect, improved liver enzyme levels and ameliorated cardiac dysfunction. Taken together, important outcomes from this study are two-fold. First, flaxseed could be an effective strategy against various aspects

of cardiac injury exhibited in an obese condition of cardio-metabolic syndrome, and second, this rodent model may be valuable for obesity studies where the confounding factors of hypertension or atherosclerosis are to be excluded.

Introduction

Flaxseed is one of the richest plant sources of the n-3 PUFA alpha linolenic acid (ALA). Recently, flaxseed supplementation decreased myocardial infarct (MI) size, reduced the incidence of arrhythmias, limited cardiac fibrosis, and prevented excessive left ventricular dilation (1). However, these actions of dietary flaxseed were achieved in a surgically induced model of MI, the coronary artery ligation-induced MI (2). The capacity for dietary flaxseed to reduce MI's in a naturally occurring model of MI would represent important information more relevant to the clinical situation.

Although there are several small animal models that manifest cardiac damage, each has its own advantages and limitations (3). The widely studied $ApoE^{-/-}$ and $LDLR^{-/-}$ mice models develop severe dyslipidemia and atherosclerosis but do not exhibit MIs (4). The *db/db* and *ob/ob* murine models develop obesity but again do not demonstrate myocardial ischemia (4). Rat models such as the spontaneously hypertensive obese rat, a rat fed a high fat/high sucrose diet, as well as the genetically manipulated rat models such as fatty Zucker and Zucker diabetic fatty rats exhibit aspects of metabolic syndrome and cardiac dysfunction but they do no develop atherosclerosis or myocardial ischemia (4, 5). Only JCR:LA-*cp* rat model has been reported to spontaneously exhibit cardiovascular dysfunction associated with metabolic syndrome, MI, stroke, hypertension, atherosclerosis and heart failure (4, 6-10). The JCR:LA-*cp* is a unique rat strain with autosomal recessive corpulent trait affecting the leptin receptor gene. Homozygous (cp/cp) JCR rats have a

total absence of functional leptin receptors and are obese-prone, while heterozygous (cp/?) are lean-prone and metabolically normal (11).

Overweight and obese individuals are at a 75% risk of developing primary hypertension (12). Hypertension, a leading cause of death, is a strongly modifiable cardiovascular risk factor (13). The cellular mechanisms contributing to the obesity-related hypertension development remain less clear. However, the renin-angiotensin-aldosterone system, increased sympathetic activity, high sodium diet, leptin resistance, and endothelial dysfunction are believed to be involved as the causative factors (12). Obesity also accounts for the increased risk of atherosclerotic vascular disease (14). Endothelial dysfunction, inflammation, adipocyte imbalance and oxidative stress are some of the cellular mechanisms involved in linking obesity and atherosclerosis (14). The JCR:LA-*cp* obese rat model of metabolic syndrome is purported to develop hypertension and atherosclerosis (12). However, due to complications of different diets, limited data and the use of different control rats for comparison, controversy exists whether obese JCR:LA-*cp* rats manifest high blood pressure (BP) or are normotensive (11, 15). Thus, a more comprehensive, carefully controlled study is warranted to clarify the status of ischemic lesions, hypertension and atherosclerosis in this model of obesity and metabolic syndrome – the JCR:LA-*cp* rats. The objectives of the present study were thus a) to verify if a high-fat, high sucrose diet produces sex-dependent pathological effects; b) to investigate whether JCR:LA-*cp* rat model develops hypertension and atherosclerosis and if these parameters exhibit sex differences in their expression; c) to study whether dietary flaxseed supplementation in the presence or absence of a high-fat, high sucrose diet will alter the incidence of myocardial lesions, BP hemodynamics, atherosclerosis, and cardiac function in male and female JCR:LA-*cp* rats.

Materials and Methods

Animal model and experimental protocol

This study protocol (18-022) was approved by the University of Manitoba Office of Research Ethics & Compliance and was done in accordance with the guidelines by the Canadian Council for Animal Care. Obese (*cp/cp*) and lean (*cp/?*) JCR:LA-*cp* rats were raised from the colony established at the University of Alberta (4, 8). Male and female animals of both genotypes were housed in a temperature and humidity-controlled room with a 12 h light/dark cycle. At 12 weeks of age, obese JCR:LA-*cp* rats of both sexes (each n=32) were randomized to receive either a) regular chow diet (Con), b) 10% ground flaxseed supplemented regular chow diet (CFlax), c) high-fat, high sucrose diet (HFHS), or d) 10% ground flaxseed supplemented HFHS diet (HFlax), for 12 weeks. Age-matched male and female lean (*cp/?*) JCR rats served as control animals and were given similar diets for 12 weeks.

The regular chow was a Prolab® RMH 3000 regular rodent chow, and the high-fat, high sucrose diet included AIN-93G chow with 35% fat (lard) and 36% carbohydrate (mostly sucrose) (TestDiet, Richmond, IN). The ground flaxseed was BakePur milled ground flaxseed obtained from Pizzey Ingredients, Russell, Manitoba, Canada. Throughout the study duration, animals had free access to water and food. Food intake was measured daily and then averaged to obtain weekly estimates. Body weights were measured every week. After 12 weeks of dietary intervention, the protocol was completed and the 24-week old JCR:LA-cp rats were terminated.

Blood pressure monitoring

Blood pressures monitoring (BP) was carried out at three-time points: baseline (12 weeks old), after 6 weeks on the diets (18 weeks old), and at the completion of the study (24 weeks old).

BP recording was performed using the CODA non-invasive blood pressure acquisition system for rats (Kent Scientific, Torrington, CT, USA) as previously described (16). Briefly, rats were placed in a rodent restrainer and the occlusion cuff was placed at the base of the tail and the Volume Pressure Recording (VPR) sensor cuff was adjacent to the occlusion cuff. Animals were allowed to acclimatize for 15 min in this position. Heating pads were preheated to 35 °C and rats were warmed for 5 min prior to the recording session. The occlusion cuff was inflated to 250 mm Hg and deflated over 15 s to measure blood pressure. The VPR sensor cuff detects changes in the tail volume as the blood returns to the tail during the deflation of the occlusion cuff. 15 μL was set as the minimum volume change. Each recording session consisted of 15 inflation and deflation cycles per set, of which the first 5 cycles were acclimation and were not used in the analysis. Animals were trained one day before the actual BP measurements.

Staining and analysis of atherosclerotic lesions

Aortic atherosclerotic lesions were analyzed en face, as described previously (17). Briefly, aortas were dissected, formalin-fixed, and pinned longitudinally on black wax plates. Aortas were stained with Oil Red O and Sudan IV. Digital images were obtained using a Nikon camera and analyzed using ImageJ version 1.52a software (NIH, USA).

Electrocardiography

An electrocardiogram was recorded for 2 min before performing transthoracic echocardiography in stabilized animals under isoflurane anesthesia. ECG tracings were obtained and analyzed using a BIOPAC data acquisition system.

Transthoracic echocardiography

All experimental rats were weighed and anesthetized with 3% isoflurane in a chamber, and then kept under 1.5–2% isoflurane throughout the procedure. An echocardiogram was obtained for 24-week old animals by 2D guided M-mode and Doppler modalities with a 13 MHz probe (Vivid E9; GE Medical Systems, Milwaukee, WI, USA) as described earlier (1). 2D M-mode parasternal short-axis view images were obtained to determine systolic functional parameters such as the percentage of left ventricle (LV) ejection fraction (EF), fractional shortening (FS), end-systolic volume (ESV) and end-diastolic volume (EDV). Doppler measurements included isovolumic relaxation time (IVRT), mitral valve (MV) E wave, A wave, and E wave deceleration time. The cardiac structural parameters such as interventricular septal thickness (IVS), LV posterior wall thickness (LVPW) and LV internal diameter (LVID) at diastole and systole were determined from parasternal short-axis view images. All echocardiographic images were analyzed to calculate the listed parameters using EchoPAC software (GE Medical Systems, Milwaukee, WI). All measurements were performed blinded and averaged from three cardiac cycles to account for inter-beat variability.

Biological sample collection and analysis

After 12 weeks of feeding, 24-week old animals were fasted overnight (16 hours) and then anesthetized with isoflurane (5%) the following morning. The depth of anesthesia was assessed by a pedal withdrawal reflex. The blood sample was collected from the inferior vena cava by opening the thoracic cavity and the heart was immediately excised. Lungs, heart, and liver organs were isolated, rinsed in PBS and weighed. The plasma was separated by centrifugation (3000 RPM, 4ºC for 15 min) and stored at -80ºC for subsequent biochemical analysis. The fasted plasma biochemical profile was assessed using cobas c 111 (Roche) for total cholesterol, low-density

lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C), triglycerides (TG), glucose, creatinine, urea, alanine aminotransferase (ALT), and aspartate aminotransferase (AST).

Total fatty acids were extracted from the plasma samples and derivatized as previously described (1). The fatty acid methyl esters were separated on a DB225MS column (30 m X 0.25 mm diameter and 0.25 μm film thickness; Agilent Technologies Canada Inc., Mississauga, Ontario) using a Varian 450 GC with FID. The temperature program was 70°C for 2 minutes, the temperature was raised to 180°C at 30°C/minute, held for 1 minute, raised to 200°C at 10°C/minute, held for 2 minutes, then raised to 220°C at 2°C/minute and held for 10 minutes before finally raising to 240°C at 20°C/minute and holding for 15 minutes. Total run time was 46.67 minutes, and samples were run with a 20:1 split ratio and a 1.3ml/min column flow. Hydrogen was used as the carrier gas. Components were identified by comparison with authentic standards (Nu-Chek Prep, Elysian, MN)

Heart histology

Each whole heart tissue was cut into three thick slices from apex to base along the long axis using a tissue slicer (Rodent heart slicer matrix HSRS002:1, Zivic Instruments). After OCT embedding, 7-μm thin sections were obtained using a cryostat (Microm HM 550), and then sections were stained with hematoxylin and eosin (ab245880) from Abcam with the following modification in the protocol. Briefly, tissue slides were fixed in 4% paraformaldehyde for 15 min. After this, tissue sections on slides were stained with hematoxylin (10 min), rinsed in deionized water, placed in bluing reagent (15 sec), rinsed in deionized water and 99% alcohol, stained with eosin (3 min), dehydrated through 3 changes of 99% alcohol, cleared in xylene and mounted. The

heart sections were examined blindly, and myocardial lesions were identified as previously described (7, 18): Stage 1, areas of early necrosis; Stage 2, areas of cell lysis with chronic inflammatory cell infiltration; Stage 3, nodules of chronic inflammatory cell infiltration; and Stage 4, old scarred lesions. Only Stage 2 lesions were identified in this study as stage 1 and stage 2 lesions are very recent, while stage 4 lesions were not detected in the present study. The number of lesions from each heart section was aggregated and the average was calculated for each group (n-8). The images were analyzed using NIS-Elements AR Analysis 4.60, Nikon Instruments.

Statistical analysis

Data were analyzed using 2-factor ANOVA with one factor as genotype and sex. The interaction effect of genotype and diet over time was analyzed with 2-factor repeated measures ANOVA with genotype and diet as factors. Additional information was extracted by separately analyzing male and female data using repeated measures 2-factor ANOVA. The between-group analysis was done by post hoc Tukey's or Sidak multiple comparisons test. The non-parametric data for heart lesion frequency was analyzed by Kruskal-Wallis test and Dunn's multiple comparisons test. Data distribution was assessed by the Kolmogorov–Smirnov test, D'Agostino–Pearson omnibus normality test and Shapiro–Wik normality test. All data are expressed as mean ± S.E.M and analyzed using GraphPad Prism v 7 (GraphPad Software, California, USA). Statistical significance was accepted when $p<0.05$.

Results

Experimental diets

The nutritional composition of the experimental diets is presented in Table 8. The ash, protein, fiber, fat and carbohydrate contents were similar between the Con and CFlax and the

HFHS and HFlax diets. As the latter diets are comprised of higher amounts of fat, the digestible energy provided by the HFHS and HFlax was higher than the Con and CFlax diets (Table 8).

Table 8. Nutritional composition of the experimental diets

Diets	Ash, %	Protein, %	Fibre, %	Fat, %	CHO, %	Protein, kcal/100g	Fat, kcal/100 g	CHO, kcal/100 g	Energy, kcal/g
Con	6.69	25.58	3.99	7.46	55.36	102.32	67.14	221.44	3.9
CFlax	6.58	25.88	4.45	7.09	55.07	103.52	63.81	220.28	3.8
HFHS	2.55	18.35	0.78	37.18	40.20	73.4	334.62	160.8	5.6
HFlax	2.92	20.25	2.42	35.82	37.67	81	322.38	150.68	5.5

Ash, protein, fiber, carbohydrates, and fat (g/100 g diet) are represented as a percentage of total nutrients, measured by proximate analysis (as fed). Caloric content of fat, protein, and CHO constituents in the diet were calculated as 9, 4, and 4 kcal/g. Fiber content of the CHO component was subtracted to yield the total caloric content of CHO. Energy was calculated as the sum of energies of protein, carbohydrate, and fat. CHO, carbohydrates; protein, crude protein; fiber, crude fiber; fat, crude fat. N=3.

Dietary intake and body weights over time in JCR:LA-*cp* rats

The average food intake during the 12 weeks dietary intervention period is shown in Figure 30. The interaction effect of genotype and diet was significant as evident from the 2-way ANOVA analysis. The food intake was significantly higher in obese male JCR:LA-cp rats on the Con and CFlax as compared to the obese male animals on HFHS and HFlax groups, but there were no

significant differences between the food intake of Con and CFlax, and HFHS and HFlax groups. The lower food consumption in the HFHS and HFlax groups could be due to the higher caloric content of these diets when compared with the Con and CFlax. Lean male JCR rats had significantly lower food intake compared to obese male animals. A similar trend was observed for obese and lean female JCR rats. A prominent sex-dependent effect was observed as lean male JCR:LA-*cp* rats on all the four diets had significantly higher food intake compared to lean female JCR rats. However, for obese JCR rats such significant differences existed only for the Con and CFlax group as the average intake was comparable between male and female obese JCR rats on the HFHS and HFlax diets.

Over the course of 12 weeks of dietary intervention, body weight gain was significantly higher in obese male JCR:LA-*cp* rats compared to age-matched lean males, as shown in Figure 31. There was a significant interaction effect of diet and genotype over time as analyzed using 2-way ANOVA with diet and genotype as independent variables. Body weight gain was comparable across the diet groups for the first 8 weeks in obese male JCR:LA-*cp* rats. For the last 4 weeks, the rate of body weight gain in obese male JCR rats on the HFHS and HFlax diets was higher than obese male JCR rats on the Con and CFlax diets, with a mean terminal weight difference of 10-18%. A similar impact of diet and genotype was observed for female JCR:LA-*cp* rats. Obese females had significantly higher body weights compared to lean female JCR rats. After the first 6 weeks, obese female JCR rats on HFHS and HFlax diets had significantly higher body weights compared to obese females on Con and CFlax diets. However, terminal weights were not different between dietary groups. A sex dependent effect was observed as obese and lean male JCR:LA-*cp* rats had significantly higher body weights compared to age-matched obese and lean females.

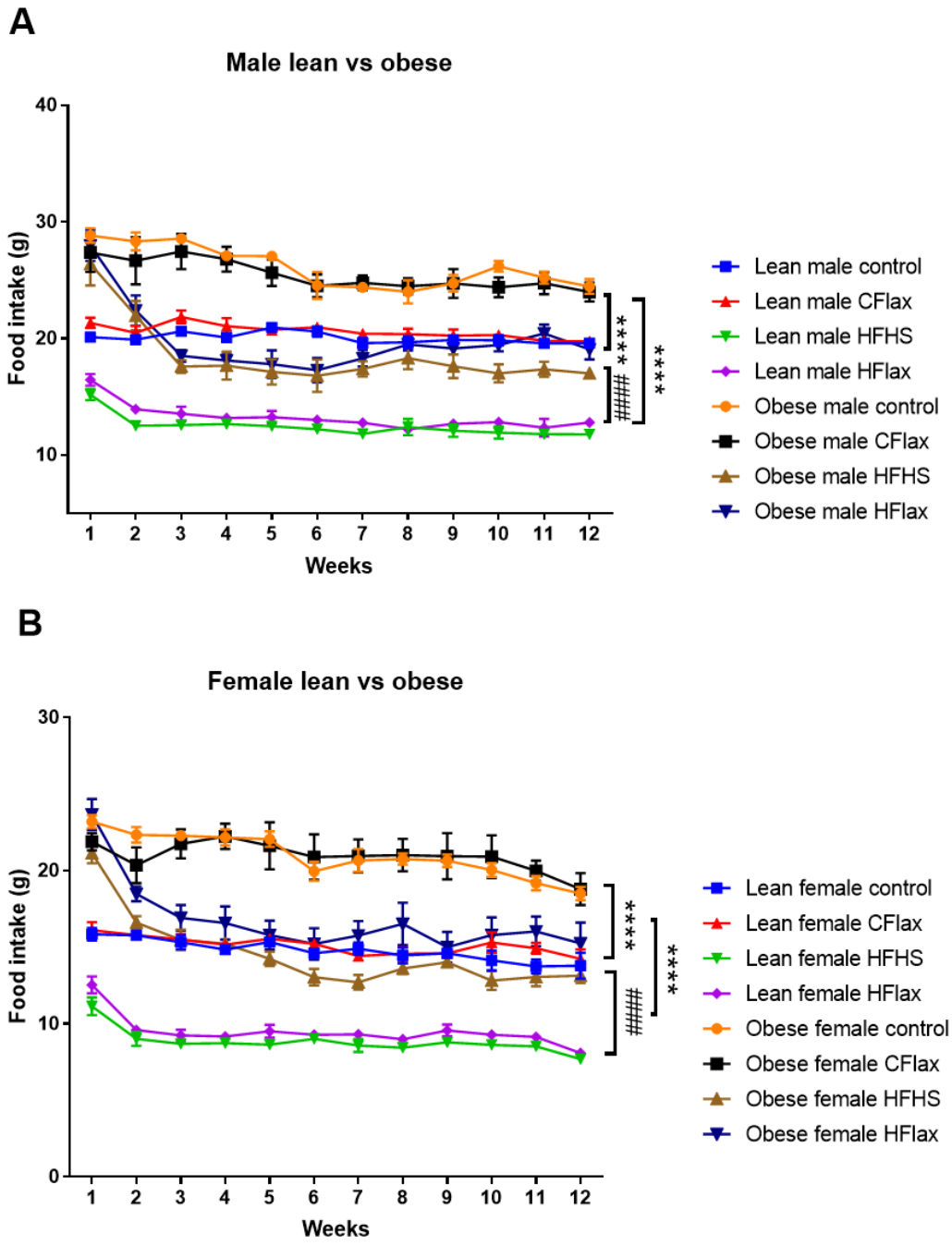


Figure 30. Effect of genotype, diet, and sex on the food intake of JCR:LA-*cp* rats. All values presented are mean±SE. A. Male data; B. Female data; *p<0.0001 versus lean JCR rats on all the four diets, 2-way ANOVA; #p<0.0001 versus respective lean JCR rats on the same diet, 2-way ANOVA. N=8.

