Long-term consumption of wild rice (*Zizania palustris L.*) in combination with phytosterols prevents atherosclerosis in LDL receptor-knock-out mice

by

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A Thesis

Submitted to the Faculty of Graduate Studies of

The University of Manitoba

in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

Department of Human Nutritional Sciences

University of Manitoba

Winnipeg

ABSTRACT

Atherosclerosis is the primary underlying pathology of CVD. Dietary treatment may be considered as one of the initial steps in the prevention of atherosclerosis. Replacing refined carbohydrate source of a cholesterol- enriched diet with antioxidant rich whole grain and inclusion of phytonutrition in the diet such as wild rice and phytosterols may reduce cardiovascular risk factors. The wild rice (*Zizania palustris* L.), an annual plant native to aquatic areas of the northern America, receives much attention by researchers because of its potent nutritional and phytochemical contents. Furthermore, another dietary component with cardiovascular benefits is the inclusion of plant sterols in our daily diet. The aim of this study was to investigate the antiatherogenic activity of wild rice in combination with phytosterols in LDL-r-KO mice. Male LDL-r-KO mice were divided into 4 groups receiving one of the following experimental diets for 20 weeks: 1. Atherogenic diet, 2.Wild rice (as the main source of dietary carbohydrates) diet, 3. 2% Phytosterols-enriched diet and 4. Diet containing both wild rice and 2 % phytosterols. Blood samples were collected through jugular vein during study, and at sacrifice through cardiac puncture; the heart and fecal materials were collected and used for biochemical and histological examinations. The supplementation of wild rice in combination with phytosterols to an atherogenic diet for up to 20 weeks significantly reduced the total plasma concentrations of cholesterol (TC) in LDLr-KO mice. However, there was no significant difference in triglyceride (TG) in wild rice in combination with phytosterols after 20 week exposure of diet. Further, wild rice in combination with phytosterols resulted in increased fecal excretion of cholesterol. Also, there was reduction in the development of atherosclerotic lesion in the group of mice supplemented with wild rice

in combination with 2% phytosterols (w/w). Our data support that combination of plant sterols and wild rice does not have additive effect in lowering cardiovascular risk.

ACKNOWLEDGMENTS

My last remaining task is to acknowledge all those people that have contributed to the work described in this thesis. First, I deeply thank my supervisor, Dr. Mohammed Moghadasian, whose expertise, understanding, and patience, added considerably to my graduate experience. Without his encouragement and constant guidance, I would not have finished this dissertation. I would also like to thank my committee members, Dr. Trust Beta, and Dr. Usha Thiyam for taking the time to support me during my thesis endeavors.

I am thankful to the Canadian Centre for Agri-Food Research in Health and Medicine (CCARM) for providing us the great facility for the use of research, as well as Agri-Food Research and Development Initiatives (ARDI) for their generous financial support that allow the success of this study. I am also grateful to my sponsor, the Saudi Cultural Bureau in Ottawa, for their generous financial support as studentship for the duration of my degree. Additional thanks for the R.O. Burrell staff for their help with animal care.

My sincere indebtedness goes to those people who are behind the written, published, and distributed books, and journals that I have referenced from in this dissertation and to my friends, particularly, Naser Ibrahim and Ala'a Eideh who have all provided me with support, guidance, friendship, and who have enriched my experience along the way. I also want to thank my former and present colleagues, at the Pathology Research Laboratory, for all their help, support, interest and valuable hints. Special thanks to our lab technician, Khuong Le for his constant guidance and patient as well as,

the postdoctoral fellowship Dr. Surendiran Gangadaran for all his support and encouragement to lead me through the research project.

Finally, most of all my love and deepest admiration goes to my mother, father, sisters, especially, Samira and brothers. I thank them all for their extraordinary support over all these years, without their love, encouragement and endless support, I would not have finished this thesis.

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LIST OF ABBREVIATIONS

$^{\bullet}$No	Nitric oxide
ABCA1	ATP binding cassette A1
ACAT	Acyl CoA: cholesterol acyltransferase
ACVD	Atherosclerotic cardiovascular disease
Apo	Apolipoprotein
CVD	Cardiovascular disease
CYP7A1	Cholesterol 7-α hydroxylase
DF	Dietary fiber
DHP	Dihydroxyacetone phosphate
DPPH	2,2-diphenyl-1-picrylhydrazyl
FDA	Food and Drug Administration
FFAs	Free fatty acids
GPO	Glycerophosphate oxidase
H_2O_2	Hydrogen peroxide
HDL	High -density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl-co-enzyme A
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase
LDL	Low-density lipoprotein

LDLr-KO	Low-density lipoprotein -receptor-knock-out
LPL	Lipoprotein lipase
MDA	Malondialdehyde
MetS	Metabolic syndrome
ORAC	Oxygen radical absorbance capacity
PH	Power of hydrogen
PPARα	Peroxisome proliferator activated receptor-α
RCT	Reverse cholesterol transport
SD	Standard deviation
SOD	Superoxide dismutase
TC	Total cholesterol
TG	Triglyceride
VLDL	Very -low density lipoprotein
WDR	Wild rice
WHO	World Health Organization

1. Introduction

Natural products and their derivatives represent more than 50% of the agents used in medical clinics throughout the world (Farnsworth et al., 1985; Cragg, 2005a). Humans use about 1 to 10% of 250000 to 500000 species of plants on earth as food and medicine (Cowan, 1999). With current advances in biological sciences, the number of plants with medical properties is ever increasing. The role of diet in preventing and controlling non-communicable diseases (Rajaraman, 2010), especially chronic diseases (WHO, 2003) have been reported in several epidemiological studies. Intake of improper diet and decreased physical activities are linked with increased incidence of a number of chronic disease including obesity, diabetes mellitus, cardiovascular disease (CVD), hypertension, stroke, and some type of cancer (WHO, 1990).

Many genetic and environmental risk factors have been reported to be involved in the development of chronic diseases. For example, elevated levels of serum cholesterol have been reported as a key risk factor for the development of atherosclerosis (Glass and Witztum, 2001). Hence the most common treatments for atherosclerosis are therapies directed at lowering cholesterol levels (Andersson et al., 2010). Plasma levels of cholesterol can be significantly reduced by inhibition of cholesterol absorption within the intestine and thereby inhibiting the atherosclerosis development. This reduction in cholesterol absorption can be achieved by the improvement of dietary pattern such as plant sterol in our healthy diet (Moghadasian et al., 1997; Moghadasian, 2006). In addition to plant sterols, other dietary components such as dietary fiber, garlic products,

soy proteins, n-3 fatty acids and others have been also reported to reduce the risk of chronic diseases (Abdullah et al., 2007; El-Sabban and Abouazra, 2008; Mellen et al., 2008; Othman et al., 2008; Riediger et al., 2008).

Whole grains are rich sources of dietary fiber, vitamins, minerals, phytosterols, and antioxidant phenolic compounds (Okarter and Liu, 2010). The health benefits of whole grains have been suggested to contribute to the synergistic health effects such as blood lipid lowering components. The major whole grains are wheat, rice and corn (Slavin, 2004). In 2006, wild rice was recognized as a whole grain by the U.S. Food and Drug Administration.

Dietary fiber in whole grain has been shown to promote better health. A growing body of evidence from both animal and human studies suggests that the consumption of sufficient amounts of dietary fiber may reduce the risks of obesity (Slavin, 2005), CVD (Mozaffarian et al., 2003), type II diabetes (Panahi et al., 2007) and colorectal cancer (Bingham et al., 2003). The refining process of whole grains to remove germ and bran, results in loss of functional compounds such as vitamins, minerals, dietary fiber and other phytochemicals (Adom et al., 2005; Slavin et al., 1999). Unlike many conventional whole grains, wild rice is not routinely refined.

Wild rice (*Zizania palustris* L.) was historically consumed by Native Americans and Canadians as a staple food (Lorenz, 1981). It is rich in minerals, vitamins, protein, starch, dietary fiber and various antioxidant phytochemicals and it is low in fat. In addition, nutritional analysis shows that wild rice has several phenolic compounds, such as ferulic acid (Qiu et al., 2010). The beneficial effect of wild rice was first reported in

late 1924. Since then, many studies have been discussing its agricultural and nutritional features (Anderson, 1976; Thomas, 1988; Dore, 1969; Archibold, 2003)

Furthermore, cardiovascular benefits of long-term consumption of wild rice have been reported. Zhang et al., (2009), reported that incorporation of wild ice into the atherogenic diet suppressed the increase in serum triglycerides and total cholesterol, and the decrease in high-density lipoprotein (HDL) cholesterol levels. Also increases in superoxide dismutase (SOD) activity and reductions in malondialdehyde concentrations in the serum and liver tissues of wild rice-fed animals have been reported (Zhang et al., 2009). Rats fed wild rice (WRD) for 8 weeks had reduced total cholesterol (TC), triglyceride (TG) and free fatty acids (FFA) concentrations, and a raised HDL cholesterol level as compared to city diet (CD) group. Also, serum leptin concentration was significantly inhibited (Han et al., 2012). More recently, we reported cholesterol-lowering and anti-atherogenic effects of wild rice in LDL-r-KO mice (Surendiran et al., 2013). The supplementation of wild rice to an atherogenic diet for over 24 weeks significantly reduced the plasma concentrations of low-density lipoproteins (LDL) and very low-density lipoprotein (VLDL) in LDL receptor-knock-out mice (LDLr – KO mice). Responsiveness to the wild rice differed between male and female mice; female mice responded better to cholesterol-lowering effects of wild rice. This gender-related variation may be due to the presence of endogenous estrogen. Incorporation of wild rice in the diet distinctly increased its total phenolic and dietary fiber contents compared to the conventional white rice diet. However, the plasma and erythrocyte SOD and catalase activities were not significantly improved. The hypocholesterolemic properties of wild

rice mainly attributed to the increased rate of fecal cholesterol excretion (Surendiran et al., 2013).

Manitoban wild rice is mainly grown in Manitoba's natural lakes, streams and rivers. It is also known as 'Canadian Lake Wild Rice', the name is based on its origin and growing nature. On Manitoba, wild rice is harvested from natural bodies of water as opposed to being cultivated or paddy-grown in other areas. There are two major growing areas in Manitoba: east of Lake Winnipeg in and around the Whiteshell area, and northwest area around The Pas and Flin Flon. In average, Manitoba contributes approximately 25% of Canada's annual wild rice production (Manitoba Agriculture and Commodities).