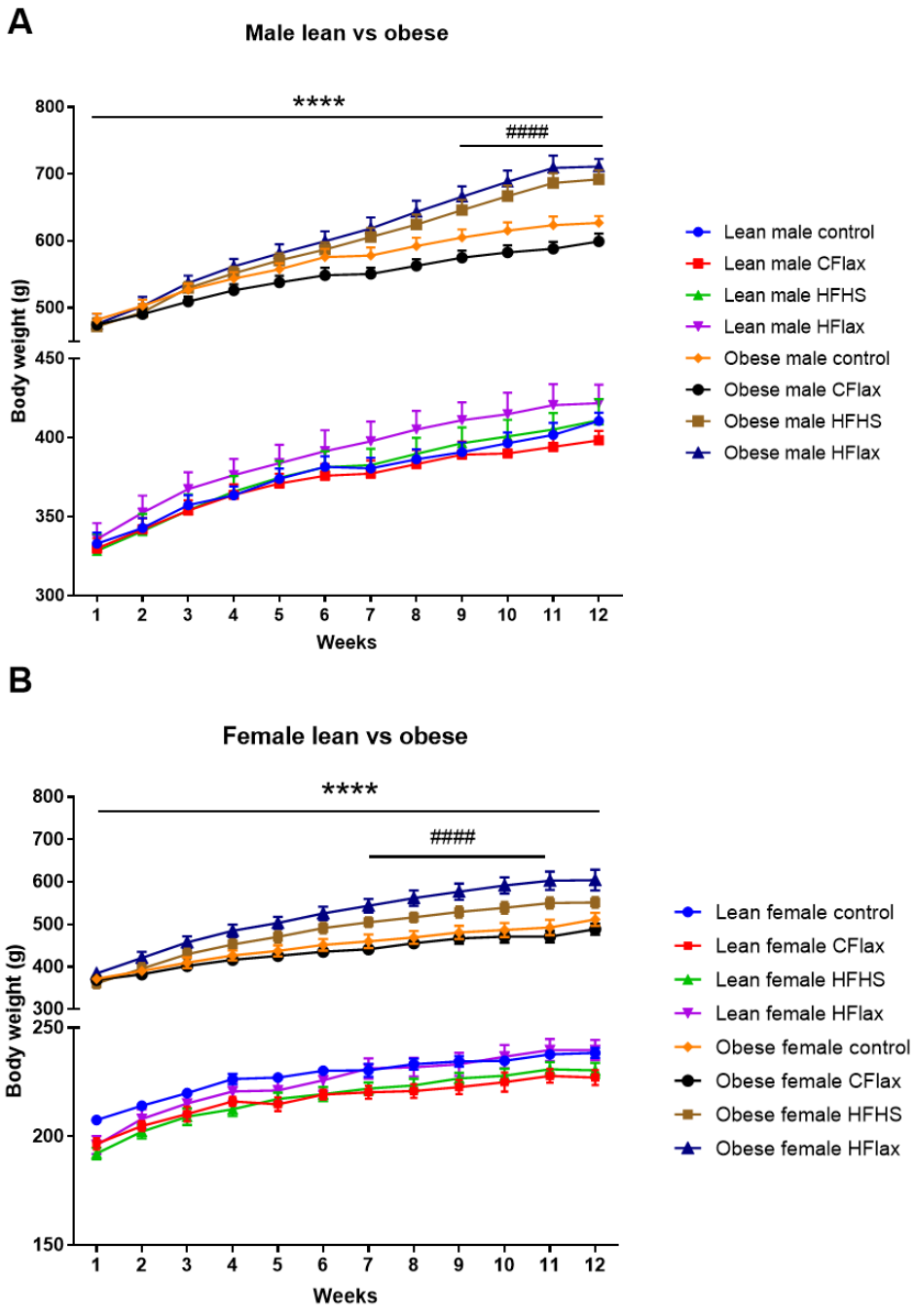


Figure 31. Effect of genotype, diet, and sex on body weights of JCR:LA-*cp* rats. All values presented are mean±SE. A. Male data; B. Female data; *p<0.0001 versus respective lean JCR rats on the same diet, 2-way ANOVA; $^{\#}p$<0.0001 versus respective obese JCR rats on the Con and CFlax, 2-way ANOVA. N=8.

Organ dimensions

Morphological examination revealed increased heart weight in male and female obese JCR rats compared to the respective lean controls (Table 9). The ratio of heart weight to tibial length (index of cardiac hypertrophy) was also significantly higher in obese JCR rats compared to the lean animals in all diet groups, as shown in Table 9. A significant sex-dependent effect was observed as female JCR rats of both genotypes had significantly lower values compared to the respective male JCR rats.

Although no effect of genotype, sex, or diet was observed on lung weight, alterations were observed in liver weight. Male and female obese JCR rats had significantly higher liver weights compared to the respective lean animals. A significant effect of diet and sex was observed using 2-way ANOVA analysis with sex and diet as variables. Male obese JCR rats on the HFHS diet had significantly higher liver weight compared to the obese animals of same sex on the Con diet. Dietary intervention with ground flaxseed for 12 weeks significantly reduced liver weight of male obese JCR rats in the HFlax group compared to the animals of the same sex and genotype on the HFHS diet (Figure 32). A predominant effect of sex was noted as female lean and obese JCR rats had lower liver weights compared to the male JCR rats of respective genotypes (Table 9 and Figure 32).

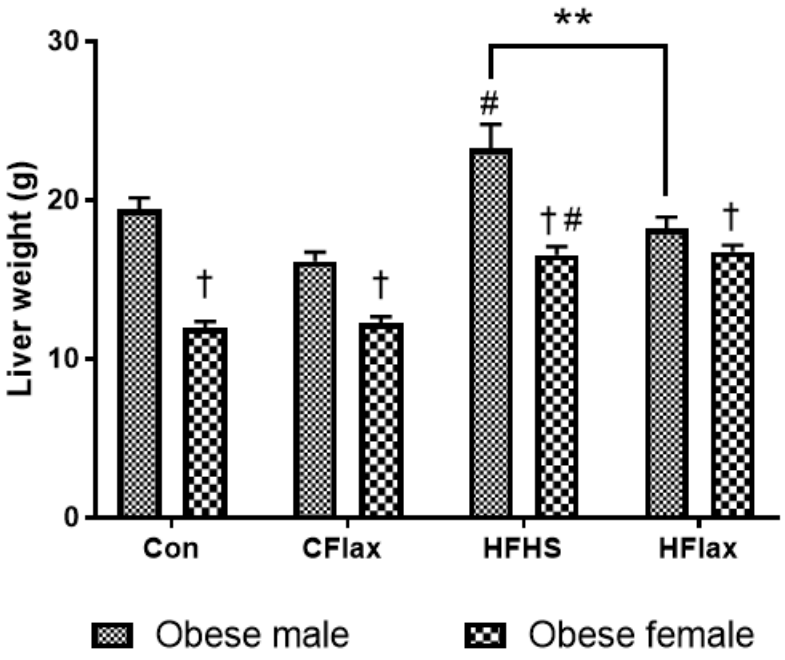


Figure 32. Effect of diet and sex on liver weight. All values presented are mean ± SE. $^{\#}p<0.05$ versus Con diet. $^{**}p<0.01$ versus JCR rats on the HFHS diet. ǂ $p<0.05$ versus respective male JCR rats on the same diet, 2-way ANOVA. N=8.

Table 9. Biometric characteristics of animals

Parameters	Genotype	Sex	Diet			
			Con	CFlax	HFHS	HFlax
Heart weight, g	Lean	Male	0.88±0.03[a]	0.89±0.02[a]	0.88±0.02[a]	0.93±0.03[a]
		Female	0.63±0.01[a‡]	0.60±0.02[a‡]	0.60±0.00[a‡]	0.63±0.01[a‡]
	Obese	Male	1.14±0.03[a,b****]	1.10±0.01[a****]	1.18±0.02[b,c****]	1.22±0.02[c****]
		Female	0.93±0.02[a****‡]	0.90±0.02[a****‡]	0.99±0.03[a,b****‡]	1.06±0.04[b****‡]
Heart weight/Tibial length, g/cm	Lean	Male	0.20±0.01[a]	0.21±0.01[a]	0.20±0.01[a]	0.21±0.01[a]
		Female	0.17±0.01[a‡]	0.17±0.01[a‡]	0.16±0.00[a‡]	0.16±0.00[a‡]
	Obese	Male	0.26±0.01[a,b****]	0.25±0.00[a****]	0.26±0.01[a,b****]	0.27±0.00[b****]
		Female	0.23±0.01[a,b****‡]	0.22±0.00[a****‡]	0.25±0.01[a,b****]	0.26±0.01[b****]
Lung weight, g	Lean	Male	4.81±0.32[a]	5.72±0.64[a]	4.72±0.18[a]	5.30±0.26[a]

		Female	5.11±0.56[a]	4.90±0.43[a]	5.02±0.21[a]	4.38±0.31[a]
	Obese	Male	4.77±0.19[a]	4.56±0.28[a]	4.83±0.25[a]	5.11±0.43[a]
		Female	4.54±0.15[a]	4.74±0.19[a]	4.88±0.36[a]	5.31±0.50[a]
Liver weight, g	Lean	Male	8.69±0.25[a]	8.62±0.19[a]	8.17±0.26[a]	8.12±0.32[a]
		Female	6.17±0.36[a,b‡]	6.49±0.40[a‡]	5.10±0.16[b‡]	5.39±0.20[a,b‡]

Values are presented as mean ± SE. The liver and lung weights were calculated as a ratio of dry/wet weights. Row means without a common superscript letter (a, b, and c) differ significantly, $p<0.05$. ****$p<0.0001$ versus respective lean JCR rats on the same diet, 2-way ANOVA. ‡$p<0.05$ versus respective male JCR rats on the same diet, 2-way ANOVA. Con, control diet; CFlax, 10% ground flaxseed supplemented control diet; HFHS, high-fat high sucrose diet; HFlax, 10% ground flaxseed supplemented high-fat high sucrose diet. N=8.

Plasma biochemistry

Plasma total cholesterol levels were significantly higher in male and female obese JCR rats on all the four diets compared to the respective age-matched lean controls. 2-way ANOVA analysis showed a significant interaction effect between sex and diet. Interestingly, female lean JCR rats in all of the four diet groups had higher plasma cholesterol values compared to the male lean JCR rats, as shown in Figure 33. Importantly, dietary flaxseed supplementation for 12 weeks significantly decreased total plasma cholesterol levels in male obese JCR rats on the CFlax diet by ~24% compared to the Con diet. A similar effect of flaxseed was observed for low-density lipoprotein-cholesterol levels (LDL-C). Male obese JCR rats in the CFlax group had a significant reduction of ~37% in LDL-C levels compared to the Con group (Figure 33).

Plasma high-density lipoprotein-cholesterol (HDL-C) and triglycerides (TG) levels were also significantly higher in obese JCR rats compared to age-matched lean JCR rats (Table 10). A sex-dependent effect was noted for lean and obese female JCR rats for HDL-C values compared to the respective male JCR rats on the similar diets. No such impact of genotype, sex or diet was observed for plasma glucose levels.

Plasma creatinine and urea levels were measured for assessing the renal function of JCR:LA-*cp* rats. A significant interaction effect of genotype and diet was observed using 2-way ANOVA with genotype and diet as independent factors. Of interest, male and female obese JCR rats on the HFHS and HFlax exhibited significantly lower values of plasma creatinine compared to the respective lean controls on the same diets (Table 10). Although no impact of genotype or sex was observed, a similar effect of diet on serum urea levels was also found. Male and female JCR rats of both genotypes on the HFHS and HFlax diet had significantly lower values of plasma urea compared to the respective male animals on the same diets (Table 10).

A substantial decrease in liver weight was observed as a result of flaxseed supplementation. Plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were estimated as markers of hepatic function. Male and female obese JCR rats on the HFHS diet had significantly higher plasma levels of ALT and AST compared to respective lean JCR rats on the same diet. A significant effect of diet was observed using 2-way ANOVA with diet and genotype as factors. Notably, male obese JCR rats on the HFlax diet had a significant ~49% and ~39% reduction in ALT and AST, respectively, compared to the male obese JCR rats on the HFHS diet (Figure 34).

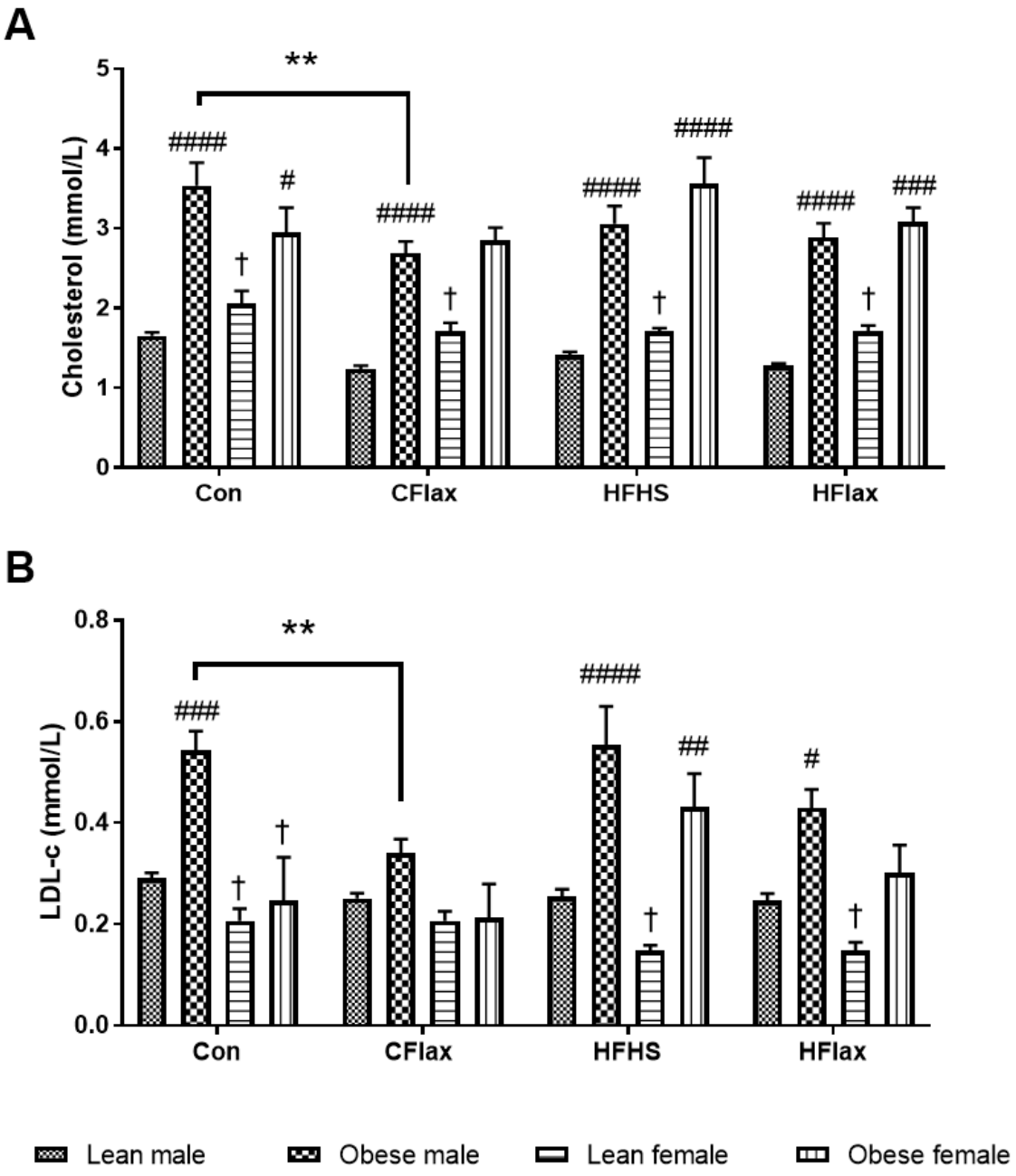


Figure 33. Effect of genotype, sex and diet on lipid levels. All values presented are mean±SE. (A), cholesterol; and (B), LDL-C, low-density lipoprotein-cholesterol. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, and $^{\#\#\#\#}p<0.0001$ versus respective lean JCR rats on the same diet, 2-way ANOVA. $^{**}p<0.01$ versus JCR rats on the Con diet. ǂ $p<0.05$ versus respective male JCR rats on the same diet, 2-way ANOVA. N=8.