Therefore, the purpose of this study was to investigate the effectiveness of Manitoban wild rice in combination with plant sterol on atherosclerosis risk factors in LDL-r-KO mice. Plasma and fecal lipid profiles were determined to assess the effect of wild rice combination with plant sterol on lipid metabolism. Also, histological examination of the hearts and lesion area were analyzed.

2. Literature review

2.1. Cardiovascular diseases

Cardiovascular diseases (CVD) are among the leading causes of illness and mortality in Canada and in many parts of the western world (Genest et al., 2009). It is a general term for several different forms of diseases that result in the heart and circulatory system. World Health Organization (WHO) reports that the number of mortality from CVD will be increased to almost 23.6 million people in 2030 (WHO, 2011). In Canada, CVD accounts for one-third of mortality in adult men and women (Genest et al., 2009). Cardiovascular disease is a chronic disease caused by ranging from structural defects, to infection, inflammation, environment and genetics. There are many known non-modifiable CVD risk factors such as, age, sex and family history; other risk factors can be controlled (modifiable risk factors) include smoking, alcohol, physical activity, diabetes mellitus, obesity, high blood pressure and high blood lipids (hypercholesterolemia) (Scarpellini and Tack, 2012).

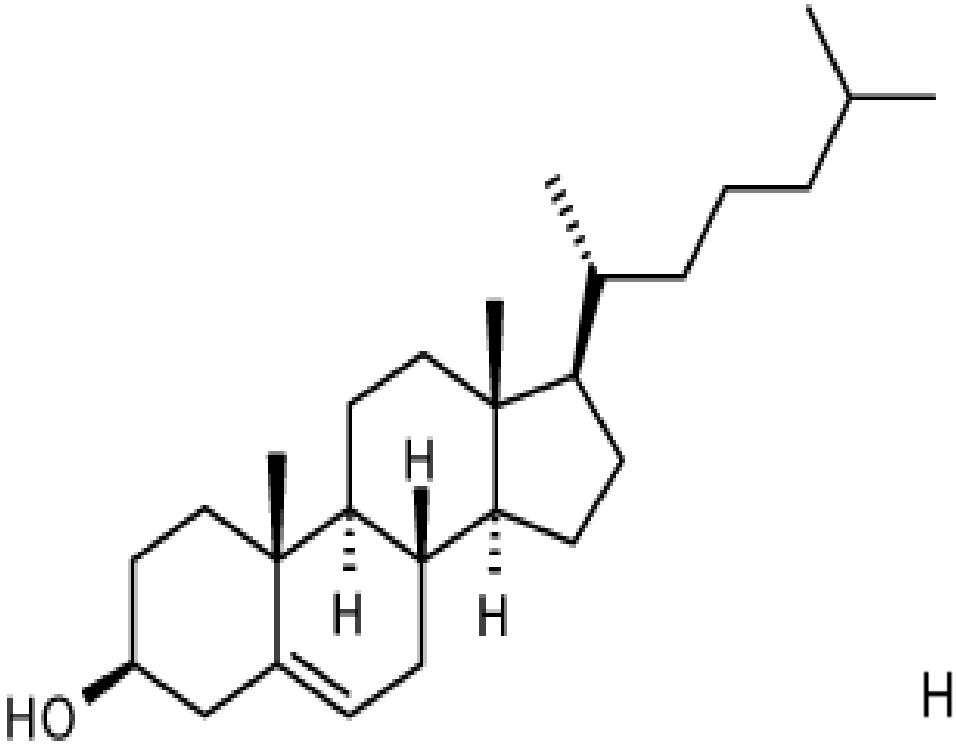
2.2. Cholesterol

Cholesterol is a type of sterol that contains 27 carbon atoms, 46 hydrogen and one "hydroxyl" group. It is an unsaturated lipid containing 2 double bonds. **Figure 1** illustrates the chemical structure of cholesterol. Cholesterol is essential for cellular function and biochemical reactions in the body (Pucadyil and Chattopadhyay, 2006). Further, cholesterol plays an integral role in human cells and perform many functions including, but not limited to, biosynthesis of steroid hormones, bile acids, and vitamin D and transporting substances through the blood from the liver to the tissues (Hanukoglu, 1992). Our body cholesterol pool comes from two sources either being absorbed from the

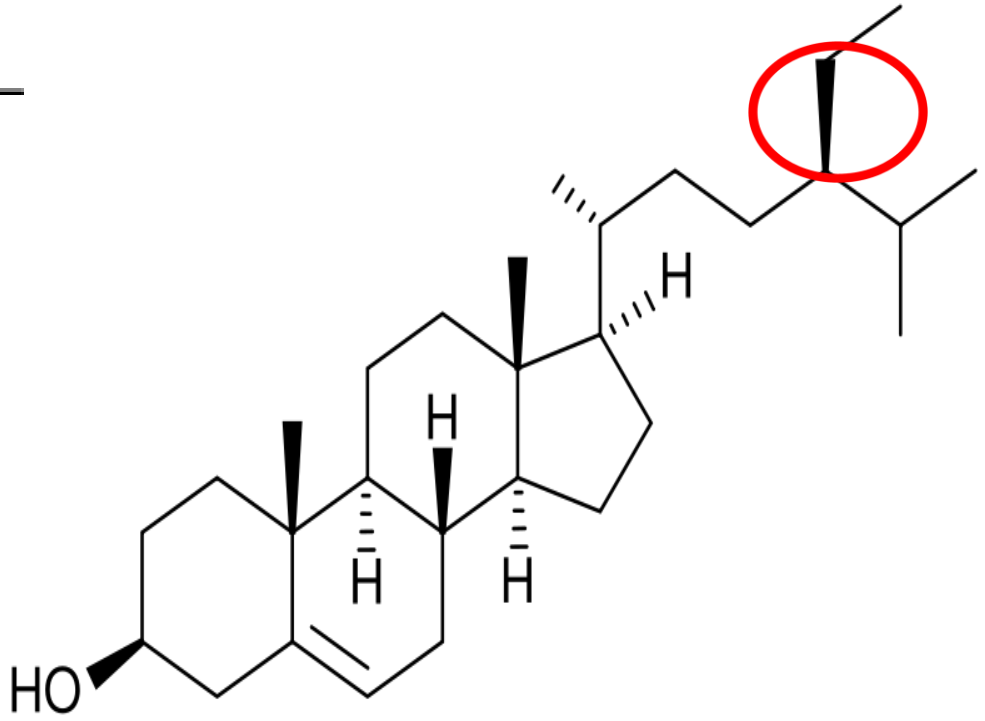
diet or synthesized in the body. Approximately 70% of whole body cholesterol is made by the liver and the other 30% comes from dietary sources. **Table 1** summarizes major sources of dietary cholesterol (Health Canada, 2010a). However, a high level of cholesterol in the blood is a significant risk factor for cardiovascular disease. Certain genetic abnormalities can raise cholesterol levels such as familial hypercholesterolemia (Kolovou et al., 2011). Therefore, it is recommended that cholesterol intake should not exceed the 300 mg/day level for healthy people and less than 200 mg/day for cardiovascular subjects (American Heart Association Nutrition Committee et al., 2006). Cholesterol is not water-soluble; therefore, it needs lipoproteins to be carried out in the blood, of which the four most common are chylomicrons, high density lipoprotein (HDL), low density lipoprotein (LDL) and very low density lipoprotein (VLDL).

Figure 1: cholesterol and plant sterols structure

Extra methyl or ethyl group



loretselohC



Plant sterols

Table 1: Common sources of dietary cholesterol

Animal	Food	Cholesterol content (ave. per 100g serving)
Beef	Ground beef, extra lean, well done	78mg
	Brain, fried	1995mg
	Liver, fried	381mg
	Tenderloin, lean, broiled	78mg
	Animal Fat	109mg
Pork	Bacon, fried	113mg
	Brain, braised	2552mg
	Liver, fried	355mg
	Ham, cured, lean, roasted	55mg
	Animal Fat	95mg
Chicken	Breast or thigh, skinless, roasted	75mg
	Egg, whole, poached	364mg (approx. 2 large eggs)
	Egg yolk, cooked	119mg (1 yolk=17g)
	Liver, fried	564mg
	Animal Fat	85g
Fish/Seafood	Atlantic salmon, farmed, baked	63mg
	Walleye, baked	110mg
	Shrimp, boiled	195mg
	Snow Crab, boiled	71mg
	Lobster, boiled	72mg

Other	Lamb, forshank, lean, cooked	70mg
	Caribou, cooked	109mg
	Deer, roasted	112mg
	Bison, roasted	82mg
	Goose, meat only, roasted	96mg

Adopted from (Kapourchali et al., 2013)

2.2.1. Chylomicrons

Chylomicrons are a type of lipoprotein produced in the small intestinal cells and transport exogenous lipids, including (85-92%) triglycerides, (6-12%) phospholipids, (1-3%) cholesterol and (1-3%) proteins (Hussain, 2000). Their main function is to carry triglycerides and other fats to the liver and other tissues in the body.

2.2.2. High density lipoprotein

High density lipoprotein (HDL) or good cholesterol plays the role of the primary extracellular acceptor thus making efflux of cholesterol via the membrane transports. HDL transports cholesterol from the body to the liver. This role of HDL is thought to be critical to reverse cholesterol transport (RCT) (AbuMweis and Jones, 2008) and is helpful to at least partially explain why risk of atherosclerosis is inversely correlated with HDL cholesterol levels. Further increasing HDL level has been found to exert several potential anti-atherogenic effects (Hersberger and von Eckardstein, 2003). Low levels of HDL (less than 1.0 mmol\L for men and less than 1.3 mmol\L for women) (Health Canada, 2009) have increased rates for heart disease.

2.2.3. Low- density lipoprotein and very- low density lipoprotein

Low-density lipoprotein (LDL) transports the cholesterol from the liver to the tissues of the body. In the bloodstream, LDL is formed from very low density lipoproteins (Pierce, 1954; Gitlin et al., 1958). Therefore, those with higher levels of LDL-C and VLDL-C tend to have more risk of CVD (Austin et al., 1988). The LDL level should be less than 3.4mmol\L (Health Canada, 2009).