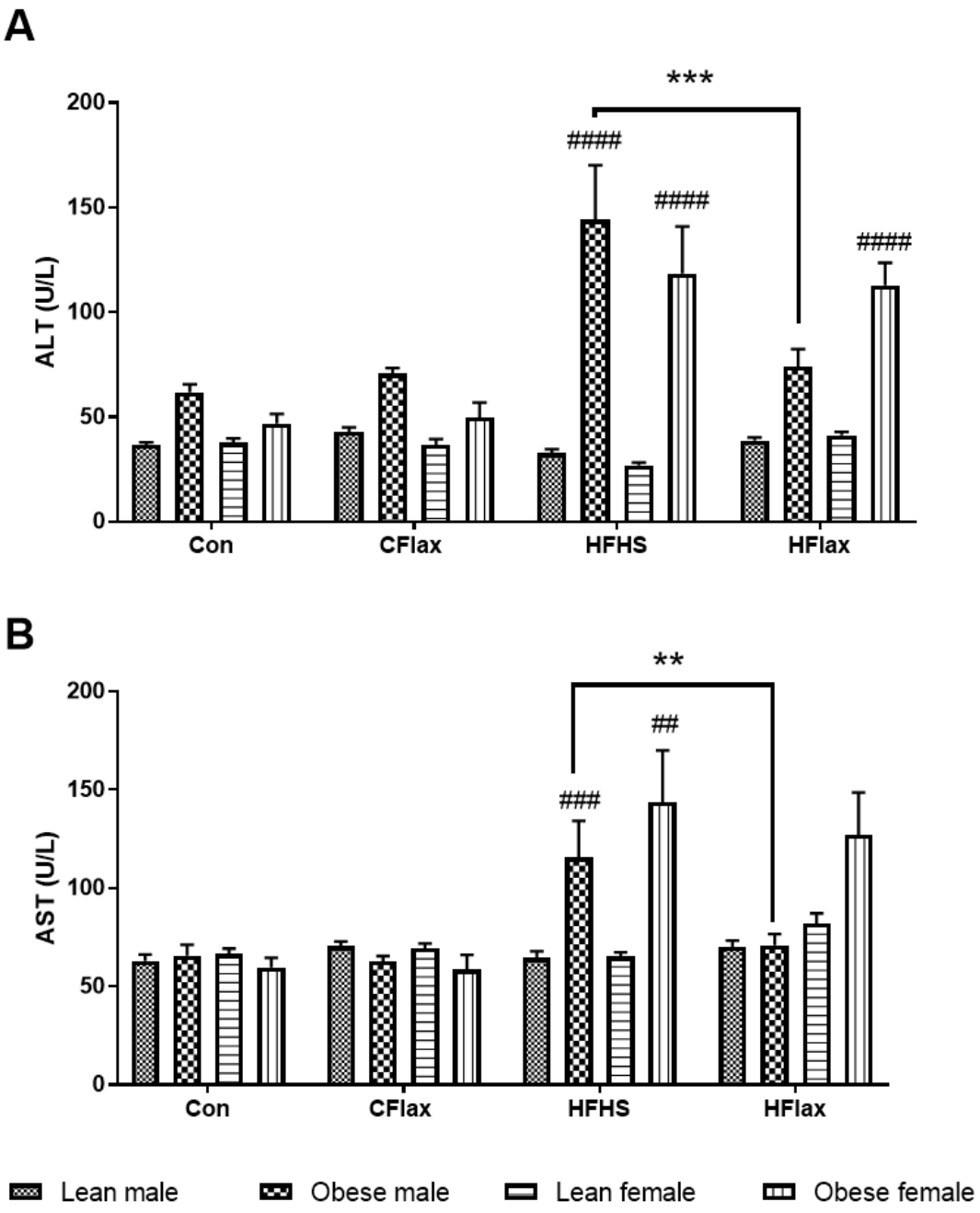


Figure 34. Effect of genotype and diet on liver enzymes. All values presented are mean±SE. (A), ALT, alanine aminotransferase; and (B) AST, aspartate aminotransferase. $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, and $^{\#\#\#\#}p<0.0001$ versus respective lean JCR rats on the same diet, 2-way ANOVA. $^{**}p<0.01$, and $^{***}p<0.001$ versus JCR rats on the HFHS diet, 2-way ANOVA. N=8.

Table 10. Plasma biochemistry.

Parameters	Genotype	Sex	Diet			
			Con	CFlax	HFHS	HFlax
HDL-C, mmol/L	Lean	Male	1.44±0.04[a]	1.08±0.03[b]	1.11±0.03[b]	1.07±0.02[b]
		Female	1.75±0.13[a‡]	1.52±0.12[a‡]	1.51±0.03[a‡]	1.49±0.07[a‡]
	Obese	Male	1.96±0.16[a**]	2.00±0.08[a****]	1.90±0.13[a****]	2.03±0.10[a****]
		Female	1.33±0.20[a‡]	1.44±0.26[a,b‡]	2.05±0.10[b]	1.93±0.09[a,b]
TG, mmol/L	Lean	Male	0.42±0.03[a,b]	0.37±0.02[a]	0.44±0.03[a,b]	0.49±0.03[b]
		Female	0.49±0.06[a]	0.39±0.05[a]	0.41±0.03[a]	0.49±0.04[a]
	Obese	Male	3.79±0.70[a****]	2.86±0.26[a,b****]	2.56±0.16[a,b****]	2.43±0.16[b****]
		Female	5.50±0.76[a****]	5.12±0.97[a****‡]	2.56±0.17[b*]	3.17±0.34[a,b**]
Glucose, mmol/L	Lean	Male	10.12±0.53[a]	9.89±0.38[a]	9.44±0.40[a]	9.20±0.43[a]

		Female	8.77±0.43[a]	8.05±0.53[a‡]	7.74±0.53[a]	7.97±0.48[a]
	Obese	Male	10.88±0.76[a]	10.50±0.76[a]	10.67±0.29[a]	9.66±0.65[a]
		Female	11.47±0.70[a]	11.08±0.75[a*]	10.15±0.41[a]	10.01±0.99[a]
Creatinine, µmol/L	Lean	Male	31.80±1.97[a]	31.58±1.46[a]	35.21±1.21[a,b]	38.85±1.80[b]
		Female	42.49±3.93[a‡]	37.95±2.40[a]	47.36±2.85[a‡]	45.19±1.12[a]
	Obese	Male	29.86±1.69[a]	25.91±1.39[a,b]	24.00±1.48[b****]	25.99±1.09[a,b****]
		Female	29.78±1.50[a**]	25.85±1.60[a*]	30.21±2.76[a***‡]	30.43±1.54[a**]
Urea, mmol/L	Lean	Male	7.73±0.37[a]	7.48±0.36[a]	5.25±0.21[b]	4.81±0.25[b]
		Female	7.44±0.38[a]	7.77±0.49[a]	5.45±0.19[b]	5.61±0.33[b]
	Obese	Male	7.58±0.66[a]	6.93±0.31[a]	4.26±0.07[b]	4.30±0.15[b]
		Female	6.48±0.58[a]	5.95±0.42[a*]	4.16±0.14[b]	4.05±0.26[b]

Values presented are mean ± SE. Row means without a common superscript letter (a, b, and c) differ significantly, $p<0.05$.HDL, high-density lipoprotein-cholesterol; TG, triglycerides. Con, control diet; CFlax, 10% ground flaxseed supplemented control diet; HFHS, high-fat high sucrose diet; HFlax, 10% ground flaxseed supplemented high-fat high sucrose diet. N=8.

* $p<0.05$, **$p<0.01$, ***$p<0.001$, ***$p<0.0001$ versus respective lean JCR rats on the same diet, 2-way ANOVA.

ǂ $p<0.05$ versus respective male JCR rats on the same diet, 2-way ANOVA.

Plasma fatty acid profile

A comprehensive list of the observed changes in the plasma fatty acids after 12 weeks of feeding with the different diets is compiled in Table 11. Using a 2-way ANOVA test with genotype and diet as independent variables revealed significant differences in the plasma levels of myristic acid (C14:0), pentadyclic acid (C15:0), palmitic acid (C16:0), palmitoleic acid (C16:1), heptadecanoic acid (C17:0), *cis*-10- heptadecanoic acid (C17:1), stearic acid (C18:0), oleic acid (C18:1), *cis*-vaccenic acid (C18:1n7), linoleic acid (C18:2 n-6), gamma-linoleic acid (C18:3 n-6), arachidic acid (C20:0), eicosadienoic acid (C20:2 n-6), dihomo-gamma-linolenic acid (C20:3 n-6), arachidonic acid (C20:4 n-6), eicosatrienoic acid (C20:3 n-3), behenic acid (C22:0), adrenic acid (C22:4), osbond acid (C22:5 n-6), docosapentaenoic acid (C22:5 n-3), lignoceric acid (C24:0), and nervonic acid (C24:1). A significant interaction between genotype and diet was observed as obese male JCR rats had significantly higher levels of total saturated fatty acids (SFAs) and lower levels of monounsaturated fatty acids (MUFAs) compared to the lean male JCR rats. Conversely, lean female and obese male JCR rats on the CFlax diet had significantly lower levels of total SFAs and higher levels of MUFA compared to the obese male JCR rats on the Con diet (Table 11).

A 3-way ANOVA with diet, genotype, and sex as independent variables demonstrated significantly higher levels of the total n-3 polyunsaturated fatty acid (PUFAs) in the male and female obese animals on the CFlax diet compared to the obese and lean JCR rats on the Con diet (Table 11). Flaxseed supplementation resulted in significantly higher levels of alpha-linolenic acid (ALA, C18:3 n-3) in the male and female JCR rats of both genotypes on the CFlax and HFlax diets compared to the Con and HFHS diets, as shown in Figure 35. A significant increase was also observed in the levels of long-chain n-3 PUFAs, EPA (C20:5), in both sexes of lean and obese

JCR rats on the CFlax compared to the Con diet. However, no such increase in the levels of EPA was observed in the JCR rats of both sex and genotype on the HFlax diet compared to the HFHS diet. Interestingly, a modest but significant decrease in the levels of another long-chain n-3 PUFAs, DHA (C22:6), was noted in lean male and female and obese male JCR rats on the CFlax diet compared to the Con diet, as presented in Figure 36. Conversely, a significant increase in the DHA levels was observed in the lean JCR rats of both sexes and obese male JCR rats on the HFlax compared to the HFHS diet. As a result of the increase in the total n-3 PUFA levels due to flaxseed supplementation in the diet, a significant lowering of the n-6 to n-3 ratio was found in all JCR rats on the CFlax and HFlax diets compared to the Con and HFHS diets, respectively (Figure 36). A strong sex dependent effect was noted as lean female JCR rats had significantly lower values of n-6/n-3 ratios compared to the male lean JCR rats on the Con, HFHS, and HFlax diets.

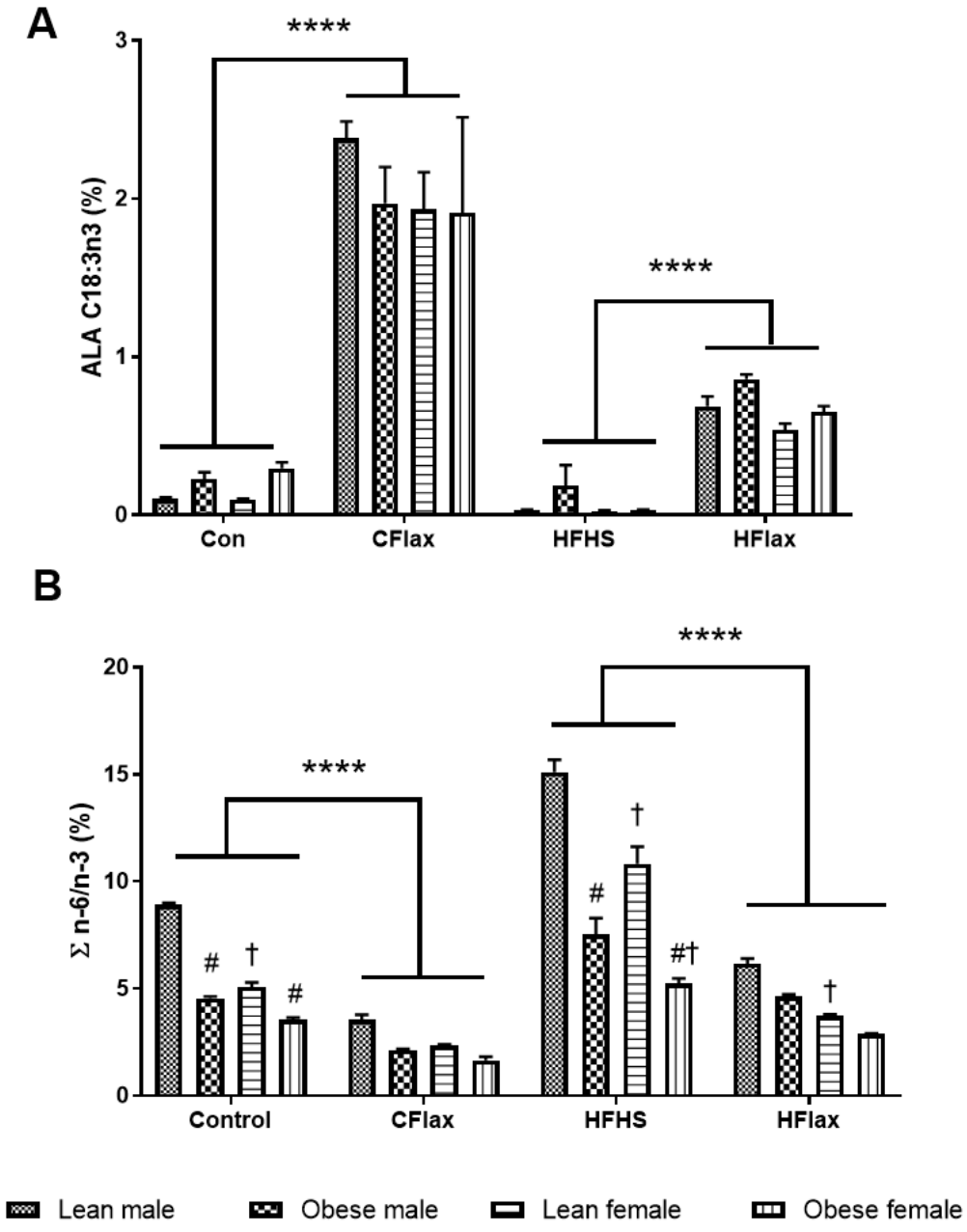


Figure 35. Effect of genotype, sex and diet on plasma fatty acids. All values presented are mean±SE. (A), ALA (C18:3 n-3); and (B) ratio of n-6 and n-3 PUFAs (∑n-6/n-3). ****p<0.0001, ***p<0.001, **p<0.01, and *p<0.05 versus respective JCR rats on different diets, 2-way ANOVA. ǂ p<0.05 versus respective male JCR rats on the same diet, 2-way ANOVA. # p<0.05 versus respective lean JCR rats on the same diet, 2-way ANOVA. N=8.

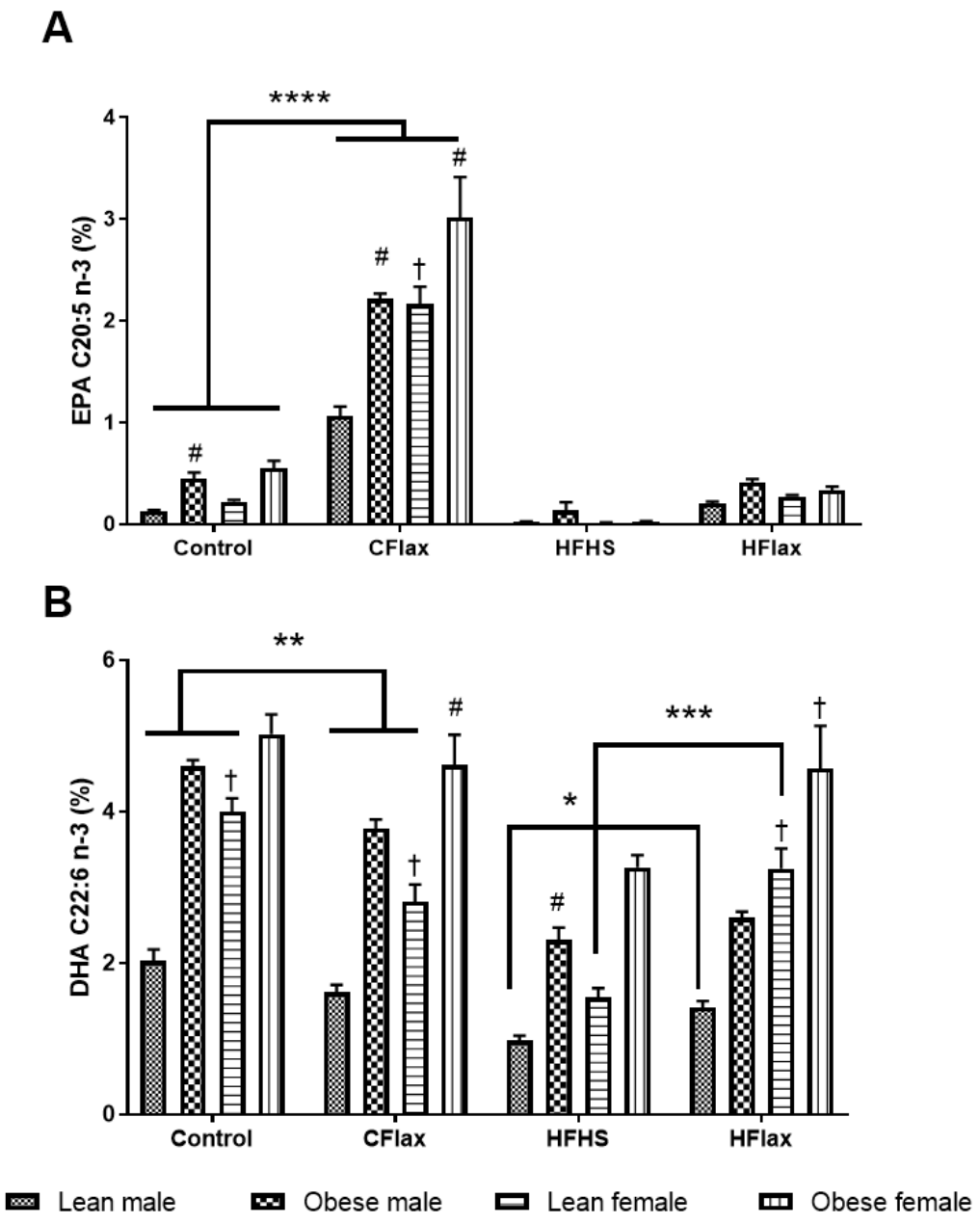


Figure 36. Effect of genotype, sex and diet on EPA and DHA. All values presented are mean±SE. (A), EPA (C20:5 n-3); and (B) DHA (C22:6 n-3). ****p<0.0001, ***p<0.001, **p<0.01, and *p<0.05 versus respective JCR rats on different diets, 2-way ANOVA. ǂ p<0.05 versus respective male JCR rats on the same diet, 2-way ANOVA. # p<0.05 versus respective lean JCR rats on the same diet, 2-way ANOVA. N=8.

Table 11. Circulating fatty acid analysis.

Fatty acids	Genotype	Sex	Diet			
			Con	CFlax	HFHS	HFlax
C14:0 (Myristic acid)	Lean	Male	0.08±0.01[a]	0.10±0.01[a]	0.12±0.02[a]	0.12±0.02[a]
		Female	0.09±0.01[a]	0.08±0.01[a]	0.10±0.00[a]	0.08±0.01[a]
	Obese	Male	0.24±0.03[a****]	0.20±0.02[a*]	0.21±0.03[a]	0.17±0.02[a]
		Female	0.20±0.02[a***]	0.15±0.02[a,b]	0.12±0.01[b¼ǂ]	0.13±0.01[b]
C15:0 (Pentadyclic acid)	Lean	Male	0.13±0.01[a]	0.15±0.01[a]	0.06±0.01[b]	0.07±0.00 [b]
		Female	0.09±0.01[a¼ǂ]	0.09±0.00[a¼ǂ]	0.05±0.00[b]	0.05±0.00[b]
	Obese	Male	0.13±0.01[a]	0.11±0.01[a*]	0.07±0.00[b]	0.06±0.00[b]
		Female	0.09±0.00[a¼ǂ]	0.09±0.01[a]	0.05±0.00[b]	0.05±0.00[b]
C16:0	Lean	Male	11.25±0.47[a]	10.35±0.39[a]	11.66±0.48[a]	11.35±0.41[a]

(Palmitic acid)		Female	10.28±0.38[a]	8.82±0.15[b]	9.65±0.19[a,b]	9.47±0.33[a,b]
	Obese	Male	17.69±0.63[a****]	15.65±0.18[b,c****]	15.90±0.33[b****]	14.20±0.14[c**]
		Female	13.42±0.79[a*ǂ]	11.81±1.33[aǂ]	10.73±0.26[aǂ]	10.91±0.83[aǂ]
C16:1 (Palmitoleic acid)	Lean	Male	0.09±0.01[a]	0.11±0.01[a]	0.13±0.01[a]	0.09±0.01[a]
		Female	0.18±0.01[a,c]	0.24±0.02[b]	0.24±0.01[b,c]	0.22±0.01[b,c]
	Obese	Male	1.06±0.12[a****]	1.18±0.14[a****]	0.75±0.09[a***]	0.66±0.12[a**]
		Female	1.12±0.13[a****]	0.97±0.19[a,b***]	0.49±0.05[b]	0.45±0.10[b]
C17:0 (Heptadecanoic acid)	Lean	Male	0.34±0.03[a]	0.34±0.01[a]	0.16±0.01[b]	0.14±0.01[b]
		Female	0.32±0.03[a]	0.34±0.03[a]	0.14±0.01[b]	0.14±0.01[b]
	Obese	Male	0.24±0.02[a**]	0.22±0.02[a,b***]	0.17±0.01[b]	0.18±0.01[b]
		Female	0.22±0.01[a**]	0.22±0.02[a**]	0.13±0.00[b]	0.14±0.01[b]
C17:1	Lean	Male	48.19±3.20[a]	49.22±1.94[a]	44.89±1.89[a]	47.72±2.16[a]

(*cis*-10-Heptadecanoic acid)		Female	40.77±2.28[a]	47.33±1.45[a]	46.23±0.78[a]	44.80±2.35[a]
	Obese	Male	24.91±1.99[a****]	33.50±1.69[b***]	31.93±1.50[b**]	34.23±1.01[b**]
		Female	30.64±3.53[a]	33.82±5.73[a]	44.11±1.75[a]	40.25±4.34[a]
C18:0 (Stearic acid)	Lean	Male	9.52±0.68[a]	9.52±0.37[a]	12.31±0.63[b]	11.62±0.35[b]
		Female	15.56±0.69[a‡]	13.35±0.38[b‡]	14.79±0.31[a,b]	15.21±0.56[a,b‡]
	Obese	Male	12.57±0.21[a**]	11.55±0.37[a]	13.40±0.69[a]	13.70±0.22[a]
		Female	15.04±0.54[a]	15.20±0.64[a‡]	14.43±0.44[a]	15.55±1.23[a]
C18:1 (Oleic acid)	Lean	Male	3.22±0.19[a]	3.28±0.19[a]	6.21±0.11[b]	5.90±0.31[b]
		Female	2.72±0.10[a]	2.43±0.05[a]	4.91±0.11[b]	4.55±0.24[b]
	Obese	Male	7.55±0.69[a****]	5.73±0.28[b***]	8.38±0.29[a**]	7.07±0.23[a,b]
		Female	8.22±0.98[a****]	6.38±1.32[a**]	5.62±0.35[a‡]	5.75±0.25[a]
	Lean	Male	0.89±0.06[a,b]	0.95±0.04[a]	0.77±0.01[b]	0.74±0.03[b]

C18:1n7 (*cis*-Vaccenic acid)		Female	0.70±0.08[a]	0.83±0.11[a]	0.61±0.01[a]	0.68±0.10[a]
	Obese	Male	1.39±0.02[a]	1.18±0.05[a,b]	0.90±0.02[b]	1.07±0.24[a,b]
		Female	0.88±0.06[a]ǂ	0.84±0.10[a]ǂ	0.69±0.03[a]	0.70±0.04[a]
C18:2 n-6 (Linoleic acid)	Lean	Male	9.87±0.40[a]	12.26±0.51[b]	7.26±0.22[c]	7.92±0.28[c]
		Female	8.73±0.43[a]	9.09±0.33[a]ǂ	5.71±0.53[b]	5.87±0.24[b]
	Obese	Male	13.56±1.00[a***]	11.33±0.53[a,b]	9.35±0.31[b,c]	8.51±0.23[c]
		Female	9.99±0.76[a]ǂ	8.70±0.79[a]ǂ	4.36±0.19[b]ǂ	5.48±0.56[b]ǂ
C18:3 n-6 (Gamma-linolenic acid)	Lean	Male	0.03±0.01[a]	0.03±0.00[a]	0.03±0.01[a]	0.02±0.01[a]
		Female	0.05±0.01[a]	0.04±0.00[a]	0.05±0.01[a]	0.05±0.02[a]
	Obese	Male	0.07±0.00[a**]	0.05±0.01[b]	0.04±0.00[b]	0.04±0.01[b]
		Female	0.09±0.02[a]	0.06±0.01[a,b]	0.04±0.00[b]	0.03±0.00[b]
C20:0	Lean	Male	0.05±0.02[a]	0.05±0.00[a]	0.07±0.01[a]	0.07±0.01[a]

(Arachidic acid)		Female	0.05±0.00[a]	0.06±0.01[a]	0.08±0.01[a]	0.07±0.01[a]
	Obese	Male	0.05±0.00[a]	0.05±0.01[a]	0.06±0.01[a]	0.06±0.00[a]
		Female	0.04±0.00[a]	0.03±0.00[a]	0.04±0.00[a**]	0.04±0.00[a*]
C20:1 (Eicosenoic acid)	Lean	Male	0.19±0.05[a]	0.19±0.02[a]	0.19±0.02[a]	0.18±0.01[a]
		Female	0.19±0.07[a]	0.25±0.13[a]	0.14±0.00[a]	0.28±0.13[a]
	Obese	Male	0.14±0.02[a]	0.11±0.03[a]	0.16±0.01[a]	0.09±0.03[a]
		Female	0.11±0.01[a]	0.09±0.01[a]	0.12±0.01[a]	0.21±0.11[a]
C20:2 n-6 (Eicosadienoic acid)	Lean	Male	0.22±0.01[a]	0.14±0.01[b]	0.08±0.00[c]	0.07±0.01[c]
		Female	0.13±0.01[a‡]	0.09±0.01[a,b‡]	0.08±0.01[b]	0.08±0.01[b]
	Obese	Male	0.25±0.01[a]	0.17±0.01[b]	0.20±0.01[c]	0.17±0.01[b]
		Female	0.13±0.02[a‡]	0.10±0.01[a,b‡]	0.08±0.00[b‡]	0.10±0.01[a,b‡]
C20:3 n-6	Lean	Male	0.15±0.01[a,c]	0.33±0.01[b]	0.20±0.01[c]	0.20±0.02[c]

(Dihomo-gamma-linolenic acid)		Female	0.20±0.02[a]	0.31±0.02[a]	0.26±0.03[a]	0.26±0.04[a]
	Obese	Male	1.56±0.05[a****]	1.13±0.05[b,c****]	1.38±0.09[a,b****]	1.00±0.06[c****]
		Female	0.74±0.08[a****ǂ]	0.66±0.03[a****ǂ]	0.34±0.03[bǂ]	0.51±0.05[a,b**ǂ]
C20:4 n-6 (Arachidonic acid)	Lean	Male	12.68±1.30[a]	6.74±0.44[b]	13.23±0.57[a]	10.14±0.53[a]
		Female	14.37±0.84[a]	8.31±0.36[b]	13.17±0.65[a]	12.39±1.20[a]
	Obese	Male	11.95±0.47[a]	7.73±0.29[b]	12.75±0.73[a]	12.88±0.37[a]
		Female	11.95±0.33[a]	8.90±0.72[b]	13.37±0.52[c]	12.56±1.30[a]
C20:3 n-3 (Eicosatrienoic acid)	Lean	Male	0.09±0.07[a]	0.07±0.01[a]	0.15±0.03[a]	0.20±0.05[a]
		Female	0.02±0.01[a]	0.06±0.01[a]	0.12±0.01[b]	0.17±0.02[b]
	Obese	Male	0.02±0.00[a]	0.08±0.01[a]	0.37±0.05[b**]	0.32±0.05[b]
		Female	0.01±0.00[a]	0.08±0.01[b]	0.11±0.01[b,cǂ]	0.14±0.01[c]
C22:0	Lean	Male	0.10±0.02[a]	0.09±0.01[a]	0.13±0.02[a]	0.14±0.01[a]

(Behenic acid)		Female	0.16±0.01[a]	0.13±0.00[a]	0.16±0.01[a]	0.21±0.02[b]ǂ
	Obese	Male	0.10±0.00[a]	0.12±0.00[a,b]	0.13±0.01[a,b]	0.16±0.02[b]
		Female	0.08±0.00[a*]	0.08±0.01[a]	0.12±0.00[a]	0.12±0.03[a***]
C22:4 (Adrenic acid)	Lean	Male	0.13±0.02[a]	0.05±0.00[b]	0.20±0.01[c]	0.08±0.02[a,b]
		Female	0.14±0.01[a]	0.04±0.01[b]	0.30±0.02[c]ǂ	0.10±0.02[a,b]
	Obese	Male	0.15±0.01[a]	0.07±0.01[b]	0.14±0.02[a]	0.15±0.02[a]
		Female	0.17±0.02[a]	0.06±0.01[b]	0.32±0.02[c]ǂ	0.14±0.02[a]
C22:5 n-6 (Osbond acid)	Lean	Male	0.03±0.01[a]	0.03±0.00[a]	0.19±0.02[a]	0.03±0.01[a]
		Female	0.06±0.00[a]	0.01±0.00[a]	0.82±0.05[c]ǂ	0.06±0.02[a]
	Obese	Male	0.07±0.01[a]	0.06±0.01[a]	0.09±0.02[a***]	0.04±0.00[a]
		Female	0.06±0.01[a]	0.02±0.00[a]	0.67±0.09[b]ǂ	0.06±0.02[a]
C22:5 n-3	Lean	Male	0.29±0.01[a]	0.87±0.09[b]	0.23±0.02[a]	0.58±0.04[c]

(Docosapentaenoic acid)		Female	0.34±0.03[a]	0.73±0.06[b]	0.20±0.02[a]	0.82±0.05[b,c]
	Obese	Male	0.78±0.05[a***]	1.57±0.02[b****]	0.34±0.10[c]	0.81±0.02[a]
		Female	0.68±0.07[a]	1.42±0.23[a***]	0.25±0.01[b]	0.86±0.06[a]
C24:0 (Lignoceric acid)	Lean	Male	0.16±0.06[a]	0.21±0.03[a]	0.17±0.02[a]	0.16±0.02[a]
		Female	0.32±0.04[a‡]	0.34±0.03[a‡]	0.16±0.01[b]	0.24±0.03[a,b]
	Obese	Male	0.18±0.02[a,b]	0.21±0.02[a]	0.13±0.01[b]	0.21±0.03[a]
		Female	0.12±0.02[a***]	0.12±0.01[a****]	0.08±0.01[a]	0.12±0.02[a*]
C24:1 (Nervonic acid)	Lean	Male	0.17±0.06[a]	0.16±0.03[a]	0.21±0.03[a]	0.24±0.02[a]
		Female	0.35±0.01[a‡]	0.30±0.01[a]	0.29±0.02[a]	0.30±0.04[a]
	Obese	Male	0.20±0.01[a]	0.20±0.02[a]	0.33±0.02[b*]	0.31±0.00[b]
		Female	0.13±0.01[a***]	0.13±0.01[a*]	0.26±0.00[a]	0.24±0.07[a]
∑SFA	Lean	Male	21.60±1.21[a]	20.77±0.76[a]	24.68±1.17[a]	23.61±0.80[a]

		Female	26.86±1.00[a]	23.21±0.54[b]	25.12±0.45[a,b]	25.47±0.93[a,b]
	Obese	Male	31.19±0.63[a****]	28.10±0.54[b****]	30.08±0.66[a,b**]	28.74±0.36[a,b**]
		Female	29.22±1.29[a]	27.70±1.87[a]	25.70±0.68[a]	27.05±2.07[a]
∑MUFA	Lean	Male	52.70±3.00[a]	53.88±1.57[a]	52.70±1.76[a]	54.93±1.71[a]
		Female	44.79±2.28[a]	51.22±1.33[a,b]	52.56±0.81[b]	50.71±2.30[a,b]
	Obese	Male	35.10±1.57[a****]	41.75±1.12[b**]	42.61±1.71[b**]	43.58±0.76[b**]
		Female	41.07±2.67[a]	42.18±4.44[a]	51.41±1.40[a]	47.56±4.45[a]
∑n-6	Lean	Male	23.13±1.66[a]	19.66±0.48[a,b]	21.18±0.54[a,b]	18.42±0.81[b]
		Female	23.66±1.19[a]	17.87±0.59[b]	20.39±0.32[a,b]	18.75±1.07[b]
	Obese	Male	27.62±0.79[a]	20.52±0.28[b]	23.95±0.78[c]	22.77±0.30[b,c]
		Female	23.14±1.04[a]	18.48±1.09[a]	19.18±0.68[a‡]	18.86±1.83[a]
∑n-3	Lean	Male	2.60±0.18[a,c]	5.67±0.43[b]	1.41±0.07[a]	3.01±0.18[c]

		Female	4.67±0.20^{a}ǂ	7.70±0.31^{b}ǂ	1.91±0.14^{c}	5.05±0.31^{a}ǂ
	Obese	Male	6.07±0.19^{a****}	9.62±0.32^{b****}	3.34±0.47^{c**}	4.90±0.12^{a**}
		Female	6.56±0.40^{a}	11.62±1.63^{b**}	3.69±0.17^{a}	6.53±0.55^{a}
∑PUFA	Lean	Male	25.72±1.84^{a}	25.33±0.85^{a}	22.59±0.58^{a}	21.43±0.96^{a}
		Female	28.33±1.30^{a}	25.56±0.83^{a,b}	22.30±0.39^{b}	23.80±1.38^{b}
	Obese	Male	33.69±0.95^{a***}	30.14±0.59^{a,b}	27.29±1.07^{b}	27.67±0.40^{b*}
		Female	29.70±1.41^{a}	30.10±2.59^{a}	22.88±0.73^{a}	25.38±2.38^{a}

Values are presented as mean ± SE. Row means without a common superscript letter (a, b, and c) differ significantly, $p<0.05$.

****$p<0.0001$ ***$p<0.001$ **$p<0.01$ *$p<0.05$ versus respective lean JCR rats on the same diet, 2-way ANOVA.

ǂ$p<0.05$ versus respective male JCR rats on the same diet, 2-way ANOVA.

N=8. Con, control diet; CFlax, 10% ground flaxseed supplemented control diet; HFHS, high-fat high sucrose diet; HFlax, 10% ground flaxseed supplemented high-fat high sucrose diet.

Myocardial lesions

As shown in Table 12, there were no significant differences in the frequency of early stage myocardial lesions in lean male and female JCR:LA-*cp* rats on the different diets. Stage 2 lesions with areas of chronic inflammatory cell infiltration and myocardial cell lysis were larger in male and female obese JCR rats, but statistical significance was obscured due to high variability. However, male and female obese JCR rats on the flaxseed supplemented HFlax diet displayed significantly lowered early stage myocardial lesion frequency compared to the respective animals in the HFHS group, as shown in Figure 37 and 38. No genotype or sex-dependent effect was observed for lean or obese animals.

Table 12. Frequency of myocardial lesions in lean animals on different diets.

Genotype	**Sex**	**Diet**			
		Con	**CFlax**	**HFHS**	**HFlax**
Lean	Male	1.25±0.63[a]	0.75±0.48[a]	2.67±0.88[a]	1.50±0.87[a]
	Female	0.40±0.24[a,b]	0.20±0.20[a]	1.20±0.20[b]	0.80±0.20[a,b]

Values are presented as mean±SE. Row means without a common superscript letter (a, b, and c) differ significantly, $p<0.05$. N=8. Con, control diet; CFlax, 10% ground flaxseed supplemented control diet; HFHS, high-fat high sucrose diet; HFlax, 10% ground flaxseed supplemented high-fat high sucrose diet.