2.3. Atherosclerosis

Atherosclerosis is the primary underlying pathology of CVD and the most common cause of death in the western world. It is characterized by the accumulation of cholesterol deposits in macrophages in large and medium sized arteries and leads to atherosclerotic lesions in the arterial wall, gradually resulting in luminal narrowing and eventually leading to impairment of blood flow (Hansson, 2005).

There are many genetic and environmental risk factors that have been identified by epidemiologic studies and they include increased levels of serum cholesterol. Circulating cholesterol concentrations have been recognized as a major risk factor of atherosclerosis. A positive relationship between total and LDL-cholesterol levels and atherosclerosis and inversely associated with HDL-cholesterol has been reported (Badimon and Vilahur, 2012).

2.4. Management of hypercholesterolemia

Statins are first choice treatment for all patients with the condition a potent class of cholesterol lowering drugs that have been proven to significantly reduce cardiovascular mortality in hyper-cholesterolemic patients (Karalis et al., 2012). Common lipid-lowering agents illustrated in **Table 2** lower cholesterol with few side effects; however, effectiveness varies from person to person (Pahan, 2006). In addition, lifestyle modification has been shown to lower cholesterol (Mannu et al., 2013). The change will be focused on dietary therapy, weight reduction, and increased physical activity (Mannu et al., 2013). Approximate and cumulative LDL-C reduction achievable by lifestyle modification is illustrated in **Table 3** (Jenkins et al., 2000). Cholesterol

lowering properties of numerous studies agents are discussed below; however, the combination of wild rice and plant sterols in cholesterol lowering effect has not been studied and it will be investigated it in this study.

Table 2: Common lipid-lowering agents

Drugs name	Benefits
Statins	Inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase
Fibrates	Decrease triglycerides; increase HDL
Ezetimibe	Inhibits intestinal cholesterol absorption
Colesevelam	Decrease LDL
Torcetrapib	Inhibits cholesterol ester transfer protein
Avasimibe	Inhibits acyl-CoA: cholesterol acyltransferase
Implitapide	Inhibits microsomal triglyceride transfer protein
Niacin	Decreases LDL and triglycerides; increases HDL

(Pahan, 2006)

Table 3: Approximate and cumulative LDL-C reduction achievable by lifestyle modification

Dietary Component	Dietary Change	Approximate LDL-C Reduction
Major Interventions		
Saturated Fat	<7% of calories	8-10%
Dietary cholesterol	<200 mg/d	3-5%
Weight Reduction	lose 10 lbs	5-8%
Other LDL-Lowering Options		
Viscous fiber	5-10 g/d	3-5%
Plant Sterol/Stanol Esters	2 g/d	6-15%
Cumulative Estimate		20-30%

Adapted with permission from Jenkins et al., 2000, permitted on Oct 27, 2013

2.4.1. Plant sterols

Plant sterols are at the top of the dietary agents in inhibition of intestinal cholesterol absorption. It is a type of sterols occurring naturally in plants, such as nuts, cereals and legumes (AbuMweis and Jones, 2008; Heggen et al., 2010). Plant sterols and cholesterol have similar chemical structures; however, they are differing markedly in their synthesis and intestinal absorption (Rozner & Garti, 2006). **Figure 1** illustrates the chemical structures of plant sterol. The various effects of plant sterols which lower the level of cholesterol are well known for humans as well as animal models. The sterol metabolism of the liver might be impacted by the restraints put in place due to cholesterol absorption in the intestine (AbuMweis and Jones, 2008). This can be analyzed by identification of changes in the expression of hepatic genes. It has been reported that consumption of 2 grams of plant sterols per day, may decrease LDL cholesterol levels by 13% (Moghadasian and Frohlich, 1999).

Health Canada recently approved addition of plant sterols to a limited number of foods such as, mayonnaise, margarine, calorie-reduced margarine, salad dressing, yogurt and yogurt drinks, and vegetable and fruit juices (Health Canada, 2010b). Consumption of 2g of plant sterols daily can reduce LDL-C by 10% to15 % without affecting HDL-C. A 10% LDL-C lowering in response to plant sterol consumption is estimated to reduce heart disease risk by 25% (Rideout et al., 2012).This phenomenon can be investigated through identification of alterations in hepatic gene expression after long-term phytosterols treatment in a responsive animal model. In Xu et al., (2008) study, there were efforts made to utilize previous experiences with phytosterols therapy in apo E-KO

mice and to determine alterations in hepatic gene expression following long-term administration of dietary phytosterols using cDNA microarray techniques. Phytosterols treatment was associated with significant alterations in the expression of 132 genes involved in different biological processes. Among them, genes related to steroid metabolism, especially cholesterol synthesis and bile acid synthesis were significantly affected. These observations may suggest mechanisms for maintaining the normal cholesterol homeostasis necessary for physiological functions of cells.

2.4.2. Dietary fiber

Another dietary agent reported to lower cholesterol levels is dietary fiber (DF) (Brown et al., 1999). There are 2 types of dietary fiber, soluble fiber includes pectin, gums, and β-glucans, whereas insoluble dietary fiber include cellulose, hemicellulose and lignin (Papathanasopoulos and Camilleri, 2010). Soluble fiber has been reported to help lower blood cholesterol (Kaczmarczyk et al., 2012). β-glucans from oats and barely have shown to have cholesterol-lowering effects (Talati et al., 2009; Ripsin et al., 1992). The potential cholesterol lowering effect of β-glucans approved the American and European authorities health claims for oats and barley. Health Canada (2010c; 2012) also approved health claims for oat and barely fiber in reducing cholesterol. Numerous epidemiological studies have reported an inverse association between WG ingestion and risk of CVD (Mellen et al., 2008). Jensen et al., (2004), reported 15 % reduction in coronary heart disease (CHD) in male with 25 g of WG daily (Jensen et al., 2004). Also, 28% reduction in coronary artery disease (CAD) was observed in atherosclerosis risk in communities study after 3 servings of WG/d (Steffen et al., 2003). Moreover, the Nurse's Health Study reported high intake of whole grain was associated with a lower risk of ischemic stroke

(Liu et al., 2000). Overall, one can conclude that approximately consumption of 3 grams of β-glucans daily may result in 7% reduction in LDL-C and 6% in TC (Harland, 2012).

2.4.3. Soy protein

Cholesterol-lowering properties of soy protein has been reported in several studies (Anderson et al., 1995). More recently, clinical trials have reported a reduction of up 6% in LDL cholesterol and 7% in total cholesterol after 40 g/day supplementation of soy protein (Wofford et al., 2012). Other investigators who studied the effect of 35g/d of soy on women, who were diagnosed with metabolic syndrome (MetS) found significantly improved in LDL and VLDL cholesterol (Bakhtiary et al., 2012). A dose – response study in hypercholesterolemic men used 20, 30, 40, or 50 g/d of soy protein for 6 weeks found that consumption as little as 20 g soy protein per day lowered non-HDL cholesterol and apo B by approximately 2.6% and 2.2%, respectively (Teixeira et al., 2000). A previous study reported that consumption of 25 g soy protein/d resulted in lower total cholesterol concentrations (Bakhit et al., 1994). This is the basis of health claims approved by the USA and Europe that intake of 25 grams of soy protein is associated with improved blood cholesterol.

2.4.4. Other dietary components

Cholesterol-lowering effect of garlic powder has been reported in several studies. A meta-analysis of total of 26 studies performed to investigate the influence of garlic on serum lipid parameters, reported that serum total cholesterol and triglyceride levels in the garlic groups was reduced by 0.28 mmol/L and 0.13 mmol/L respectively (Zeng et al., 2012). Moreover, a study on hyperlipidaemic patients reported that consumption of 800 mg garlic powder was associated with in a reduction of 12% in total cholesterol and 17%

in triglyceride (Mader, 1990). However, another clinical trial reported that the intake of 900 mg/d of garlic powder for 12 weeks in patients with hypercholesterolemia was not effective in lowering cholesterol levels in these subjects (Isaacsohn et al., 1998). However, it appears that several factors may influence cholesterol-lowering effects of garlic products; for example, formulation and the method of preparation.

2.5. Wild rice

Wild rice (*Zizania* spp.) is aquatic grass from North part of America, including the provinces of Saskatchewan, Manitoba in Canada and some parts of Asia (Oelke, 2004). It served as a native staple food to the locals for10,000 years (Aiken,1988; Steeves, 1952). Wild rice is also known as Canadian rice, Indian rice, water oats, or blackbird oats (Steeves, 1952). **Table 4** summarizes the names of 25 species and varieties of wild rice. There are four species of wild rice distributed in North America and in eastern Asia belonging to the genus *Zizania.* While *Zizania aquatic* L., *Zizania palustris* L., *Zizania texana* A.S. Hitch are the native species of North America, *Zizania latifolia* Turcz basically originates from China, Japan and Vietnam (Oelke, 1993).

Z. palustris grows predominantly in the Great Lakes region in shallow lakes and rivers of the United States and Canada (Oelke & Dicker, 2000). It is historically consumed as a staple food for Native Americans and now serves as an economically important specialty commercial crop (Aiken, 1988; Oelke, 1993; Oelke, 2004). The other annual species, *Z. aquatica* is grown along the St. Lawrence River, eastern and south eastern United States coastal areas, and in Louisiana (Oelke, 1993). *Z. texana*, which is perennial with small seeds, grows in a small area in Texas (Oelke, 2004). Neither *Z.*

aquatica nor *Z. texana* is harvested for food due to their thin and small seeds production (Oelke, 1993; Xu et al., 2010). Lastly, *Z. latifolia* is a perennial plant widely grown in south-eastern Asia (Guo et al., 2007; Zhai et al., 2001). It is often infected by fungus *Ustilago esculenta P. Henn*, and produces nutritious shoots that have been harvested as an edible aquatic vegetable (Guo et al., 2007; Oelke, 2004).

2.5.1. Nutritional composition of wild rice

Since late 20th century, utilization of wild rice has been gaining popularity among consumers. Due to its unique flavor and texture, wild rice is used in a wide variety of gourmet food products. It is usually served as rice or mixed grains, soup, breakfast cereal, muffin, or salad (Mäkelä et al., 1998; Oelke & Dicker, 2000). Wild rice is an expensive gourmet food, especially the naturally grown Canadian wild rice. In 2006, wild rice was recognized as a whole grain by the Food and Drug Administration (FDA), USA.