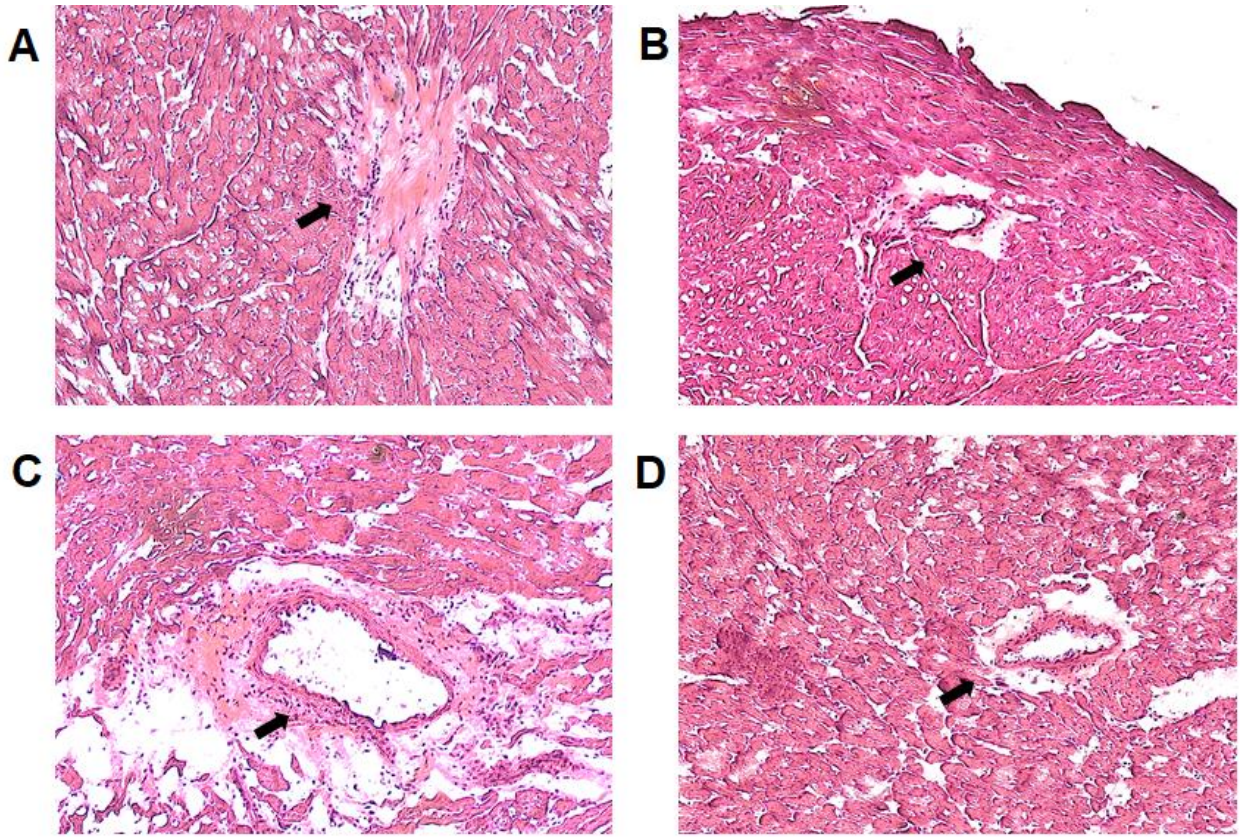


Figure 37. Representative micrographs of myocardial lesions in male and female obese JCR:LA-*cp* rats. (A) Male obese JCR:LA-*cp* rats on HFHS; (B) Male obese JCR:LA-*cp* rats on HFlax; (C) Female obese JCR:LA-*cp* rats on HFHS; and (D) Female obese JCR:LA-*cp* rats on HFlax. Black arrowheads indicate ischemic lesions characterized by myocardial cell lysis and inflammatory cell infiltration. All images were captured at a magnification of 10X after hematoxylin and eosin staining of whole heart sections.

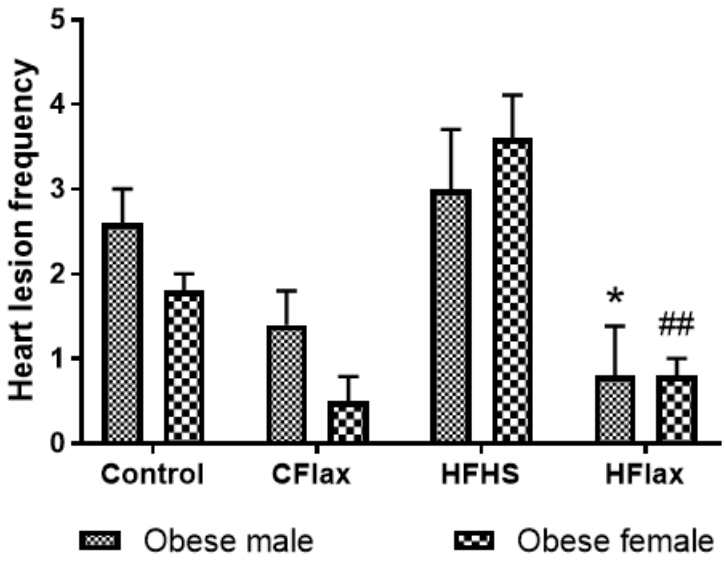


Figure 38. Effect of flaxseed on the frequency of myocardial lesions. All values presented are mean±SE. *p<0.05 versus male obese JCR rats on the HFHS, Kruskal-Wallis, Dunn's multiple comparisons test. $^{\#\#}p$<0.01 versus female obese JCR rats on the HFHS diet, Kruskal-Wallis test, Dunn's multiple comparisons test. All images were captured at a magnification of 10X after hematoxylin and eosin staining of heart sections.

Cardiac structure

Echocardiographic examination was performed to study the alterations in the cardiac structure of male and female obese JCR:LA-*cp* rats after 12 weeks of feeding on different diets compared to the age-matched lean controls. There was a significant effect of genotype after 2-way ANOVA analysis with diet and genotype as independent variables. As shown in Table 13, male and female obese JCR:LA-*cp* rats had higher values for left ventricle mass, left ventricle posterior wall thickness in diastole (LVPWd) and in systole (LVPWs), interventricular septum thickness in diastole (IVSd) and in systole (IVSs), and left ventricle internal diameter in diastole (LVIDd) and in systole (LVIDs) compared to the age-matched lean controls on the same diets. A prominent sex-

dependent effect was noted as female lean and obese JCR rats had lower values for LVIDs and LVIDd, respectively, compared to the male obese JCR rats on the same diet, as shown in Table 13 and Figure 39. A 2-way ANOVA analysis with sex and diet as factors demonstrated a significant effect of diet. Lean female JCR rats on a flaxseed supplemented high fat high-sucrose diet (HFlax) had significantly lower LVIDs compared to the JCR rats of the same genotype and sex on a HFHS diet (Figure 39).

Systolic and diastolic function

24-week old male and female obese JCR rats displayed no signs of systolic dysfunction. Indices of ejection phase performances such as ejection fraction and fractional shortening were comparable between obese JCR rats and age-matched lean controls (Table 13). Paradoxically, values for stroke volume (SV), cardiac output (CO), end-diastolic volume (EDV), and end-systolic volume (ESV) were significantly higher in obese JCR rats compared to the age-matched lean JCR rats. A significant interaction effect of sex and diet was confirmed using 2-way ANOVA with sex and diet as independent factors. Female lean JCR rats had significantly lower values for EDV and ESV compared to the respective male lean JCR rats (Table 13 and Figure 39). A strong effect of diet was observed after dietary supplementation with ground flaxseed for 12 weeks. Female lean animals on the HFlax diet had significantly higher FS and lower ESV compared to the animals of the same genotype and sex in the HFHS group (Figure 39).

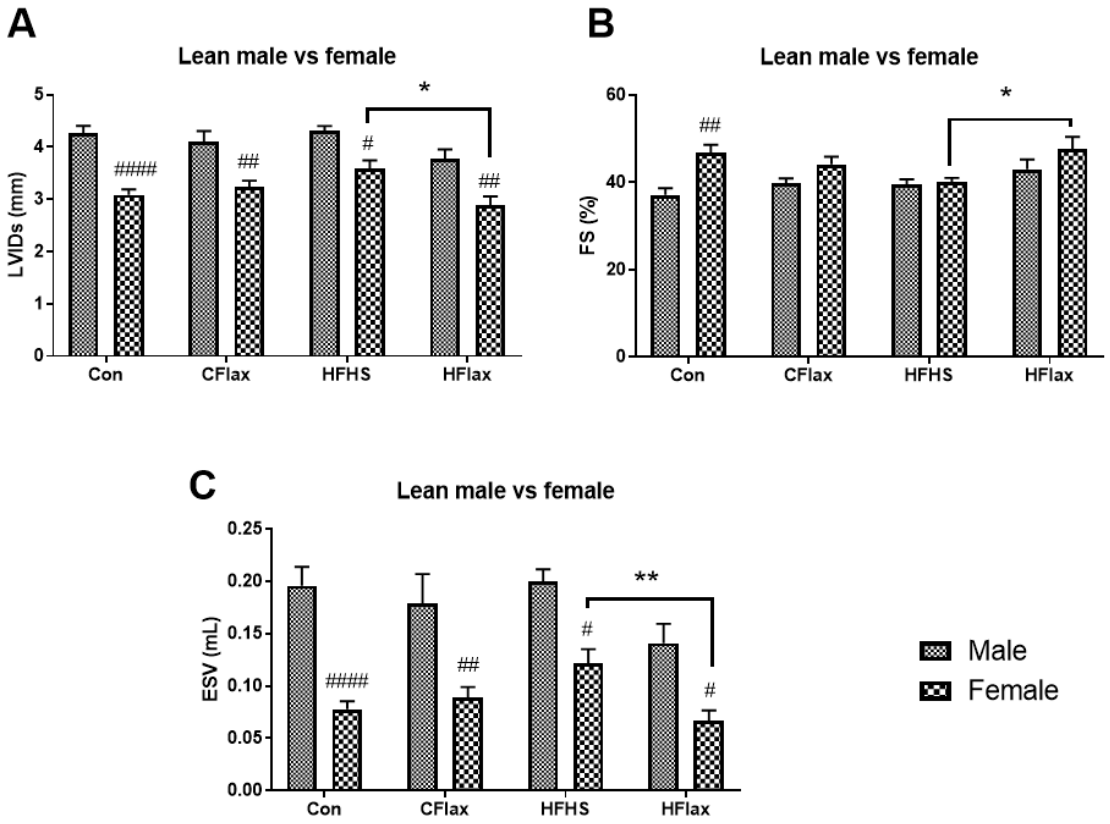


Figure 39. Effect of sex and diet on cardiac structure and function. All values presented are mean ± SE. (A), LVIDs, LV internal diameter in systole; (B) FS, LV fractional shortening; and (C) ESV, LV end-systolic volume. $^{\#\#\#\#}p<0.0001$, $^{\#\#\#}p<0.001$, $^{\#\#}p<0.01$, and $^{\#}p<0.05$ versus respective male JCR rats on the same diet, 2-way ANOVA. $^{**}p<0.01$, and $^{*}p<0.05$ versus female JCR rats on the HFHS diet, 2-way ANOVA. N=8.

Mitral inflow early (E wave) diastolic and late atrial (A wave) filling velocities were estimated by Doppler echocardiography. Diastolic function parameters such as E wave, deceleration time, A wave, ratio of E wave to A wave were similar between all the groups, irrespective of genotype, sex, and diet (Table 13). However, mild diastolic dysfunction in obese JCR rats was characterized by an altered isovolumetric relaxation time (IVRT). Prolonged IVRT denotes increased myocardial stiffness due to fibrosis. No impact of genotype or sex was noted in

a 2-way ANOVA analysis on IVRT values. However, flaxseed supplementation in the HFlax group significantly lowered IVRT values in male and female obese JCR rats compared to the JCR rats of respective sex in the HFHS group, as shown in Figure 40.

Collectively, these data indicate that obese JCR:LA-*cp* rats do not develop systolic dysfunction even when fed a high-fat high-sucrose diet for 12 weeks. However, the HFHS diet did lead to moderate systolic dysfunction in lean females and mild diastolic dysfunction in obese JCR rats which was improved by the flaxseed supplemented diet.

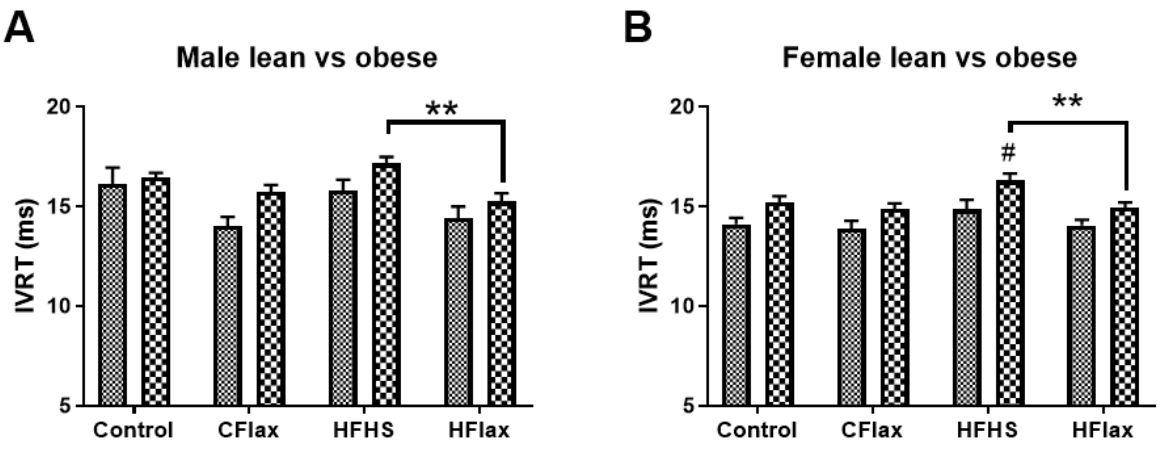


Figure 40. Effect of diet and genotype on IVRT. All values presented are mean±SE. (A), IVRT, isovolumetric relaxation time of lean male versus obese JCR rats; and (B) IVRT of female lean versus obese JCR rats. $^{\#}p<0.05$ versus respective lean JCR rats on the same diet, 2-way ANOVA. $^{**}p<0.01$ versus female JCR rats on the HFHS diet, 2-way ANOVA. N=8.

Electrocardiography

Electrocardiography demonstrated no changes in the ECG waves irrespective of genotype, sex, and diet. Similarly, no changes were observed for cardiac arrhythmias in any of the experimental groups.

Table 13. Echocardiographic measurements.

Parameters	**Genotype**	**Sex**	**Diet**			
			Con	**CFlax**	**HFHS**	**HFlax**
LVPWd, mm	Lean	Male	1.43±0.09	1.52±0.07	1.53±0.08	1.52±0.08
		Female	1.39±0.05	1.29±0.05	1.36±0.04	1.33±0.04
	Obese	Male	1.81±0.08*	1.98±0.12**	1.82±0.08	1.84±0.05
		Female	1.60±0.08	1.85±0.10****	1.80±0.05***	1.91±0.11****‡
LVPWs, mm	Lean	Male	2.14±0.06	2.27±0.09	2.27±0.10	2.30±0.14
		Female	2.39±0.04	2.13±0.08	2.13±0.07	2.16±0.07
	Obese	Male	2.66±0.11*	2.86±0.10**	2.67±0.11	2.78±0.15
		Female	2.60±0.13	2.96±0.13****	2.88±0.09	2.96±0.14
IVSd, mm	Lean	Male	1.25±0.03	1.29±0.05	1.21±0.05	1.30±0.03

		Female	1.17±0.08	1.17±0.05	1.22±0.05	1.19±0.06
	Obese	Male	1.34±0.06	1.52±0.05	1.46±0.06*	1.56±0.08*
		Female	1.28±0.06	1.35±0.05‡	1.29±0.08‡	1.43±0.07‡
IVSs, mm	Lean	Male	2.02±0.05	2.15±0.08	2.15±0.06	2.11±0.08
		Female	1.99±0.04	1.92±0.04	1.88±0.09	2.00±0.09
	Obese	Male	2.44±0.08**	2.52±0.11*	2.50±0.06*	2.57±0.09**
		Female	2.26±0.13	2.54±0.07**	2.31±0.16	2.49±0.13*
LVIDd, mm	Lean	Male	6.76±0.10	6.80±0.30	7.17±0.14	6.64±0.16
		Female	5.81±0.16‡	5.80±0.12‡	6.00±0.18‡	5.51±0.14‡
	Obese	Male	7.69±0.13**	7.21±0.20	7.96±0.08*	7.82±0.17***
		Female	6.93±0.16****‡	6.69±0.08**	7.01±0.20***‡	7.10±0.15****‡
LVIDs, mm	Obese	Male	4.75±0.10	4.31±0.17	4.91±0.11	4.67±0.22**

		Female	3.85±0.23	3.26±0.14	3.86±0.25	3.86±0.18**
EDV, mL	Lean	Male	0.71±0.03	0.74±0.10	0.83±0.04	0.67±0.05
		Female	0.47±0.04‡	0.46±0.03‡	0.51±0.04‡	0.40±0.03‡
	Obese	Male	1.01±0.05*	0.85±0.06	1.11±0.03*	1.06±0.06***
		Female	0.76±0.05***	0.68±0.02**	0.79±0.06***	0.81±0.04****
ESV, mL	Obese	Male	0.26±0.02	0.20±0.02	0.29±0.02	0.26±0.03**
		Female	0.15±0.02	0.09±0.01	0.16±0.03	0.15±0.02*
EF, %	Lean	Male	72.38±1.81	75.88±1.46	75.63±1.25	79.13±2.33
		Female	83.13±1.47‡	80.75±1.65	76.75±1.05	83.38±2.47
	Obese	Male	74.25±0.94	76.38±1.59	74.00±1.45	76.13±1.97
		Female	80.63±2.20	86.75±1.36‡	81.13±2.22	81.63±1.90
FS, %	Obese	Male	38.50±0.80	40.13±1.38	38.25±1.13	40.25±1.85

		Female	44.50±2.34	51.25±1.76‡	45.25±2.27	45.88±2.13
SV, mL	Lean	Male	0.51±0.02	0.56±0.07	0.63±0.04	0.53±0.04
		Female	0.39±0.03	0.37±0.02‡	0.39±0.03‡	0.34±0.03‡
	Obese	Male	0.75±0.04**	0.65±0.05	0.82±0.03	0.80±0.04***
		Female	0.61±0.03***	0.59±0.02***	0.63±0.04****	0.66±0.04****
HR, BPM	Lean	Male	370.00±16.12	377.40±9.24	362.50±4.30	373.80±9.87
		Female	389.30±5.77	393.10±7.10	379.30±7.64	379.30±6.11
	Obese	Male	326.00±5.00*	327.00±8.58**	347.10±9.67	345.00±8.57
		Female	364.90±4.36	410.60±27.38	367.10±8.43	358.80±6.89
CO, mL/min	Lean	Male	0.19±0.01	0.21±0.02	0.23±0.01	0.20±0.02
		Female	0.15±0.01	0.15±0.01	0.15±0.01‡	0.13±0.01‡
	Obese	Male	0.24±0.01	0.21±0.01	0.28±0.01	0.28±0.01*

		Female	0.22±0.01**	0.24±0.01****	0.23±0.01***	0.24±0.01****
LV mass, g	Lean	Male	1.05±0.03	1.08±0.04	1.11±0.03	1.06±0.03
		Female	0.92±0.02‡	0.91±0.01‡	0.95±0.02‡	0.90±0.02‡
	Obese	Male	1.29±0.05**	1.32±0.06**	1.37±0.05***	1.38±0.04****
		Female	1.12±0.04**	1.16±0.04****	1.17±0.03****	1.25±0.05****
Mitral E, mm/s	Lean	Male	86.50±2.48	87.88±2.54	87.63±3.26	91.63±2.92
		Female	95.50±2.03	92.38±2.80	97.38±1.92	91.88±3.09
	Obese	Male	98.75±4.63	96.88±3.87	101.40±3.22	99.88±4.40
		Female	97.13±2.94	97.63±2.93	99.25±3.29	102.90±3.79
DT, ms	Lean	Male	41.63±2.09	34.25±2.86	38.38±3.91	40.88±4.94
		Female	33.13±3.10	32.25±2.78	38.25±1.29	34.75±1.82
	Obese	Male	50.38±2.94	47.13±5.85	39.50±2.61	45.38±4.90

		Female	38.00±2.28	32.88±3.32	38.50±1.81	40.88±2.83
Mitral A, mm/s	Lean	Male	55.13±4.19	66.75±4.35	54.25±4.38	58.50±4.63
		Female	69.00±3.54	69.38±3.02	67.13±4.30	62.13±3.85
	Obese	Male	53.38±3.59	57.50±5.50	69.75±5.48	65.13±5.40
		Female	68.38±2.51	68.75±6.02	70.25±4.66	72.50±3.97
E/A	Lean	Male	1.61±0.09	1.36±0.07	1.71±0.20	1.61±0.09
		Female	1.39±0.05	1.35±0.04	1.49±0.08	1.49±0.05
	Obese	Male	1.91±0.13	1.80±0.19	1.55±0.17	1.63±0.17
		Female	1.41±0.06	1.53±0.19	1.46±0.07	1.45±0.10

Values presented are mean ± SE. N=8. LV, left ventricle; LVPWd, LV posterior wall thickness in diastole; LVPWs, LV posterior wall thickness in systole; EDV, LV end-diastolic volume; ESV, LV end-systolic volume; LVIDd, LV internal diameter in diastole; LVIDs, LV internal diameter in systole; IVSd, LV interventricular septum thickness in diastole; IVSs, LV interventricular septum thickness in systole; EF, LV ejection fraction; FS, LV fractional shortening; E, early diastolic filling velocity; A, late atrial filling velocity; E/A, ratio of early [E] and late

atrial [A] ventricular filling velocity; IVRT, isovolumetric relaxation time; DT, deceleration time; HR, heart rate; SV, stroke volume; CO, cardiac output. Con, control diet; CFlax, 10% ground flaxseed supplemented control diet; HFHS, high-fat high sucrose diet; HFlax, 10% ground flaxseed supplemented high-fat high sucrose diet.

* $p<0.05$, ** $p<0.01$, *** $p<0.001$, *** $p<0.0001$ versus respective lean JCR rats on the same diet, 2-way ANOVA.

ǂ $p<0.05$ versus respective male JCR rats on the same diet, 2-way ANOVA.

The effect of genotype on blood pressure in JCR:LA-*cp* rats

Systolic, diastolic, and mean arterial blood pressure values were obtained at baseline in 12-week-old JCR:LA-*cp* rats prior to any dietary intervention. Baseline systolic blood pressures (SBPs), diastolic blood pressures (DBPs), and mean arterial blood pressures (MABPs) were not significantly different in 12-week old obese male and female JCR rats compared with age-matched lean males and females, as shown in Table 14. There was no significant interaction between genotype and sex as data were similar when analyzed using 2-way ANOVA in which one factor was genotype and the other was sex.

After 6 weeks of dietary intervention, blood pressure monitoring of 18 weeks old JCR:LA-*cp* rats revealed no differences in SBPs, DBPs, or MABPs between lean and obese genotypes (Figure 41). However, a sex dependency on BP was identified at 6 weeks by 2-way ANOVA with sex and diet as independent variables. The lean female JCR rats had significantly lower SBPs in the Con, HFHS, and HFlax groups as compared to lean males. However, no such changes were noted for DBPs. In addition, obese female rats had significantly lower SBPs, DBPs, and MABPs in the Con group as compared to obese males. No clear trend for an effect of diet on SBP, DBP or MABP was observed.

The possibility that longer periods of dietary intervention may elicit an effect on BP in the JCR:LA-*cp* rats was studied. At 24 weeks of age, obese JCR rats exhibited no differences in SBPs, DBPs, and MABPs, as compared to age-matched lean controls, regardless of diet (Figure 42). However, once again a significant effect of sex was confirmed by 2-way ANOVA with sex and diet as factors. Female obese JCR:LA-*cp* rats had significantly lower SBPs and MABPs for the CFlax group as compared to obese male rats. In addition, the lean female JCR:LA-*cp* rats had significantly lower SBPs in the CFlax and HFHS groups as compared to lean males.

Table 14. Baseline blood pressure values of 12 weeks old JCR:LA-*cp* rats.

SBP (mm Hg)								
	Male				**Female**			
	Con	CFlax	HFHS	HFlax	Con	CFlax	HFHS	HFlax
Lean	140±5	146±6	150±6	137±3	123±5	139±5	132±5	135±7
Obese	128±6	135±4	139±3	141±4	127±5	123±5	136±3	132±4
DBP (mm Hg)								
	Male				**Female**			
	Con	CFlax	HFHS	HFlax	Con	CFlax	HFHS	HFlax
Lean	96±4	100±6	103±5	91±3	83±5	96±4	89±4	88±5
Obese	85±5	90±4	92±3	93±3	82±4	82±4	90±2	89±3
MABP (mm Hg)								
	Male				**Female**			
	Con	CFlax	HFHS	HFlax	Con	CFlax	HFHS	HFlax
Lean	111±4	116±6	118±6	106±3	96±5	110±5	103±4	104±5
Obese	99±6	104±4	107±3	108±3	97±5	95±4	106±2	103±4

Values are presented as mean±SE. Con, control diet; CFlax, 10% ground flaxseed supplemented control diet; DBP, diastolic blood pressure; HFHS, high-fat high sucrose diet; HFlax, 10% ground flaxseed supplemented high-fat high sucrose diet; MABP, mean arterial blood pressure; and SBP, systolic blood pressure. All values were >0.05 vs respective control values. N=8.

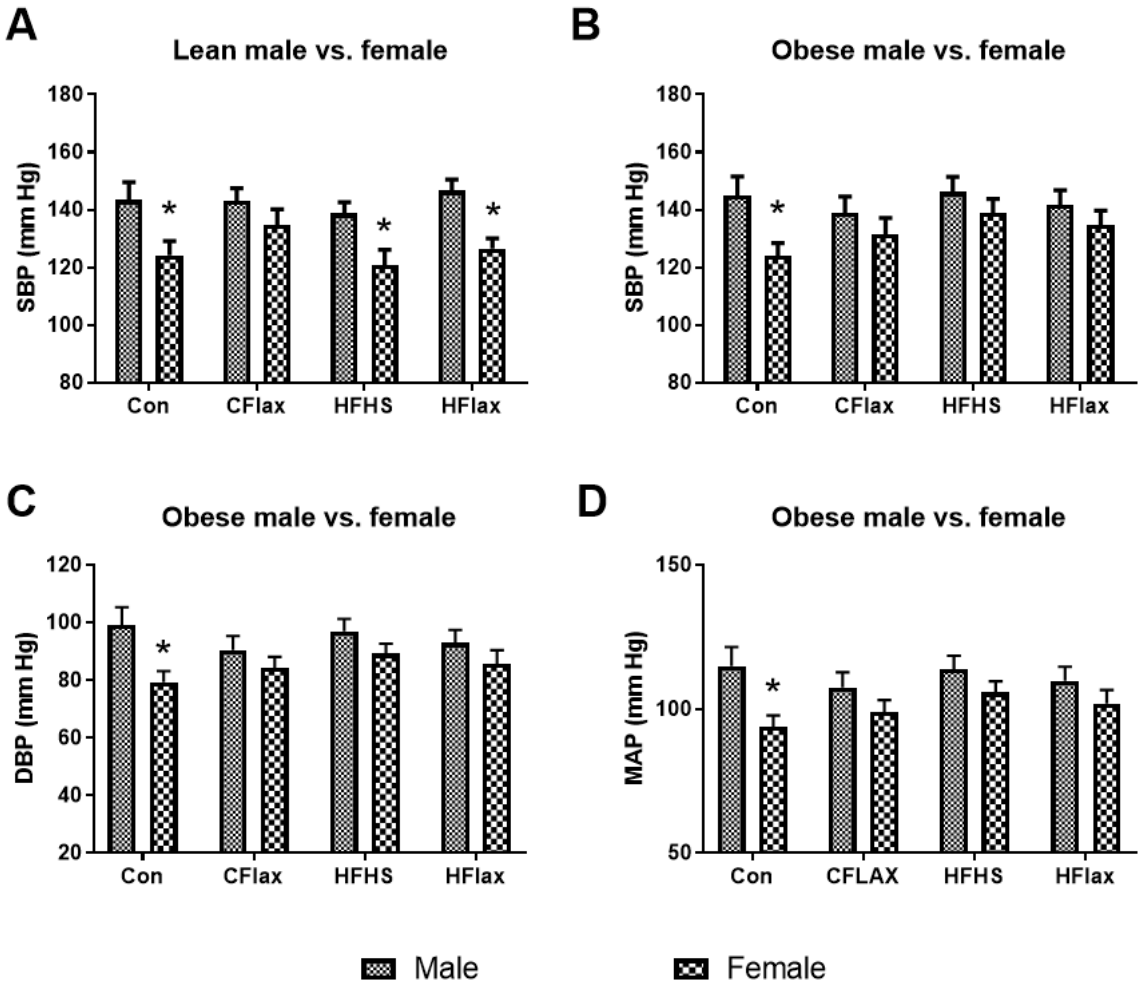


Figure 41. Effect of sex on blood pressure values of 18 weeks old JCR:LA-*cp* rats.

All values presented are mean±SE. **(A)**, SBPs of lean male versus female JCR rats; **(B)**, SBPs of obese male versus female JCR rats; **(C)**, DBPs of obese male versus female JCR rats; and **(D)**, MABPs of obese male versus female JCR rats. DBP, diastolic blood pressure; MABP, mean arterial blood pressure; and SBP, systolic blood pressure. *$p<0.05$ versus respective male JCR rats on the same diet, 2-way ANOVA. N=8.

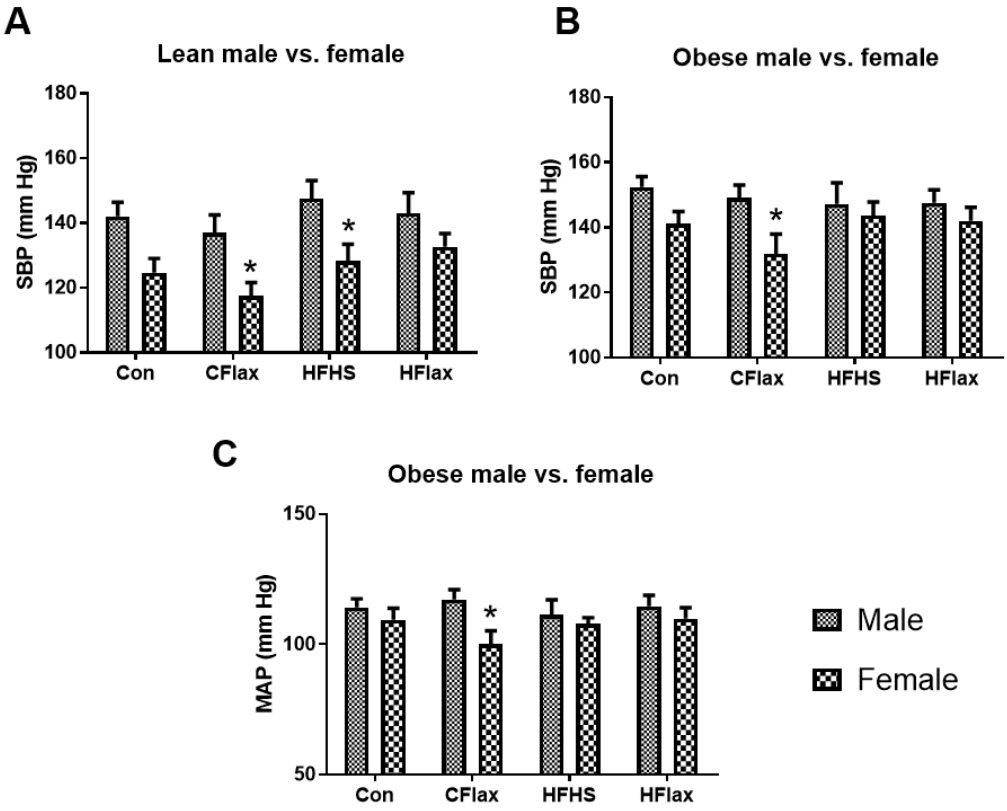


Figure 42. Effect of sex and diet on blood pressure values of 24 weeks old JCR:LA-*cp* rats. All values presented are mean±SE. **(A)**, SBPs of lean male versus female JCR:LA-*cp* rats; **(B)**, SBPs of obese male versus female JCR:LA-*cp* rats; **(C)**, MABPs of obese male versus female JCR:LA-*cp* rats. DBP, diastolic blood pressure; MABP, mean arterial blood pressure; and SBP, systolic blood pressure. *$p<0.05$ versus respective male JCR:LA-*cp* rats on the same diet, 2-way ANOVA. N=8.

A time-course analysis was performed to examine how BP values changed over 12, 18, and 24 weeks of age (Figure 43). Repeated measures 2-way ANOVA with one factor as time exhibited no change in SBPs in any of the diet groups for lean male animals. In contrast, obese male animals had significantly higher SBPs for the Con group at 24 weeks as compared to the baseline. No such changes were noted for DBPs and MABPs for both lean and obese male animals.

Lean female JCR:LA-*cp* rats had a statistically significant ~16% decrease in SBP in the CFlax at 24 weeks as compared to 12 weeks. Similar to obese male rats, female obese animals had significantly higher SBPs in the Con group at 24 weeks as compared to the baseline. No differences were observed for DBPs and MABPs for both lean and obese female JCR:LA-*cp* rats. Taken together, female JCR rats were more sensitive to the effect of ground flaxseed supplemented diet when compared with male animals.

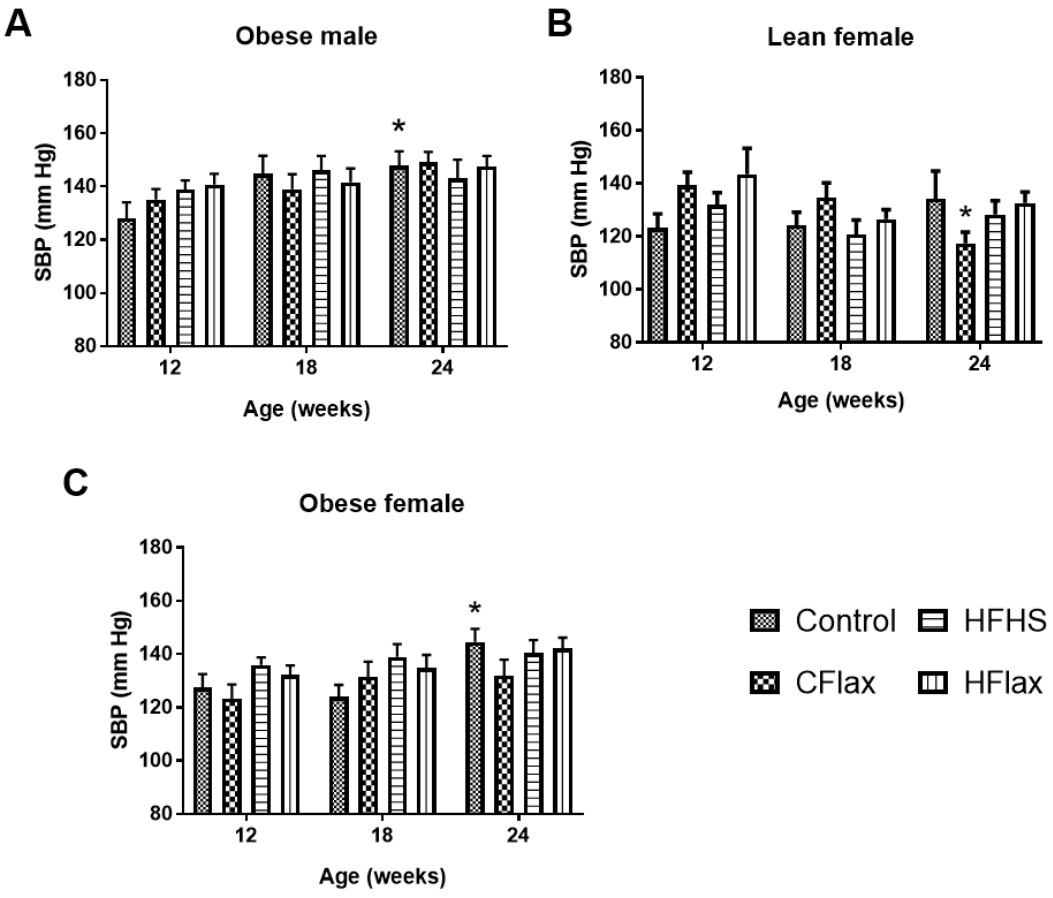
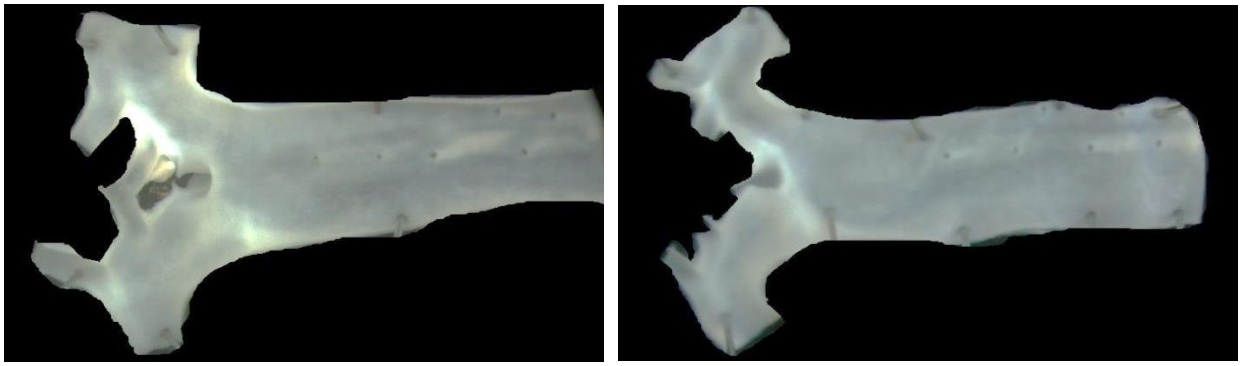


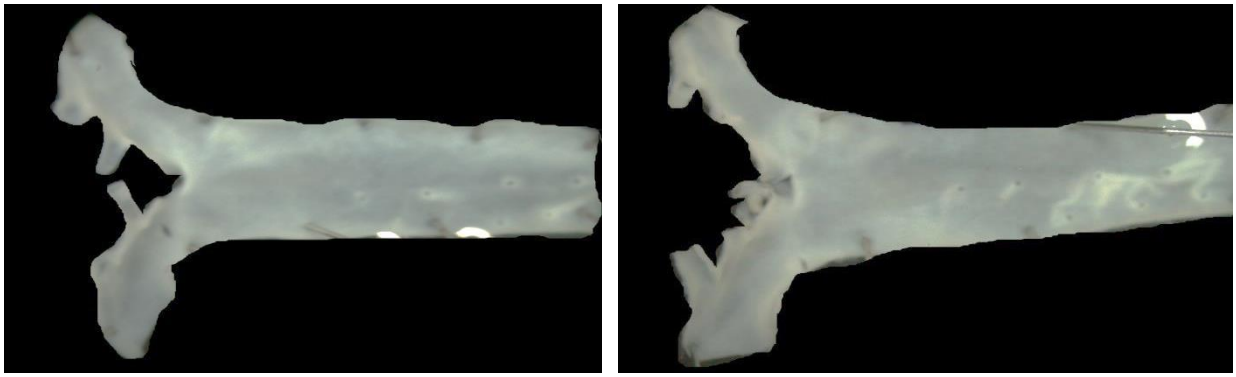
Figure 43. Temporal trends in blood pressure over a course of 12 weeks in JCR:LA-*cp* rats. All values presented are mean±SE. **(A)**, SBPs of obese male JCR rats; **(B)**, SBPs of lean female JCR rats; **(C)**, SBPs of obese female JCR rats. SBP, systolic blood pressure. *$p<0.05$ versus same group at 12 weeks, repeated measures 2-way ANOVA. N=8.