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Table 4 Different species and varieties of wild rice

S. No.	Species	Varieties
1	*Zizania* Gronov. ex L.	
2	*Zizania* sect. *Perennes* Tzvelev	
3	*Zizania aquatica* L.	*Zizania aquatica* L. var. *angustifolia* Hitchc. *Zizania aquatica* L. subsp. *angustifolia* (Hitchc.) Tzvelev *Zizania aquatica* L. var. *brevis* (Fassett) S.L.Chen *Zizania aquatica* L. subsp. *brevis* (Fassett) S.L.Chen *Zizania aquatica* var. *brevis* Fassett *Zizania aquatica* L. var. *interior* Fassett *Zizania aquatica* L. var. *subbrevis* B.Boivin
4	*Zizania aristata* Kunth	
5	*Zizania bonariensis* Balansa & Poitrass.	
6	*Zizania caduciflora* (Turcz.) Hand.-Mazz.	
7	*Zizania ciliata* Spreng.	
8	*Zizania clavulosa* Michx.	
9	*Zizania dahurica* Turcz. ex Steud.	
10	*Zizania effusa* Herb.Linn. ex Munro	
11	*Zizania fluitans* Michx.	
12	*Zizania interior* (Fassett) Rydb.	
13	*Zizania latifolia* Turcz. ex Stapf	
14	*Zizania lenticularis* Michx. ex Steud.	
15	*Zizania liliacea* Michx.	
16	*Zizania mezii* Prodoehl	
17	*Zizania microstachya* Nees ex Trin.	
18	*Zizania miliacea* Michx.	
19	*Zizania mutica* Larrañaga	
20	*Zizania natans* Bosc ex Trin.	
21	*Zizania palustris* L.	*Zizania palustris* L. subsp. *interior* (Fassett) S.L.Chen *Zizania palustris* L. f. *purpurea* Dore
22	*Zizania retzii* Spreng.	
23	*Zizania subtilis* Raspail	
24	*Zizania terrestris* L.	
25	*Zizania texana* Hitchc.	

Source: IPNI 2012

Wild rice contains high amounts of minerals, vitamins, protein, starch, dietary fiber and various antioxidant phytochemicals, along with low fat profile. Like other cereals, wild rice grain is a food commodity containing approximately 71 - 84% carbohydrate and 10 - 18% protein as the main constituents (**Table 5**) (Anderson, 1976; Dreher, 2001). The nutritional value of wild rice is comparable with other cereals, such as oats, wheat, and corn.

Wild rice is a rich source of dietary fiber which consists of 0.6 to 2.0% of crude fiber, and 5.2 % of total dietary fiber, with 3.3 % insoluble and 0.8 % of soluble fiber (Anderson, 1976; Dreher, 2001). Crude fiber consists of cellulose, hemicellulose, lignin, pectin, gum, and mucilage (Marlett & Bokram,1981; Spiller, 2001). In addition, wild rice is high in vitamins B1, B2, and E (**Table 6**) (Anderson, 1976). The mineral composition of wild rice is shown to be comparable with that in other cereals. Wild rice is a good source of calcium, iron, magnesium, potassium, phosphorus, and zinc (**Table 7**) particularly, the zinc content in wild rice is more than that in oats, wheat and corn.

Wild rice grain has 13-17% protein content (Terrell & Wiser, 1975). The essential amino acids profile of wild rice appeared to be similar to that of other cereals (Oelke, 1976). The lysine value of wild rice is about 3.8 to 5.3% of its protein content (**Table 8**), which is close to what found in oat (3.2 to 5.2 %), wheat (2.3 to 4.3 %), and corn (3.6 %) (Brinkman et al., 2006; Jensen, 1975) (Brinkman et al., 2006; Jensen, 1975).

Fat content of wild rice is less than 1%; polyunsaturated fatty acids including linoleic and α-linolenic fatty acids are the main fatty acids (Przybylski et al., 2009).

Furthermore, low n-6 /n-3 fatty acid ratio makes wild rice attractive for prevention of chronic diseases (Riediger et al., 2008).

Table 5. Nutritional composition of wild rice

Component	Content (%)
Moisture	5 – 13
Ash	1.1 – 2.0
Protein	10 – 18
Albumins – water soluble (% of total nitrogen)	10
Globulins – Salt soluble (% of total nitrogen)	10
Prolamines - alcohol soluble (% of total nitrogen)	1
Glutelins - alcohol soluble (% of total nitrogen)	79
Fat / Lipid	0.35 – 1.1
Total Carbohydrate	71 – 84
Crude fiber	0.6 – 2.0
Starch	69.3
Sugar	-
RDS	60.1
SDS	4.0
RS	5.2

(Anderson, 1976; Dreher, 2001; Surendiran et al., 2013; Kennedy, 1924; Watts & Dronzek, 1981; Zhai et al., 1994; Terrell & Wiser, 1975). RDS: Rapidly digesting starch; SDS: slowly digestible starch; RS: Resistant starch.

Table 6. Vitamin content of wild rice

Vitamin	Levels (mg/100g)
Retinol, retinal and carotinoids (Vitamin A)	-
Thiamine (Vitamin B1)	0.3 – 0.63
Riboflavin (Vitamin B2)	0.07 – 0.6
Niacin (Vitamin B3)	4.6 – 10.3
Tocopherols (Vitamin E)*	0.2 – 0.5
Ascorbic acid (Vitamin C)	-

* Total tocopherol in wild rice is 1.6-42.5 mg/100g and the amount of alpha and gamma tocopherol reported to be 1.42- 25.37 and 3.86 mg/100g, lipids respectively and that translated to be over 80% of total alpha and gamma tocopherol (Przybylski et al., 2009).

(Anderson, 1976; Swain et al., 1978; Nelson et al., 1942; Przybylski et al., 2009)

Table 7 Heavy, trace metals and mineral contents of wild rice

Minerals	Levels (mg/100g)Wild Rice
Calcium (Ca)	11 – 25
Chromium (Cr)	0.09 – 0.14
Cobalt (Co)	0.04 – 0.11
Copper (Cu)	0.10 – 0.41
Iron (Fe)	1.2 – 5.1
Lithium (Li)	0.02 – 0.04
Magnesium (Mg)	80 – 161
Manganese (Mn)	0.93 – 1.8
Nickel (Ni)	0.02 – 0.03
Phosphorus (P)	236 – 500
Potassium (K)	55 -560
Sodium (Na)	1.34 – 6.0
Zinc (Zn)	1.2 – 12.0
Lead (Pb)	0.01 – 0.02

(Anderson, 1976; Watts & Dronzek, 1981; Zhai et al., Pip, 1993; Nriagu & Lin, 1995)

Table 8. Amino acid composition of wild rice

Component	Content (g/100g)
Alanine	5 – 6.5
Arginine	7.2 – 9.0
Aspartic acid	9.8 – 11.1
Cystine	0.3 – 2.9
Glutamic acid	18.5 – 20.0
Glycine	4.5 – 5.1
Histidine	2.1 – 3.0
Isoleucine	4.1 – 4.7
Leucine	6.3 – 7.6
Lysine	3.8 – 5.3
Methionine	2.3 – 3.3
Phenylalanine	4.8 – 5.5
Proline	3.2 – 4.5
Serine	5.1 – 5.7
Theronine	3.1 – 3.8
Tryptophan	1.6 – 1.9
Tyrosine	3.1 – 4.1
Valine	5.5 – 8.8

(Anderson, 1976; Watts & Dronzek, 1981; Zhai et al., 1994; Terrell & Wiser, 1975; Oelke, 1976; Wang et al., 1978)

acid (14.1 to 18.4%), stearic acid (1.1 to 1.3%), and oleic acid (12.8 to 16.2%) (Przybylski et al., 2009).

Wild rice is a rich source of dietary antioxidants, including phenolic compounds. Total phenolic compounds of 9 different wild rice varieties were reported to vary from 419 ± 14 mg GAE/ kg to 588 ± 27 mg GAE/ kg; this level of antioxidant phenolic compounds is approximately 9 – 13 times higher than that in conventional white rice (46 ± 1 mg GAE/ kg) (Qiu et al., 2010). Ferulic acid was found to be the most abundant phenolic acid (241.58 mg/ kg – 355.41 mg/ kg) in wild rice.

2.5.2. Potential health benefits of wild rice

There was a time when the Chinese wild rice was an important grain in China and thus was an herbal medicine. The rice was used to treat diabetes and other associated with nutrition, but today in the modern day eating habits and techniques, the importance of wild rice for this purpose has disappeared (Zhai et al., 1996). There were certain experiments that were performed by (Zhang et al., 2009) to determine whether or not the intake of wild rice had desirable effects on serum lipid and antioxidant status in an animal model that is consuming a diet high in fat and cholesterol. However, the precise mechanisms for how wild rice improved serum lipid profiles in rats were not explored.

Wild rice like other cereals has high contents of starch and protein, low fat content, and a good source of dietary fiber (Anderson, 1976). Higher intake of dietary fiber has been associated with lower risk of obesity (Slavin, 2005), cardiovascular disease (Mozaffarian et al., 2003), and type II diabetes (Panahi et al., 2007).

Wild rice as a whole grain has a low-glycemic index. In addition, it has high amylose content (Hoover et al., 1996). A low glycemic high-amylose diet has been shown to improve overall well-being. In rats fed a high-amylose diet decreased body weight, increased fecal mass, fecal moisture, and lower fecal pH were observed. Furthermore, a high-amylose diet has been shown to lower blood glucose in normal and diabetic rats (Zhu et al., 2012). The amylose content of wild rice starch ranged from 21.7 to 23.8% (Watts, 1980; Lorenz, 1990; Tahara et al., 2000). As such increased wild rice intake can be recommended to increase dietary amylose intake in order to lower blood glucose in diabetes prevention and management.

Antioxidants have an important role in cardiovascular; lipid peroxidation is considered a risk factor for atherosclerosis. As the disease progresses radical scavengers are inhibited leading to greater oxidative stress and eventually to complication of diabetes such as retinopathy, and atherosclerosis (McCune and Johns, 2002). Antioxidant vitamins E, C and beta-carotene have been show to alleviate the effects of insulin resistance in overweight individuals (Vincent et al., 2012). In addition, the mineral magnesium has been shown to delay the onset of hyperglycemia in diabetes induced rats. This effect is attributed to magnesium's role as a hydroxyl radical scavenger (Abayomi et al., 2011).