Absence of atherosclerotic lesions: no effect of genotype, diet or sex

Histological analysis by staining with Sudan IV and Oil Red O revealed the absence of lipid-rich atherosclerotic plaques on aorta tissue of JCR:LA-*cp* rats. No effect of genotype, diet or sex was observed indicating that obese JCR rats do not have atherosclerosis and the high-fat high sucrose diet supplementation does not induce any aortic lesions in this rat strain (Figure 44).



Lean Male **Lean Female**



Obese Male **Obese Female**

Figure 44. Representative images of aortic tissue stained for fat-rich plaques.

Discussion

The JCR:LA-*cp* is a unique congenic rat strain that has a corpulent (*cp*) gene mutation. The *cp* rat mutation creates a stop codon in the extracellular domain of the leptin receptor (ObR), leading to a complete absence of the ObR in the plasma membrane of homozygous *cp*/*cp* rats (11). The JCR:LA-*cp* rats homozygous for this autosomal recessive *cp* gene (*cp*/*cp*) are obese, hyperphagic, insulin resistant, and hyperlipidemic whereas the heterozygous (cp/?) animals are lean and metabolically normal (6, 11).

As expected, obese JCR:LA-*cp* rats had higher body weight and food intake compared to their lean counterparts. There was an absence of any effect of flaxseed on food intake or body weight of JCR:LA-*cp* rats. Inconsistent findings have been reported from several trials of flaxseed on body weight, with some showing positive and other negative correlations (19). Flaxseed consumption may have a favorable impact on patients with higher BMI (19). The weight reducing effect of flaxseed has been attributed to its high fiber content of ~28%, which can regulate energy intake and induce satiety (20). In a recent study by Luo et al (21), flaxseed polysaccharide significantly reduced body weights in obese rats on a high-fat diet. The anti-obesity effect of flaxseed was achieved through an inhibition of inflammation and a relief of endoplasmic reticulum stress, eventually removing leptin resistance and restoring leptin function by downregulation of NPY (21). McCullough et al (22) have shown that flaxseed (and its component of ALA) induced the expression of leptin in adipose tissue. The complete absence of the ObR (leptin receptor) in the JCR:LA-*cp* animals may have negated any functional effects of an increase in leptin expression induced by flaxseed.

Obese JCR:LA-*cp* rats had higher liver weights compared to their lean counterparts. Flaxseed supplementation lowered the liver weight in male obese JCR rats. The hepatoprotective

effect of flaxseed was further validated by the lowered plasma levels of ALT and AST in the flaxseed supplemented group. The serum levels of ALT and AST are regarded as markers of hepatocellular injury (23). As a consequence of liver damage, the hepatic cell integrity is lost leading to the release of cellular enzymes such as ATL and AST into the blood (24). The pathogenesis of liver injury includes the activation of Kupffer cells and neutrophils that induces oxidative stress and releases pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β that can cause the death of hepatocytes (25). Zhang et al (25) reported decreased hepatic inflammation by flaxseed supplementation through the reduction in plasma and hepatic tissue levels of inflammatory markers. Wang et al (26) reported that flaxseed oil blocks the activation of toll-like receptor 4 in the liver and prevents the intracellular signaling pathway that results in the production of inflammatory cytokines. The SDG component of flaxseed could also exert hepatoprotective effect due to its potent antioxidant activity (27). The synergistic effect of ALA and SDG could be responsible for the potential hepatoprotective effect of flaxseed.

Consistent with the previous observation by Diane et al (28), obese JCR:LA-*cp* rats had higher heart weights compared to their lean counterparts. There was a lack of effect of flaxseed on heart weight or heart weight/tibial length. This neutral effect of flaxseed on heart weight observed in the present study is in agreement with other feeding studies (29, 30). On the contrary, Derbali et al (31) reported a decrease in heart weight in flaxseed pretreated rats compared to the isoproterenol group. The different experimental models may explain the discrepant effect.

In the present study, JCR:LA-*cp* rats failed to show cardiac dysfunction when administered a control rat chow diet. However, when administered a diet rich in fat and sugar, lean female and obese male animals showed signs of moderate systolic dysfunction and mild diastolic dysfunction, respectively. Flaxseed supplementation improved LV internal diameter as well as systolic function

parameters such as FS and ESV in lean female JCR:LA-*cp* rats. This is consistent with an earlier study that reported the attenuation of left ventricular dilatation after an MI by dietary flaxseed supplementation (1). Doppler echocardiography revealed increased IVRT in obese JCR:LA-*cp* rats compared to the lean controls. This is consistent with previous data reporting the presence of diastolic dysfunction in obese JCR rats (28, 32). Prolonged IVRT is suggestive of impaired myocardial relaxation and increased stiffness due to fibrosis (33, 34). Dietary supplementation with flaxseed significantly lowered IVRT in obese JCR:LA-*cp* rats. The improvement in myocardial stiffness could be attributed to the powerful antifibrotic effect of flaxseed, as reported by Parikh et al (1). Flaxseed oil rich in the ALA content upregulates antifibrotic miR 29b and decreases the collagen 1A1 levels [unpublished data]. Dietary flaxseed exerts an anti-inflammatory effect by decreasing the levels of TNF-α in the ischemic myocardium (1). Thus, the antifibrotic effect of flaxseed may, in part, be mediated through the reduction in myocardial inflammation. By virtue of its effect on decreasing myocardial fibrosis and thus improving IVRT, dietary flaxseed could be an effective strategy against the progression of heart failure with preserved ejection fraction (HFpEF), however, further investigations are warranted.

One of the unique characteristics of the JCR:LA-*cp* rat strain is the presence of ischemic cardiac lesions (6). Russell et al (35) described the appearance of four types of myocardial lesions from Stage 1-4. Stage 1 lesions are areas of early necrosis without inflammatory cell infiltration. These lesions are very short-lived and progress to Stage 2 or 3 lesions (36). Stage 3 lesions are small foci of chronic inflammation without cell lysis and these lesions also have a short lifespan. The absence of stage 1 and 3 lesions in the present study could be due to the short lifespan of these lesions. Stage 4 lesions are old or advanced scarred lesions that exhibit collagen fibers accumulating as animals age (36). Although Russell et al (7) reported the presence of Stage 4

lesions in the obese JCR rats, the relatively young age of rats (24-week old) used in the present study compared to the previous study (39-week old) may explain why the Stage 4 lesions were not detected in the current study. The Stage 2 lesions are of significant size and represent ischemic lesions undergoing active resolution (36, 37). The higher incidences of the Stage 2 lesions in the present study could be possibly due to the higher fat and sugar content of the diet. Russell et al (38) attributed thrombotic disease and ischemic lesions in the obese JCR:LA-*cp* rats to the presence of macrovascular disease in the animals. Other studies (6, 39) correlated the frequency of spontaneous myocardial lesions in this rat strain with hyperinsulinemia and hyperlipidemia. Flaxseed supplementation for 12 weeks reduced the frequency of the myocardial ischemic lesions in male and female obese JCR rats. This is in parallel with the earlier observation of reduced infarct size by dietary flaxseed in an MI model induced by coronary artery ligation (1). The mechanism behind the reduced myocardial lesions by flaxseed could be multifactorial. The ALA component of flaxseed exerts a strong anti-inflammatory effect and inhibits apoptosis of cardiomyocytes (40, 41). Furthermore, ALA upregulates the expression of anti-apoptotic proteins in the myocardium, decreases the production of reactive oxygen species, and inhibits the activity of the angiotensin-converting enzyme (ACE) (31, 40, 42). ACE inhibitors are known to augment the endothelium-derived relaxing factor/nitric oxide (EDRF/NO) pathway (43) and enhance the activity of bradykinin (44). Improvement in the EDRF/NO pathway and the activity of bradykinin could improve endothelial function and contribute to the reduced myocardial lesions. The SDG content of flaxseed with its powerful antioxidant effect reduces infarct size and increases the expression of endothelial NO synthase, VEGF, and angiopoietin-I (45). By upregulating these angiogenic proteins SDG may improve the myocardial oxygen supply. Thus, the effect of flaxseed on myocardial ischemic lesions could have been mediated through the synergistic effect of its two

components. This action on ischemic lesions may also explain in part the effects observed regarding myocardial dysfunction. Ischemic lesions were very small to affect cardiac electrical activity. This explains the lack of arrhythmias observed in the study.

Upon brief examination, it may be surprising to observe the absence of hypertension in grossly obese animals in the present study. However, this BP result is in agreement with the initial study of BP changes over time in JCR:LA-*cp* rats. Russell et al (46) reported that corpulent rats have lower SBPs compared to lean animals at 8 weeks of age. However, with time, SBPs rapidly rise in obese JCR:LA rats and fall in lean JCR:LA rats. Importantly, by 35 weeks of age, there are no significant differences in SBPs between lean and obese JCR:LA animals (46). Our data also aligns with other studies (47, 48) that observed no differences in SBPs between 14-16 weeks old lean and obese JCR:LA-*cp* rats. However, our data contrasts with two previous studies, one by Soler et al (49) that observed higher SBPs and DBPs in JCR:LA rats and another by Hunter et al (15) that described higher mean arterial blood pressure (MABP) of JCR:LA-*cp* animals after 12 weeks on a normal or HFHS diet. However, instead of using the appropriate controls - the lean JCR:LA-*cp* animals, both studies compared the BP values of JCR:LA-*cp* with Sprague-Dawley rats which are neither phenotypically nor genotypically similar to JCR:LA-*cp* rats. Furthermore, in the Hunter et al study (15), no data on SBPs and DBPs were reported, but only *in vitro* estimates of MABPs using pressure myography, which is not a reliable estimate of physiological BP. Finally, the shorter duration of the study (2 weeks) in the work by Soler et al (49) could also contribute to the observed dissimilarity. Our study aligns with earlier work (46-48) that JCR:LA-*cp* rats do not have hypertension despite the obesity. This lack of hypertension may be explained genetically by the original strain (the LA/N) which was normotensive (35, 50).

Leptin has a pressor effect mediated through increased SNS activity and enhanced vascular reactivity of kidneys to noradrenaline (51). Thus, it is not surprising that in both males and females, human leptin gene mutations or leptin (*ob*/*ob*) or leptin receptor (*db*/*db*) knockout rodents have lower arterial blood pressure (52, 53). However, agouti mice, which are also a model of obesity, demonstrate higher BP than *ob*/*ob* mice (51). This suggests that obesity does not invariably cause hypertension in rodents. This contrasting BP response to obesity could be attributed to the underlying genetic and neuroendocrine mechanisms. *Ob*/*ob* mice have a complete lack of leptin effect while agouti mice that develop leptin resistance secondary to hyperleptinemia may preserve the sympatho-excitatory actions of leptin (51). Diminished SNS has been observed in JCR:LA-*cp* rats with a lower catecholamine release due to stress. Of note, no significant BP differences occur between JCR genotypes in response to restrainer induced stress (54). The lower SNS activity in normotensive JCR:LA-*cp* rats despite being severely obese may imply that a functional leptin system is required for increasing SNS activity and BP. This hypothesis is supported by the observation that low SNS activity exists in Pima Indians who have a lower prevalence of hypertension but higher rates of obesity and hyperinsulinemia (55).

Both dietary interventions, the high-fat high sucrose diet and the flaxseed supplementation, had no effect on the BP. The lack of effect of flaxseed on BP may be surprising in view of the anti-hypertensive effects of dietary flaxseed demonstrated in carefully controlled trials with patients with peripheral arterial disease (56). However, this same study showed that normotensive patients remained unaffected by flaxseed (56). Therefore, the lack of effect on normotensive BP values in JCR:LA-*cp* is entirely consistent with these results.

Rodents are typically resistant to atherosclerosis on dietary intervention and even a high cholesterol (10% w/w) diet does not produce vascular lesions (57). Only genetically modified

models such as the ApoE$^{-/-}$ mice, LDLR$^{-/-}$ mice, and cholesterol ester transfer protein (CETP) transgenic rats are used for the experimental atherosclerosis studies (57). In this study of the JCR:LA-*cp* rat model of obesity, no apparent atherosclerotic vascular lesions were observed. This is consistent with a previous study by McNamee et al (58) that reported the absence of significant atherosclerotic lesions on aortic rings by either scanning electron microscopy or light microscopy. However, earlier studies on this rat strain have discovered the presence of extensive atherosclerosis in 9 months old male *cp*/*cp* rats (10, 35). This discrepancy with the present results could be because of the younger age of rats (~6 months versus 9 months) or differences in dietary interventions used in the present study.

As expected, obese JCR:LA-*cp* rats had significantly higher total plasma cholesterol levels and LDL-C compared to the lean controls. This is in accord with earlier investigations by Russell et al (4) that reported the presence of hyperlipidemia due to hepatic hypersecretion of VLDL with subsequent effects on LDL-C and HDL-C. Flaxseed supplementation reduced the plasma levels of total cholesterol and LDL-C in male obese JCR animals. The cholesterol-lowering effect of flaxseed in the present investigation agrees with previous data (59, 60). Edel et al (61) reported a reduction of 15% and 11% in LDL-C and total cholesterol in peripheral artery disease patients within 1 month of flaxseed consumption. n-3 PUFAs decrease lipogenesis by suppressing the activation of the sterol regulatory element-binding protein-1c (SREBP-1c) transcription factor (62). However, the ALA content of flaxseed did not correlate with lowered cholesterol levels (61). The higher dietary fiber content of flaxseed, which is 28% by weight, could be responsible for the cholesterol-lowering effect of flaxseed (63). The sex-dependent effect of flaxseed could be due to differences in the lipid levels. Female obese JCR rats had only a mild elevation in the plasma cholesterol levels compared to the male obese animals. The liver estrogen signaling may have

altered lipid metabolism which in turn may have resulted in the observed sex differences (64). However, this sexual dimorphism needs further investigation. Consistent with an earlier report (65), obese JCR rats had higher TGs and HDL levels. The absence of an effect of flaxseed on TGs and HDL is in agreement with previous studies (61, 66).

The current study comprehensively investigated the effect of diet as well as sex on this unique animal strain. Collectively, the presence of dyslipidemia, hepatic injury and cardiac dysfunction makes JCR:LA-*cp* rats a model of metabolic syndrome. In this study, dietary flaxseed supplementation reduced cholesterol levels, lowered markers of liver injury, decreased ischemic lesion frequency and improved systolic and diastolic function in the JCR:LA-*cp* rats. The decrease in ischemic lesions is of particular importance. It is consistent and extends previous findings from our lab that flaxseed can reduce infarct size in a different model of MI. The previous work used an artificial means of inducing an MI (ligation of the coronary artery). The present results have now importantly extended this cardioprotective effect of dietary flaxseed to a naturally occurring spontaneous model of MI. Further studies of the underlying mechanisms are warranted to generate evidences supporting flaxseed as a highly effective treatment approach in metabolic syndrome or for preventing the progression to heart failure with preserved ejection fraction. The present study also clearly demonstrates that the JCR:LA-*cp* rat model of obesity does not manifest atherosclerosis or hypertension, even in the presence of a high fat, high sucrose diet administered over an extended period of time. This strain of rat could serve as an experimental model of obesity without the confounding factor of hypertension and atherosclerosis.

References

1. Parikh M, Raj P, Austria JA, Yu L, Garg B, Netticadan T, et al. Dietary flaxseed protects against ventricular arrhythmias and left ventricular dilation after a myocardial infarction. J Nutr Biochem. 2019;71:63-71.

2. Wu Y, Yin X, Wijaya C, Huang M-H, McConnell BK. Acute myocardial infarction in rats. J Vis Exp. 2011(48):2464.

3. Kumar M, Kasala ER, Bodduluru LN, Dahiya V, Sharma D, Kumar V, et al. Animal models of myocardial infarction: Mainstay in clinical translation. Regul Toxicol Pharmacol. 2016;76:221-30.

4. Russell JC, Proctor SD. Small animal models of cardiovascular disease: Tools for the study of the roles of metabolic syndrome, dyslipidemia, and atherosclerosis. Cardiovasc Pathol. 2006;15(6):318-30.

5. Panchal SK, Brown L. Rodent models for metabolic syndrome research. J Biomed Biotechnol. 2011;2011:351982.

6. Russell JC, Graham SE, Richardson M. Cardiovascular disease in the jcr:La-cp rat. Mol Cell Biochem. 1998;188(1-2):113-26.

7. Russell JC, Graham SE, Amy RM, Dolphin PJ. Inhibition of myocardial lesions in the jcr:La-corpulent rat by captopril. J Cardiovasc Pharmacol. 1998;31(6):971-7.

8. Russell JC, Koeslag DG. Jcr:La-corpulent rat: A strain with spontaneous vascular and myocardial disease. ILAR Journal. 1990;32(3):27-32.

9. Diane A, Pierce WD, Kelly SE, Sokolik S, Borthwick F, Jacome-Sosa M, et al. Mechanisms of comorbidities associated with the metabolic syndrome: Insights from the jcr:La-cp corpulent rat strain. Frontiers in nutrition. 2016;3:44-.

10. Russell JC, Amy RM. Early atherosclerotic lesions in a susceptible rat model. The la/n-corpulent rat. Atherosclerosis. 1986;60(2):119-29.

11. Brindley DN, Russell JC. Animal models of insulin resistance and cardiovascular disease: Some therapeutic approaches using jcr:La-cp rat. Diabetes Obes Metab. 2002;4(1):1-10.

12. Lerman LO, Kurtz TW, Touyz RM, Ellison DH, Chade AR, Crowley SD, et al. Animal models of hypertension: A scientific statement from the american heart association. Hypertension. 2019;73(6):e87-e120.

13. Syme C, Shin J, Richer L, Gaudet D, Paus T, Pausova Z. Sex differences in blood pressure hemodynamics in middle-aged adults with overweight and obesity. Hypertension. 2019;74(2):407-12.

14. Lovren F, Teoh H, Verma S. Obesity and atherosclerosis: Mechanistic insights. Can J Cardiol. 2015;31(2):177-83.

15. Hunter I, Soler A, Joseph G, Hutcheson B, Bradford C, Zhang FF, et al. Cardiovascular function in male and female jcr:La-cp rats: Effect of high-fat/high-sucrose diet. Am J Physiol Heart Circ Physiol. 2017;312(4):H742-h51.

16. Wilde E, Aubdool AA, Thakore P, Baldissera L, Alawi KM, Keeble J, et al. Tail-cuff technique and its influence on central blood pressure in the mouse. Journal of the American Heart Association. 2017;6(6):e005204.

17. Mohanta S, Yin C, Weber C, Hu D, Habenicht A, Jr. Aorta atherosclerosis lesion analysis in hyperlipidemic mice. Bio-protocol. 2016;6(11):e1833.

18. Russell J, Amy R, Michaelis O, McCune S, Abraham A. Myocardial disease in the corpulent strains of rats. Frontiers in diabetes research: lessons from animal diabetes III. 1990:402-7.

19. Mohammadi-Sartang M, Mazloom Z, Raeisi-Dehkordi H, Barati-Boldaji R, Bellissimo N, Totosy de Zepetnek JO. The effect of flaxseed supplementation on body weight and body composition: A systematic review and meta-analysis of 45 randomized placebo-controlled trials. Obes Rev. 2017;18(9):1096-107.

20. Pan A, Chen M, Chowdhury R, Wu JH, Sun Q, Campos H, et al. Alpha-linolenic acid and risk of cardiovascular disease: A systematic review and meta-analysis. Am J Clin Nutr. 2012;96(6):1262-73.

21. Luo J, Qi J, Wang W, Luo Z, Liu L, Zhang G, et al. Antiobesity effect of flaxseed polysaccharide via inducing satiety due to leptin resistance removal and promoting lipid metabolism through the amp-activated protein kinase (ampk) signaling pathway. J Agric Food Chem. 2019;67(25):7040-9.

22. McCullough RS, Edel AL, Bassett CM, Lavallee RK, Dibrov E, Blackwood DP, et al. The alpha linolenic acid content of flaxseed is associated with an induction of adipose leptin expression. Lipids. 2011;46(11):1043-52.

23. Pratt DS, Kaplan MM. Evaluation of abnormal liver-enzyme results in asymptomatic patients. N Engl J Med. 2000;342(17):1266-71.

24. Ume Salma N, Serva Peddha M, Aswathanarayana Setty JL. Ameliorative effect of flaxseed (linum usitatissimum) and its protein on ethanol-induced hepatotoxicity in wistar rats. J Food Biochem. 2019;43(12):e13047.

25. Zhang X, Wang H, Yin P, Fan H, Sun L, Liu Y. Flaxseed oil ameliorates alcoholic liver disease via anti-inflammation and modulating gut microbiota in mice. Lipids Health Dis. 2017;16(1):44.

26. Wang M, Zhang X-J, Yan C, He C, Li P, Chen M, et al. Preventive effect of α-linolenic acid-rich flaxseed oil against ethanol-induced liver injury is associated with ameliorating gut-derived endotoxin-mediated inflammation in mice. J Funct Foods. 2016;23:532-41.

27. Kezimana P, Dmitriev AA, Kudryavtseva AV, Romanova EV, Melnikova NV. Secoisolariciresinol diglucoside of flaxseed and its metabolites: Biosynthesis and potential for nutraceuticals. Frontiers in genetics. 2018;9:641.

28. Diane A, Borthwick F, Wu S, Lee J, Brown PN, Dickinson TA, et al. Hypolipidemic and cardioprotective benefits of a novel fireberry hawthorn fruit extract in the jcr:La-cp rodent model of dyslipidemia and cardiac dysfunction. Food Funct. 2016;7(9):3943-52.

29. Shafie SR, Wanyonyi S, Panchal SK, Brown L. Linseed components are more effective than whole linseed in reversing diet-induced metabolic syndrome in rats. Nutrients. 2019;11(7).

30. Park JB, Velasquez MT. Potential effects of lignan-enriched flaxseed powder on bodyweight, visceral fat, lipid profile, and blood pressure in rats. Fitoterapia. 2012;83(5):941-6.

31. Derbali A, Mnafgui K, Affes M, Derbali F, Hajji R, Gharsallah N, et al. Cardioprotective effect of linseed oil against isoproterenol-induced myocardial infarction in wistar rats: A biochemical and electrocardiographic study. J Physiol Biochem. 2015;71(2):281-8.

32. Borthwick F, Warnakula S, Mangat R, Uwiera RR, Russell JC, Kelly SE, et al. Apoa-1 infusion reduces arterial cholesterol and myocardial lesions in a rat model of cardiac dysfunction and insulin resistance. Atherosclerosis. 2012;222(2):402-8.

33. Arques S, Roux E, Sbragia P, Pieri B, Gelisse R, Ambrosi P, et al. Accuracy of the isovolumic relaxation time in the emergency diagnosis of new-onset congestive heart failure with preserved left ventricular systolic function in the setting of b-type natriuretic peptide levels in the mid-range. Int J Cardiol. 2008;124(3):400-3.

34. Yu WC, Chiou KR, Lin YP, Lee WH, Huang WB, Chen CH. Non-invasive determination of left ventricular relaxation time constant by transthoracic doppler echocardiography. J Chin Med Assoc. 2004;67(7):317-22.

35. Russell JC, Amy RM. Myocardial and vascular lesions in the la/n-corpulent rat. Can J Physiol Pharmacol. 1986;64(10):1272-80.

36. Russell JC, Kelly SE, Vine DF, Proctor SD. Irbesartan-mediated reduction of renal and cardiac damage in insulin resistant jcr : La-cp rats. Br J Pharmacol. 2009;158(6):1588-96.

37. Mangat R, Warnakula S, Borthwick F, Hassanali Z, Uwiera RR, Russell JC, et al. Arterial retention of remnant lipoproteins ex vivo is increased in insulin resistance because of increased arterial biglycan and production of cholesterol-rich atherogenic particles that can be improved by ezetimibe in the jcr:La-cp rat. Journal of the American Heart Association. 2012;1(5):e003434.

38. Russell JC, Proctor SD, Kelly SE, Lohn M, Busch AE, Schafer S. Insulin-sensitizing and cardiovascular effects of the sodium-hydrogen exchange inhibitor, cariporide, in the jcr: La-cp rat and db/db mouse. J Cardiovasc Pharmacol. 2005;46(6):746-53.

39. Russell JC, Ewart HS, Kelly SE, Kralovec J, Wright JL, Dolphin PJ. Improvement of vascular dysfunction and blood lipids of insulin-resistant rats by a marine oil-based phytosterol compound. Lipids. 2002;37(2):147-52.

40. Xie N, Zhang W, Li J, Liang H, Zhou H, Duan W, et al. A-linolenic acid intake attenuates myocardial ischemia/reperfusion injury through anti-inflammatory and anti-oxidative stress effects in diabetic but not normal rats. Arch Med Res. 2011;42(3):171-81.

41. Ganguly R, Hasanally D, Stamenkovic A, Maddaford TG, Chaudhary R, Pierce GN, et al. Alpha linolenic acid decreases apoptosis and oxidized phospholipids in cardiomyocytes during ischemia/reperfusion. Mol Cell Biochem. 2018;437(1):163-75.

42. Carotenuto F, Minieri M, Monego G, Fiaccavento R, Bertoni A, Sinigaglia F, et al. A diet supplemented with ala-rich flaxseed prevents cardiomyocyte apoptosis by regulating caveolin-3 expression. Cardiovasc Res. 2013;100(3):422-31.

43. Becker RH. [ace inhibition and atherosclerosis in the animal model]. Z Kardiol. 1994;83 Suppl 4:15-20.

44. Linz W, Wiemer G, Scholkens BA. Contribution of bradykinin to the cardiovascular effects of ramipril. J Cardiovasc Pharmacol. 1993;22 Suppl 9:S1-8.

45. Penumathsa SV, Koneru S, Thirunavukkarasu M, Zhan L, Prasad K, Maulik N. Secoisolariciresinol diglucoside: Relevance to angiogenesis and cardioprotection against ischemia-reperfusion injury. J Pharmacol Exp Ther. 2007;320(2):951-9.

46. Russell JC, Amy RM. Plasma lipids and other factors in the la/n corpulent rat in the presence of chronic exercise and food restriction. Can J Physiol Pharmacol. 1986;64(6):750-6.

47. Wascher TC, Wolkart G, Russell JC, Brunner F. Delayed insulin transport across endothelium in insulin-resistant jcr:La-cp rats. Diabetes. 2000;49(5):803-9.

48. McKendrick JD, Salas E, Dube GP, Murat J, Russell JC, Radomski MW. Inhibition of nitric oxide generation unmasks vascular dysfunction in insulin-resistant, obese jcr:La-cp rats. Br J Pharmacol. 1998;124(2):361-9.

49. Soler A, Hunter I, Joseph G, Hutcheson R, Hutcheson B, Yang J, et al. Elevated 20-hete in metabolic syndrome regulates arterial stiffness and systolic hypertension via mmp12 activation. J Mol Cell Cardiol. 2018;117:88-99.

50. Richardson M, Schmidt AM, Graham SE, Achen B, DeReske M, Russell JC. Vasculopathy and insulin resistance in the jcr:La-cp rat. Atherosclerosis. 1998;138(1):135-46.

51. Mark AL, Shaffer RA, Correia ML, Morgan DA, Sigmund CD, Haynes WG. Contrasting blood pressure effects of obesity in leptin-deficient ob/ob mice and agouti yellow obese mice. J Hypertens. 1999;17(12 Pt 2):1949-53.

52. Faulkner JL, Belin de Chantemele EJ. Sex differences in mechanisms of hypertension associated with obesity. Hypertension. 2018;71(1):15-21.

53. Hall JE, da Silva AA, do Carmo JM, Dubinion J, Hamza S, Munusamy S, et al. Obesity-induced hypertension: Role of sympathetic nervous system, leptin, and melanocortins. J Biol Chem. 2010;285(23):17271-6.

54. McArthur MD, Graham SE, Russell JC, Brindley DN. Exaggerated stress-induced release of nonesterified fatty acids in jcr:La-corpulent rats. Metabolism. 1998;47(11):1383-90.

55. Weyer C, Pratley RE, Snitker S, Spraul M, Ravussin E, Tataranni PA. Ethnic differences in insulinemia and sympathetic tone as links between obesity and blood pressure. Hypertension. 2000;36(4):531-7.

56. Rodriguez-Leyva D, Weighell W, Edel AL, LaVallee R, Dibrov E, Pinneker R, et al. Potent antihypertensive action of dietary flaxseed in hypertensive patients. Hypertension. 2013;62(6):1081-9.

57. Leong X-F, Ng C-Y, Jaarin K. Animal models in cardiovascular research: Hypertension and atherosclerosis. BioMed Research International. 2015;2015:11.

58. McNamee CJ, Kappagoda CT, Kunjara R, Russell JC. Defective endothelium-dependent relaxation in the JCR:La-corpulent rat. Circ Res. 1994;74(6):1126-32.

59. Bloedon LT, Balikai S, Chittams J, Cunnane SC, Berlin JA, Rader DJ, et al. Flaxseed and cardiovascular risk factors: Results from a double blind, randomized, controlled clinical trial. J Am Coll Nutr. 2008;27(1):65-74.

60. Mandasescu S, Mocanu V, Dascalita AM, Haliga R, Nestian I, Stitt PA, et al. Flaxseed supplementation in hyperlipidemic patients. Rev Med Chir Soc Med Nat Iasi. 2005;109(3):502-6.

61. Edel AL, Rodriguez-Leyva D, Maddaford TG, Caligiuri SP, Austria JA, Weighell W, et al. Dietary flaxseed independently lowers circulating cholesterol and lowers it beyond the effects of cholesterol-lowering medications alone in patients with peripheral artery disease. The Journal of Nutrition. 2015;145(4):749-57.

62. Delarue J, LeFoll C, Corporeau C, Lucas D. N-3 long chain polyunsaturated fatty acids: A nutritional tool to prevent insulin resistance associated to type 2 diabetes and obesity? Reprod Nutr Dev. 2004;44(3):289-99.

63. Brown L, Rosner B, Willett WW, Sacks FM. Cholesterol-lowering effects of dietary fiber: A meta-analysis. Am J Clin Nutr. 1999;69(1):30-42.

64. Palmisano BT, Zhu L, Eckel RH, Stafford JM. Sex differences in lipid and lipoprotein metabolism. Mol Metab. 2018;15:45-55.

65. Russell JC, Koeslag DG, Amy RM, Dolphin PJ. Plasma lipid secretion and clearance in hyperlipidemic jcr:La-corpulent rats. Arteriosclerosis. 1989;9(6):869-76.

66. Pan A, Yu D, Demark-Wahnefried W, Franco OH, Lin X. Meta-analysis of the effects of flaxseed interventions on blood lipids. Am J Clin Nutr. 2009;90(2):288-97.

CHAPTER 3: LIMITATIONS

There are several limitations in the studies conducted during this thesis research.

With regard to the LAD ligation study:

1) As only male rats were examined for the effect of dietary flaxseed in the first study of the LAD ligation-induced MI, sex differences cannot be ruled out.
2) Given the low mortality observed in this study, the sample size could have been too small to detect differences in cardiac function. This small sample size could be due to the overestimation of the effect size while calculating the power of the study.
3) No mechanistic data was generated to explain the antiarrhythmic effect. Future work is warranted to describe the action via electrophysiological techniques.
4) If the protocol had been designed to continue for a few additional weeks beyond 8 weeks post-MI, we may have been able to assess the effect of dietary flaxseed on severely depressed cardiac function in conditions of heart failure.
5) A dose-response study to evaluate the effect of different amounts of flaxseed in this model could have added more information. Although a powerful anti-atherosclerotic effect has been observed in the other studies at the same dose investigated here, a higher amount of flaxseed in the diet may have resulted in a significant improvement in cardiac function.
6) As the primary objective was to study the effect on ventricular remodeling after permanent coronary artery occlusion, results should be interpreted with caution when extrapolating to the human condition of ischemia/reperfusion and non-ST elevation myocardial infarction where other complicating factors like atherosclerosis, cardiomyopathy, vasospasm, etc could play important modulatory roles in the pathology.

7) There was a lack of continuous assessment of specific microRNAs at different time points after the induction of MI due to limited resources and protocol design. This monitoring could have strengthened our results.

With regard to the study of JCR:LA-*cp* rats:

1) Limited heart tissue availability precluded assessment of molecular mechanisms in the JCR:LA-*cp* genetic rat model of spontaneous myocardial ischemia.
2) Discrepant plasma levels of ALA in animals on control and high-fat high-sucrose diet despite supplementation of the same amount of flaxseed warrants further investigation.
3) As humans are less likely to consume a high-fat high-sucrose diet around the clock, the feeding protocol consisting of ad libitum high-fat high-sucrose diet represents an extreme scenario.
4) Although macroscopic staining evaluation revealed an absence of atherosclerotic lesions, electron microscopy may have yielded information about the adverse vascular changes at the microscopic level.
5) As this study utilized a genetic model of leptin receptor mutation, the generalizability of our findings to the general human population is limited. However, this was a comprehensive trial examining the effect of diet in an obese rat model of cardiometabolic syndrome involving both sexes.

CHAPTER 4: SUMMARY

Dietary flaxseed has been shown to be protective against atherosclerosis and hypertension in an array of experimental and clinical studies. The work in this thesis investigated the effects of dietary supplementation of flaxseed in an experimental setting of ischemic heart disease.

The first study involved the induction of acute myocardial infarction (MI) by ligating the left artery descending coronary artery in Sprague Dawley rats. Animals were fed either ground flaxseed, alpha-linolenic acid (ALA) rich flaxseed oil, or flax lignan rich in secoisolariciresinol diglycoside (SDG) for 2 weeks before and for 8 weeks after the induction of MI. The data obtained from this study suggested that flaxseed supplementation can reduce infarct size and fibrosis, decrease the incidences of arrhythmias, and prevent adverse left ventricular remodeling after an MI.

To explore the molecular mechanisms and identify the potential bioactive component of flaxseed responsible for the observed effect in an MI, specific microRNA expression analysis and inflammation assays were performed. Flaxseed, as well as its components, decreased the expression levels of an inflammatory marker TNF-α and an extracellular matrix protein, collagen I. Flaxseed oil rich in ALA upregulated the expression of microRNAs 133a, 135a, and 29b suggesting ALA could be responsible for the antiarrhythmic, antiapoptotic, and antifibrotic effects of dietary flaxseed in an MI. Based on the positive results from the first study, the effects of dietary flaxseed on the heart were investigated further.

The second study investigated the effects of dietary flaxseed in the JCR:LA-*cp* genetic rat model of metabolic syndrome that spontaneously develops myocardial ischemic lesions. Dietary flaxseed supplementation for 12 weeks reduced plasma levels of cholesterol and liver enzymes,

decreased myocardial ischemic lesions and improved cardiac systolic and diastolic function. Flaxseed supplementation also prevented the worsening of cardiac function when animals were fed a high-fat, high-sugar diet to exacerbate metabolic and cardiac dysfunction.

Taken together, the data generated in this thesis suggest that dietary flaxseed represents a highly effective cardioprotective agent in a setting of myocardial ischemia and MI. Furthermore, flaxseed could also extend its therapeutic effect against myocardial injury in a condition of cardiometabolic syndrome. Further studies are warranted to extend these therapeutic uses of flaxseed to human conditions of myocardial ischemia and MI and to understand the molecular mechanisms responsible for these significant cardioprotective effects.