Phenolic compounds and their derivatives such as flavanoids are secondary metabolites with antioxidant activity. They help protect against oxidative stress induced diseases such as diabetes, cancer, and cardiovascular disease. A high concentration of phenolic acids can be found in the outer layer of rice grain, existing in insoluble bound form. The phenolic content of wild rice is 9-13 times higher than the phenolic content of

white rice. The radical scavenging activities of wild rice are 2-3 times higher than that of white rice.

Furthermore, methanol extracts of wild rice display up to ten times higher antioxidant activity than white rice using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and oxygen radical absorbance capacity (ORAC) methods (Qiu et al., 2010).

Zhang et al. (2009) reported the cardio-protective effects of wild rice in relation to its lipid lowering effects in rats fed with a high saturated fat diet (Zhang et al., 2009). Incorporation of wild rice into the atherogenic diet suppressed the increase in serum triglycerides and total cholesterol, and the decrease in HDL cholesterol levels. This study also reported increases in SOD activity and reductions in malondialdehyde (MAD) concentrations in the serum and liver tissues of wild rice-fed animals. Han et al. (2012) evaluated the protective potentials of wild rice against obesity and liver lipotoxicity induced by high fat atherogenic diets in rats. Incorporation of wild rice into the atherogenic diet suppressed the increase of lipid droplets accumulation, free fatty acids, and leptin, and also inhibited the decrease of lipoprotein lipase and adipose triglyceride lipase activities. Further, the wild rice enriched diet reduced the elevation of sterol-regulatory element binding protein-1c and gene expression of fatty acid syntheses and acetyl-CoA carboxylase in the liver suggesting a protective effect of wild rice. They also reported that wild rice has ameliorating effects on insulin resistance in rats fed with high fat high cholesterol diet (Han et al., 2012). It was found that wild rice treatment significantly decreased liver triglyceride and free fatty acid levels; serum lipocalin-2 , followed by an increase in adiponectin concentrations in the liver of rats.

We observed cholesterol-lowering and anti-atherogenic effects of wild rice in LDLr-KO mice (Surendiran et al., 2013). The supplementation of wild rice to an atherogenic diet for over 24 weeks significantly reduced the plasma concentrations of low density lipoproteins (LDL) and very low density lipoprotein (VLDL) in LDLr-KO mice. The responsiveness to the wild rice differed between male and female mice; female mice responded better to cholesterol-lowering effects of wild rice. This gender-related variation may be due to presence of endogenous estrogens. Further, incorporation of wild rice in the diet distinctly increased its total phenolic and dietary fiber contents compared to conventional white rice diet. However, the plasma and erythrocyte SOD and catalase activities were not significantly improved. The hypocholesterolemic properties of wild rice mainly attributed to the increased rate of fecal cholesterol excretion (Surendiran et al., 2013).

Due to the high vitamin, mineral, antioxidant content, and high fiber content of wild rice as compared to white rice increased consumption can be recommended in order to help prevent and manage chronic diseases. The full effects of wild rice in combination with plant sterols on cardiovascular risk factors have yet to be elucidated.

3. RESEARCH PLAN

3.1. Study rationale

Atherosclerosis is a complex disease associated with lipid accumulation in the vessel wall. It is considered to be a form of chronic inflammation due to interaction between modified lipoproteins, monocyte-derived macrophages, T cells, and the normal cellular elements of the arterial wall (Wang and Mazza, 2002). The particular inflammatory process can lead to the development of lesions and plaques that protrude in the arterial lumen which can further lead to plaque rupture and thrombosis. These situations can become fatal as a result of the acute complications of myocardial infarction and strokes.

Dietary treatment may be considered as one of the initial steps in the prevention of atherosclerosis (El-Sabban and Abouazra, 2008). Incorporation of novel and effective dietary supplements into the diet may be effective in the treatment of atherosclerosis (Zhang et al., 2009; Mukherjee, 2003). Plant sterols are functional lipids and as such may help in lowering levels of LDL cholesterol and help to reduce circulating levels of cholesterol (AbuMweis and Jones, 2008). Plant sterols are naturally available in plant products, such as oils, nuts, cereals and legumes (AbuMweis and Jones, 2008; Heggen et al., 2010). Furthermore, another dietary modification with cardiovascular benefits is the inclusion of wild rice (*Zizania* sp.) in daily menus. The northern wild rice (*Zizania palustris* L.), an annual plant native to aquatic areas of the northern America, Saskatchewan and Manitoba (Lorenz, 1981) receives much attention by researchers because of its potent nutritional and phytochemical contents. Wild rice is whole grain finding applications in gourmet foods due to its nutritional value and unique taste. With

regard to the nutritional values, wild rice is high in protein and starch, and low in fat. It is also a rich source of many phytochemicals such as dietary fiber, phytosterols and vitamins B1, B2, E, as well as minerals (Zhang et al., 2009)

Recently attention has been paid to increased whole grain intake which demonstrated to have beneficial impacts on cardiovascular risk. Increased levels of oxidative stress have been reported in cardiovascular disease (Manson et al., 1993). Therefore, replacing refined carbohydrate source of cholesterol- enriched diet with antioxidant rich whole grain and inclusion of phytonutrition in the diet such as wild rice and phytosterols may reduce cardiovascular risk factors. One well known dietary antioxidants are phenolic compounds including anthocyanins, which is abundant in wild rice. Although Kim et al., (2008) reported the presence in anthocyanins in wild rice, we are not able to indicate anthocyanins in different species of wild rice (Dr. Beta communication). Therefore, this study aimed to investigate the role of Manitoban wild rice in combination with phytosterols on reducing cardiovascular risk factors in experimental animals with dyslipidemia and atherosclerosis. The proposed study utilizes the well-known animal model LDL-receptor-knock-out (LDL-r-KO) mice and established experimental methods to evaluate the hypothesis.

4. STUDY HYPOTHESIS AND OBJECTIVES

4.1. Hypothesis

Combination of plant sterols and wild rice have additive effects on cholesterol reduction, resulting in prevention of atherogenesis in LDL-receptor-knock-out mice.

4.2. Objectives

The main goal of this study was to investigate the cardiovascular benefits of Manitoban wild rice in combination with plant sterols in LDL-receptor-knock-out mice. This study will specifically:

1. Determine the effects of wild rice in combination with plant sterols on plasma total cholesterol, triglyceride, VLDL, LDL, and HDL cholesterol levels;
2. Determine the effects of wild rice in combination with plant sterols on fecal lipid content.
3. Determine the effects of wild rice in combination with plant on atherosclerotic lesion development in aortic roots.

5. MATERIALS AND METHODS

5.1.Animal and diets

The experimental design of this study is summarized in **Figure 2.** Thirty two, 4 weeks old, male LDLr-KO mice were purchased from Jackson's Laboratory, USA (Bar Harbor, ME, U.S.A). Mice were housed in a controlled room temperature at 22-24 °C and a 12:12 hour light-dark cycle. Numerous studies (Teupser et al., 2003; Knowles and Maeda, 2000) have suggested LDLr-KO mice as an appropriate dyslipidemia model for atherosclerosis. After one week of acclimation, mice were randomly divided into four experimental groups (n=8 each). **Group I**: control group (C) received an atherogenic diet. **Group II**: received the same diet incorporated with 60% of wild rice (WDR). **Group III:** received an atherogenic diet supplemented with 2% phytosterolss (PS). **Group IV:** received an atherogenic diet incorporated with wild rice in combination of phytosterolss (60% WDR+ 2% PS). The diets were designed based on AIN-93G Purified Rodent Diet (Dyet # 110700) with slight modification to meet the minimum requirement of AIN-93 diet specification. Locally grown wild rice (Far North Wild Rice) was purchased from Flin Flon, MB, Canada; while phytosterols were purchased from (Sigma Chemicals, St. Louis, USA). All four diets were made by Dyets Inc. (Bethlehem, PA, U.S.A). The diets were kept in a cold room between 0 °C to 4 °C during the entire course of study.

All diets were supplemented with 0.06 % (w/w) dietary cholesterol. The digestible carbohydrate source was replaced with: a) commercial carbohydrate source used in rodent diet, such as sucrose and corn starch (control diet group); b) wild rice used as carbohydrate source (wild rice diet group); c) sucrose and corn starch used as carbohydrate source (phytosterols diet group); d) wild rice used as carbohydrate source

(WDR+ 2% PS diet group). Diet treatment protocols are summarized in **Table 9.** The composition of the experimental diet is outlined in **Table 10**. The experimental period was 20 weeks.

5.2. Ethics

This study protocol was approved by the Animal Care Committee on the use of animals in research at the University of Manitoba, Winnipeg, MB, Canada.

5.3. Statistical analysis

Data were analyzed using SPSS version 11.5 statistical software for Windows (SPSS Inc., Chicago, IL, U.S.A). Comparisons were conducted using Univariate analysis, One way ANOVA followed by Tukey test to determine significant differences among various experimental groups.

A P-value of <0.05 was considered to be statistically significant. Results were expressed as mean ± standard deviation (SD).

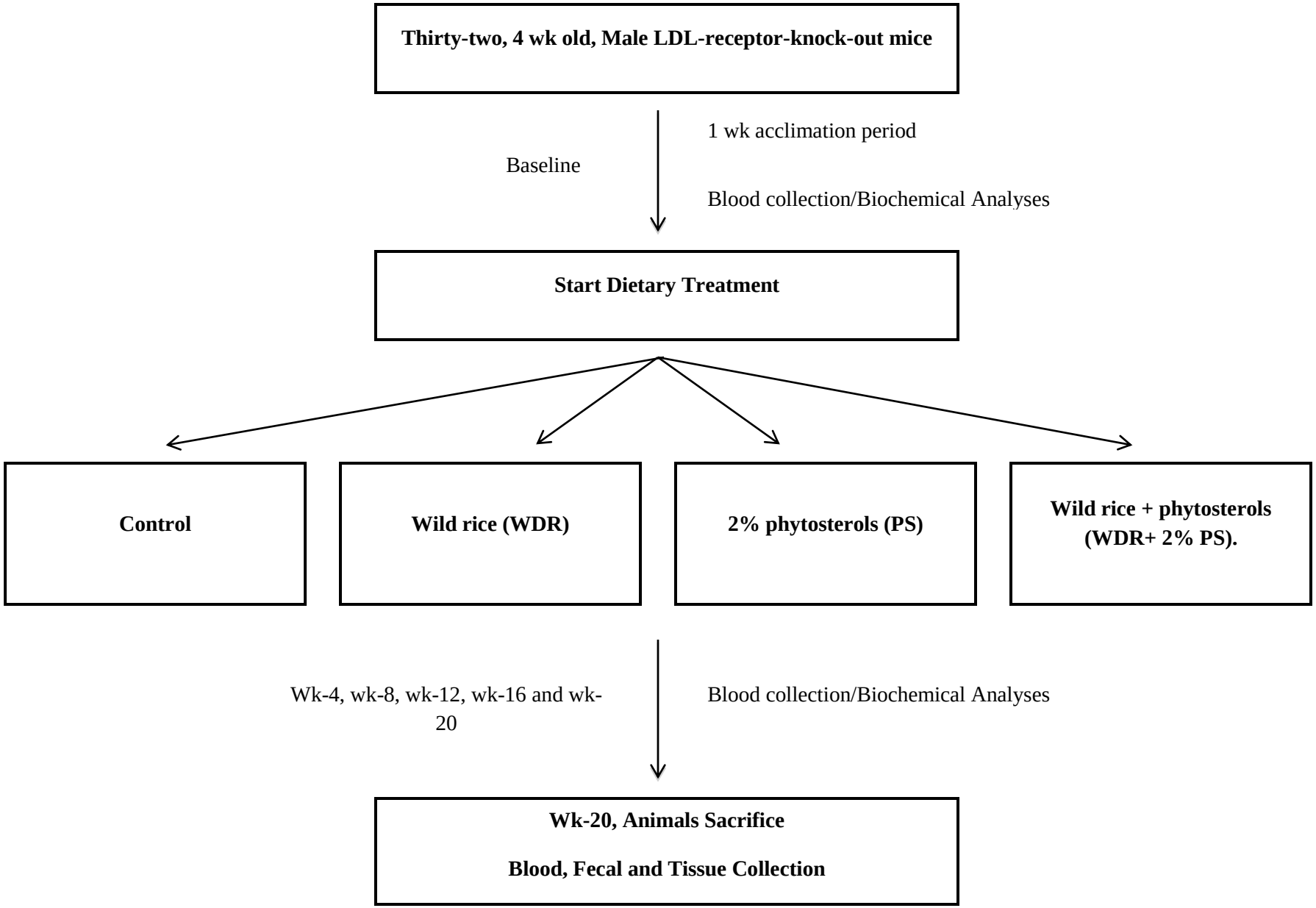


Figure 2. Experimental Design

Table 9. Summary of the experimental groups and their diet treatment protocols

Experimental Groups	Diet Protocols
Control (n=8)	Control diet (high cholesterol 0.06 % (w/w) dietary cholesterol + Sucrose and corn starch used as mean source of carbohydrate)
Wild rice (n=8)	Control diet + high cholesterol 0.06 % (w/w) dietary cholesterol+ wild rice used as mean source of carbohydrate
2% phytosterols (n=8)	Control diet + high cholesterol 0.06 % (w/w) dietary cholesterol +2% phytosterols+ sucrose and corn starch used as mean source of carbohydrate
Wild rice + phytosterols (n=8)	Control diet + high cholesterol 0.06 % (w/w) dietary cholesterol +2% phytosterolss + wild rice used as mean source of carbohydrate

Phytosterols = extract from soybean oil, high cholesterol 0.06 % (w/w) dietary cholesterol

Table 10. Experimental diet composition (g/ kg diet)

Ingredients	Control	Wild Rice	2% B-Phytosterols	WDR+ 2% PS
Casein	200.0	117.5	200.0	117.52
L-Cystine	3.0	3.0	3.0	3.0
Sucrose	100.0	0.0	100.0	0.0
Cornstarch	396.9	0.0	396.9	0.0
Dyetrose	132.0	81.9	132	81.9
Wild Rice	0.0	609.5	0.0	609.5
Soybean Oil	70.0	70.0	70.0	70.0
t-Butylhydroquinone	0.014	0.014	0.014	0.014
Cellulose	50.0	50.0	30.0	50.0
Mineral Mix	35.0	35.0	35.0	35.0
Vitamin Mix	10.0	10.0	10.0	10.0
Choline Bitartrate	2.5	2.5	2.5	2.5
Cholesterol	0.6	0.6	0.6	0.6
B-Phytosterols	0.0	0.0	20.0	20.0

6. Data collection

6.1. Food intake and body weight

Body weight and food intake were measured regularly. Twenty- four hours food intake measurement was taken every 4 weeks at identical times. The total food consumption was calculated by weighing the food given and food remaining in each cage. Body weights of the animals were measured weekly.

6.2. Blood sampling and tissue collection

Fasting blood samples were drawn at baseline, then every 4 week-period until sacrifice at week 20. All mice were fasted for approximately 12 hours prior to blood collection. Blood samples were drawn through jugular vein of lightly anesthetised animals during the study. Plasma was separated from blood samples by centrifugation at 4 °C (5000 rpm for 15 minutes) and stored at -80°C for analyses. At the end of the study, mice were euthanized using carbon dioxide gas and final blood samples were collected through cardiac puncture. The hearts, livers, kidneys spleens, and abdominal fats tissues were collected, weighed and stored at -80°C until analysis and part of the tissues were fixed in 10% buffered formalin and sectioned for future studies.

6.3. Total cholesterol

Plasma TC levels were measured using a standard enzymatic kit (Genzyme Dignostic chemicals Limited, Charlottetown, PE, Canada). The procedure involved a number of steps: the principle underlying the enzymatic assay is hydrolysis and oxidation of cholesterol esters to produce hydrogen peroxide (H_2O_2) with cholest-4-en-3-one. In the presence of peroxidase, hydrogen peroxide combined with hydrobenzoic

acid and 4-aminoantipyrine to form chromgen (Allain et al., 1974). In brief, 250 µl of TC reagent plus 3 µl each standards and plasma samples were added to a microplate reader. After 20 minutes of incubation at room temperature, readings were taken at 500 nm. All experimental samples were analyzed in duplicate and mean values were calculated.

6.4. Triglycerides (TG)

Plasma TG concentrations were quantified using a standard enzymatic kit (Genzyme Dignostic chemicals Limited, Charlottetown, PE, Canada) (Moghadasian and Frohlich, 1999). The manufacturer provided a standard solution (200 mg/dL) which was used to produce a linear response curve. Three hundred µl of reagent plus 5 µl of standards and plasma samples were added to each well and incubated at room temperature for 10 minutes. Plasma hydrolysis of triglycerides by lipoprotein lipase (LPL) resulted in glycerol and FFAs. In the presence of ATP and glycerolkinase, glycerol was phosphorylated to form glycerol-1-phoshate. Glycerol-1-phosphate was then oxidized by glycerophosphate oxidase [GPO] to dihydroxyacetone phosphate [DHP] and hydrogen peroxide [H_2O_2]. In the presence of peroxidase, a mixture of hydrogen peroxide and 4-aminoantipyrine and N-ethyl-N(3-sulphopropyl)-m-anisidine was oxidized by hydrogen peroxide to form a quinoneimine dye that could be quantified at 540 nm. All experimental samples were analyzed in duplicate and mean values were calculated.

6.5. HDL-cholesterol

Plasma HDL-cholesterol (CHDL) levels were assessed using a standard enzymatic kit (HDL Ultral Sekisui Diagnostics, LLC). The procedures of HDL-cholesterol determination were similar with that of plasma cholesterol assay as described above. All samples were measured in duplicate to obtain mean values. Also, HDL/LDL ratio was calculated.

6.6. LDL-cholesterol

Plasma LDL-cholesterol (CLDL) levels were calculated according to Friedewald formula CLDL=(TC- CHDL-TG/5) (Friedewald et al., 1972). This method provides appropriate estimation for plasma LDL-cholesterol and can be used in many cases (Gazi et al., 2006; Johnson et al., 1997).

6.7. VLDL-cholesterol

Plasma VLDL-cholesterol (CVLDL) levels were calculated according to Friedewald formula CVLDL=(TG/5) (Friedewald et al., 1972). Friedewald formula can be used to calculate either VLDL-C or LDL-C when the plasma triglyceride concentration less is than 400 mg/100 ml (Friedewald et al., 1972).

6.8. Fecal lipid extraction

Lipid extraction from the feces was conducted according to the method of Folch et al., (1957) with slight modification. Fecal samples (0.5g) were soaked overnight in 5 ml of 0.025 % $CaCl_2$. Then, samples were vortexed for 3 minutes to homogenize the softened fecal samples followed by adding 25 ml of chloroform: methanol (2:1, v/v) and

vortexed for 1 minute to extract the lipid. Lipid was separated from sample matrix by centrifugation at room temperature (5000 rpm for 10 minutes). The lipid-solvent (lower) phase was carefully pipetted out and dried under nitrogen gas at 50 °C to obtain total lipid weight. Total lipid weight was divided by fecal sample weight to calculate lipid weight as percentage of total fecal weight. Then, gas chromatography was performed to determine individual sterol components.

6.9. Histological examination

Specimens from heart tissues containing aortic roots were taken at sacrifice and fixed in 10 % formalin. Aortic roots were sectioned and stained with hematoxylin and eosin (H&E) for morphological and morphometrical analysis for atherosclerotic lesions, using light microscopy technique and Image-Pro Plus software (Moghadasian et al., 1997).

7. RESULT

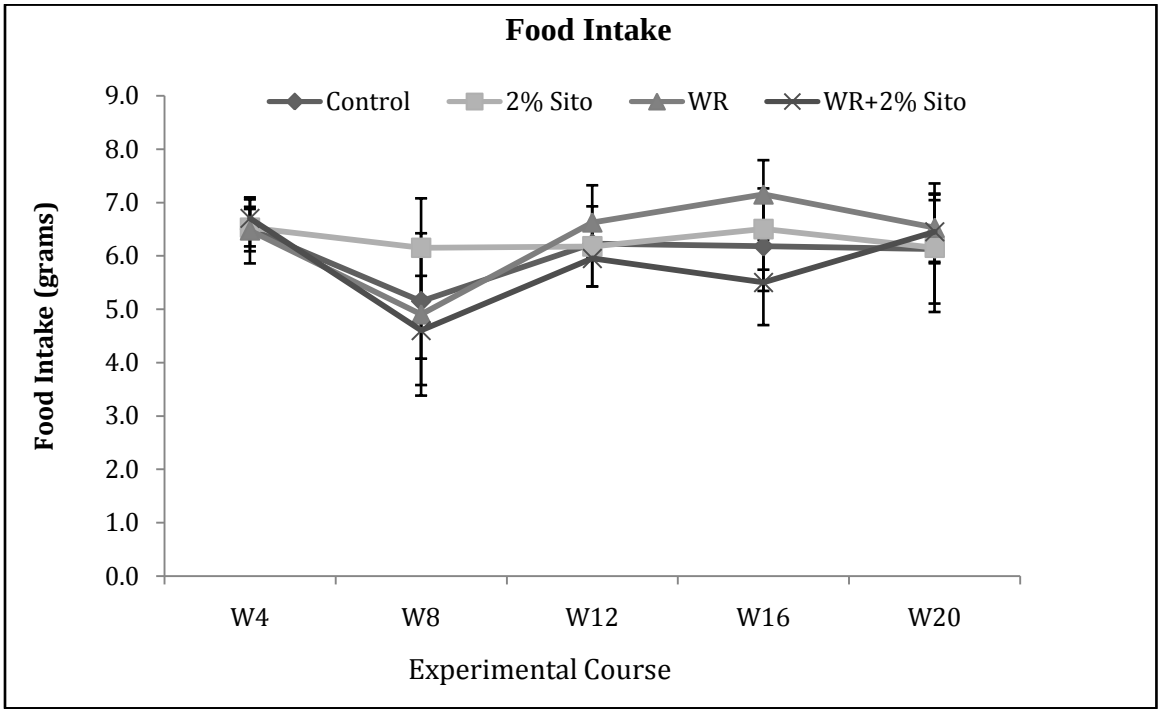
7.1.Food intake, body weights and tissue weights

Mean monthly food intake was comparable among the groups and throughout the study as seen in **Figure 3**. Final food intakes of mice were not significantly different among the four treatment groups over the entire experimental course (P=0.890).

The extent of mean body weight gain was comparable among all of the experimental groups as presented in **Figure 4.** Final body weights of mice were not significantly different among the experimental groups (P=0.109).

Table 11 shows the weight of the body organs in all of the 4 experimental groups at the end of study. All the tissues including the heart, liver, total right and left kidney, spleen and abdominal fat were weighed. The weights of kidney and abdominal fat were not significantly different in all diet groups (P=0.259, and P= 0.084 respectively). The weights of the heart, liver and spleen were significantly different between the groups (P= 0.048, P=0.051, and P=0.027 respectively). Mice treated with wild rice in combination with 2% phytosterols had significantly smaller hearts (P=0.041). Also, phytosterols treated mice had significantly smaller livers and spleens (P=0.045 and P=0.030) respectively in relative to their counterparts in the control group despite their comparable body weights.

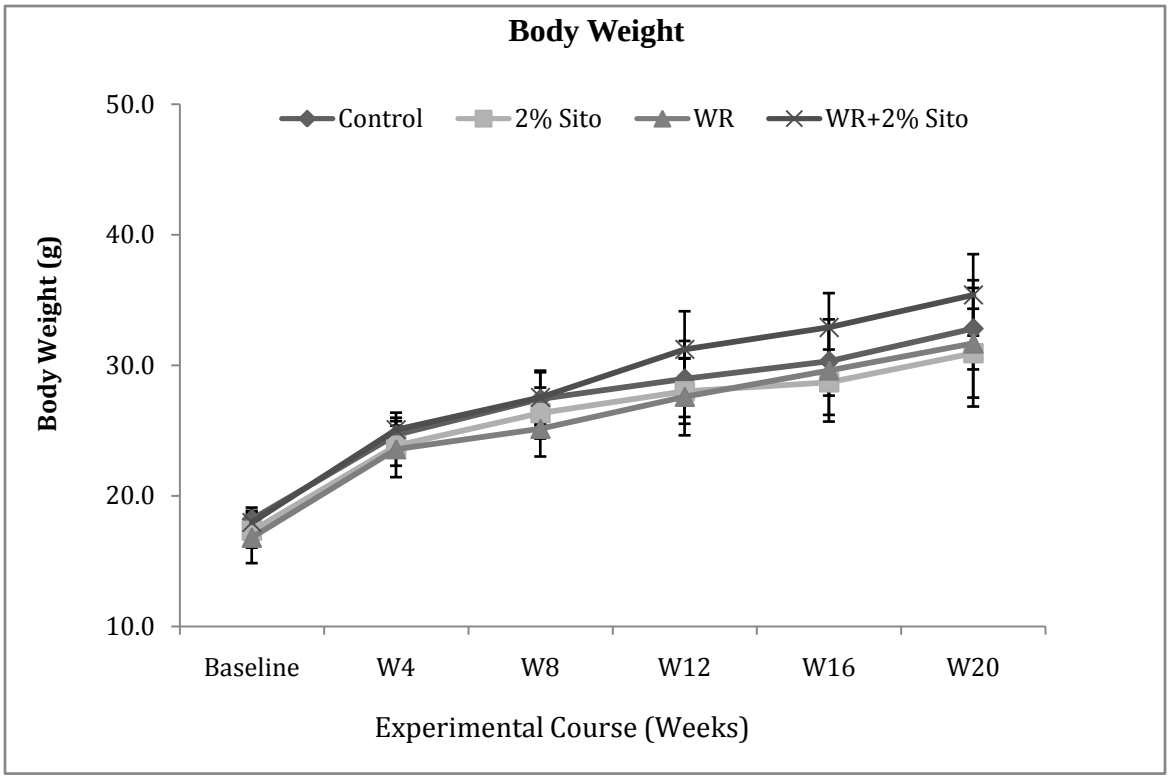
Figure 3. Food intake measurements of LDLr-KO mice after 20 weeks of experimental diet treatment. The average food intake among the four groups was comparable throughout the study.



Data are presented as mean ± standard deviation.

Control; 2% Sito= phytosterol; WR= wild rice.

Figure 4. Mean body weights of LDLr-KO mice after 20 weeks of experimental diet treatment. All the experimental groups showed a consistent weight gain.



Data are presented as mean ± standard deviation.

Control; 2% Sito= phytosterol; WR= wild rice.

Table 11. Organ weights (g) of LDLr-KO mice after 20 weeks of experimental diet treatment

Groups	Control	2% phytosterols	Wild rice	2% phytosterols and wild rice
Heart	0.2 ± 0.03	0.18 ± 0.02	0.19 ± 0.03	0.17 ± 0.02*
Liver	1.55 ± 0.29	1.23 ± 0.15*	1.33 ± 0.22	1.29 ± 0.22
Total Kidney	0.52 ± 0.05	0.42 ± 0.07	0.47 ± 0.11	0.37 ± 0.08
Spleen	0.14 ± 0.08	0.08 ± 0.01*	0.10 ± 0.03	0.08 ± 0.01
Abd Fat	1.35 ± 0.56	1.15 ± 0.82	1.56 ± 0.72	1.97 ± 0.35

Data are presented as mean ± standard deviation. Abd, abdominal
*** P < 0.05 as compared to the control group**

7.2. Total cholesterol concentrations

Figure 5 illustrates concentrations of TC in all of the experimental groups at baseline, wk 4, wk 8, wk 12, wk 16, and wk 20 of the study. Total cholesterol concentrations were comparable among all treatment groups at baseline. At wk 4, there was a significant (P=0.0001) difference in TC levels among groups. TC levels were significantly decreased in the 2% phytosterols, wild rice+2% phytosterols and wild rice treated mice as compared to the control groups (P=0.0001, P=0.0001 and P=0.006, respectively). At wk 20, TC plasma concentrations remained significantly lower in the 2% phytosterols, wild rice+2% phytosterols and wild rice group relative to the control groups (P=0.0001, P=0.001 and P=0.002, respectively).

7.3. Total triglyceride concentrations

Levels of TG at baseline and during the experimental course are illustrated in **Figure 6**. Plasma triglyceride concentrations were comparable among all treatment groups during the study.

7.3. HDL-cholesterol concentrations

Levels of plasma HDL-cholesterol in all experimental groups are illustrated in **Figure 7**. Plasma HDL- cholesterol levels were slightly higher in control mice relative to other groups. At wk 20, 2% phytosterols, wild rice and wild rice in combination with 2% phytosterols treated mice showed no significant difference in HDL-cholesterol levels (0.85 ± 0.2 mmol/L and 0.95 ± 0.2 mmol/L for 2% phytosterols and wild rice respectively, and 0.92 ± 0.1 mmol/L for wild rice in combination with 2% phytosterols) as compared to control group (1.54 ± 0.4).

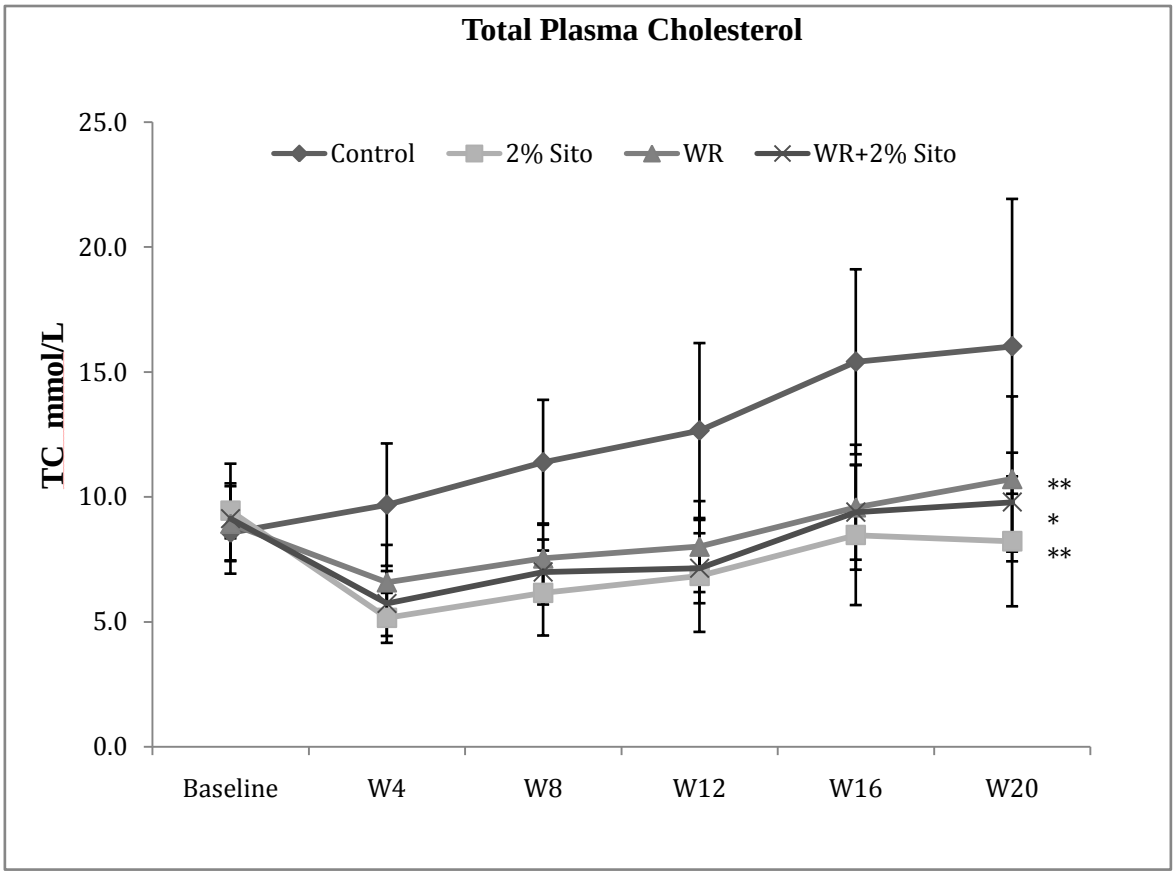
7.4. LDL-cholesterol concentrations

Figure 8 shows the levels of plasma LDL-cholesterol in the experimental groups. At week 20, plasma LDL-cholesterol levels were significantly (P=0.001) different among the groups. Significant reduction in plasma LDL-cholesterol levels were observed in 2% phytosterols, wild rice and wild rice in combination with 2% phytosterols treated mice (P=0.001, P=0.037 and P=0.008, respectively) compared to control group. Comparison of LDL:HDL cholesterol ratio between groups are presented in **Figure 9**. 2% phytosterols and wild rice in combination with 2% phytosterols treated mice had lowest LDL:HDL cholesterol ratio (1.17±0.50 and 1.37±0.66 respectively) as compared to wild rice and control group (1.52±0.6 and 1.52±0.5 respectively).

7.5. VLDL-cholesterol concentrations

VLDL-cholesterol levels are shown in **Figure 10**. Plasma VLDL-cholesterol levels were significantly (P=0.0001) different among groups at week 20. Significant reduction in plasma VLDL-cholesterol levels were observed in 2% phytosterols, wild rice and wild rice in combination with 2% phytosterols treated mice (P=0.0001, P=0.0001 and P=0.0001, respectively) compared to control group.

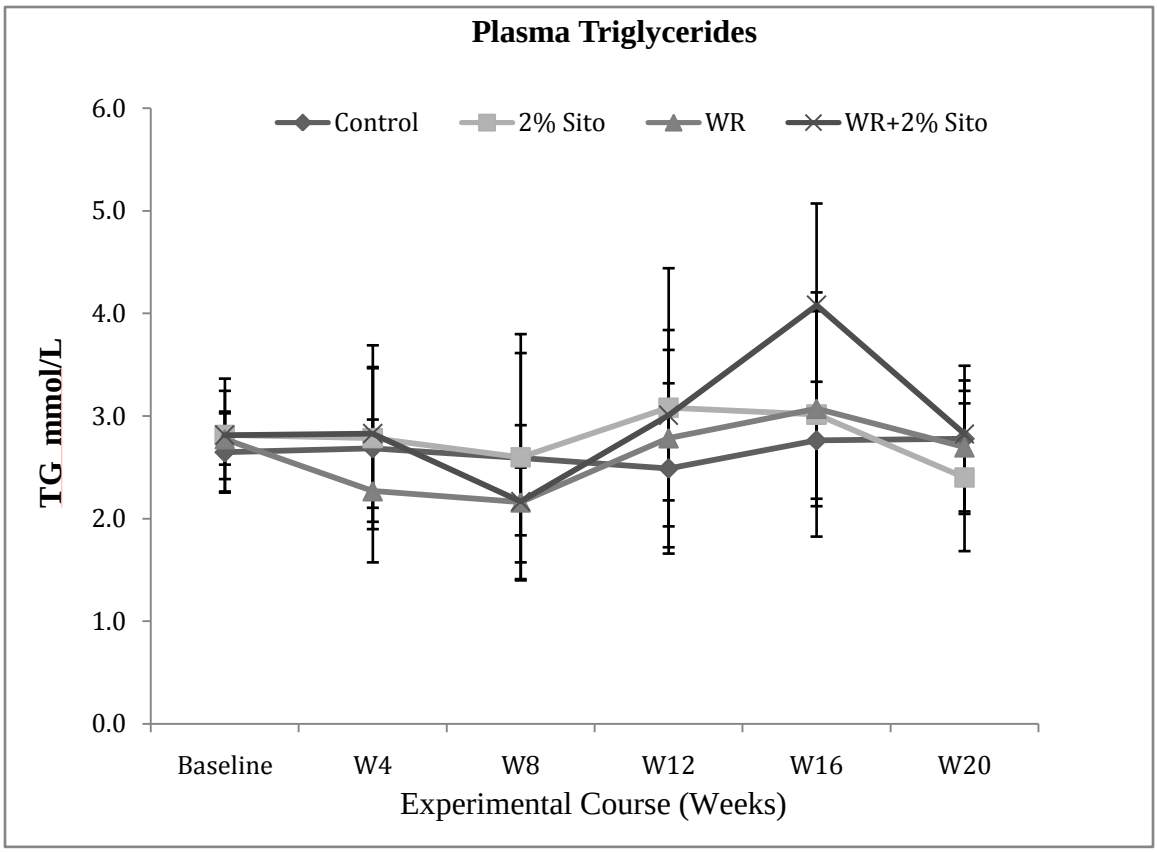
Figure 5. Total cholesterol concentration of LDL-r-KO mice at baseline and 4-wk intervals of experimental diet treatment.



Data are presented as mean ± standard deviation. Significant change *P < 0.05; **P < 0.001as compared to the control group in all weeks.

Control; 2% Sito= phytosterol; WR= wild rice.

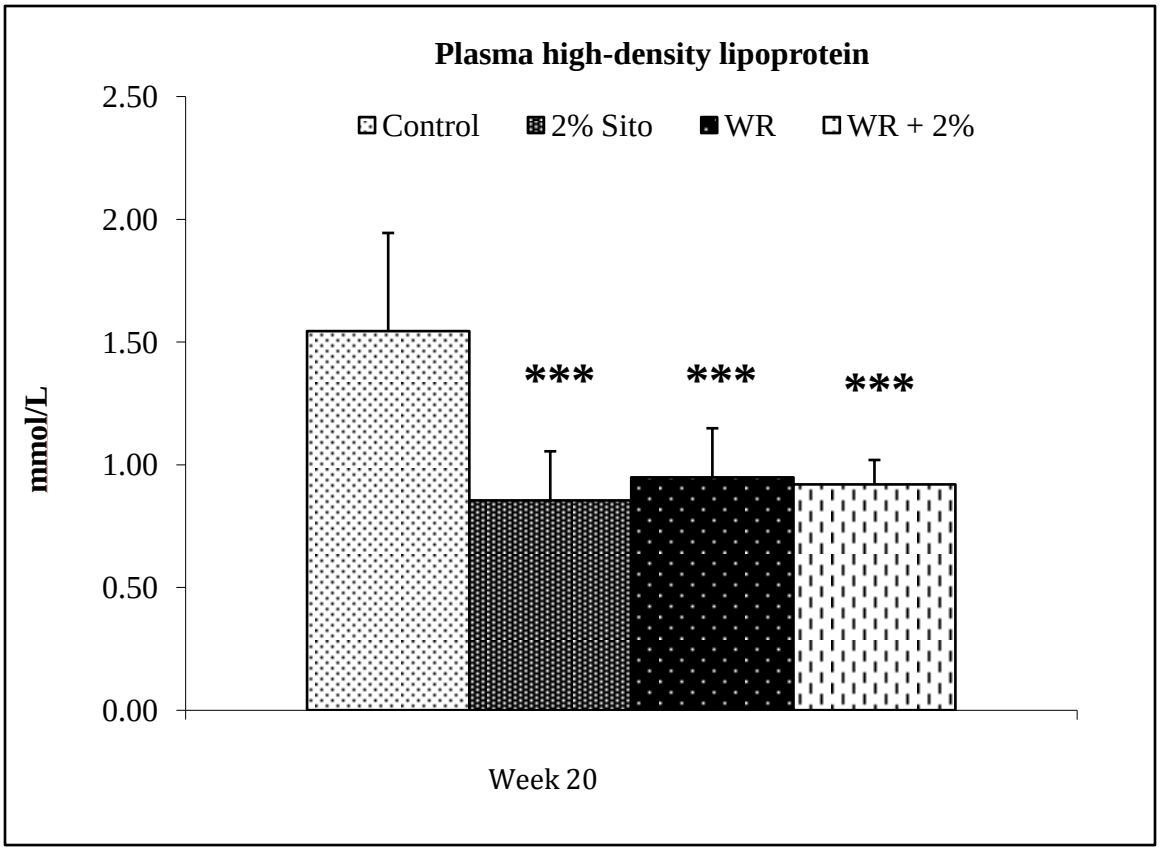
Figure 6. Triglycerides concentration of LDL-r-KO mice at baseline and 4-wk intervals of experimental diet treatment.



Data are presented as mean ± standard deviation.

Control; 2% Sito= phytosterol; WR= wild rice.

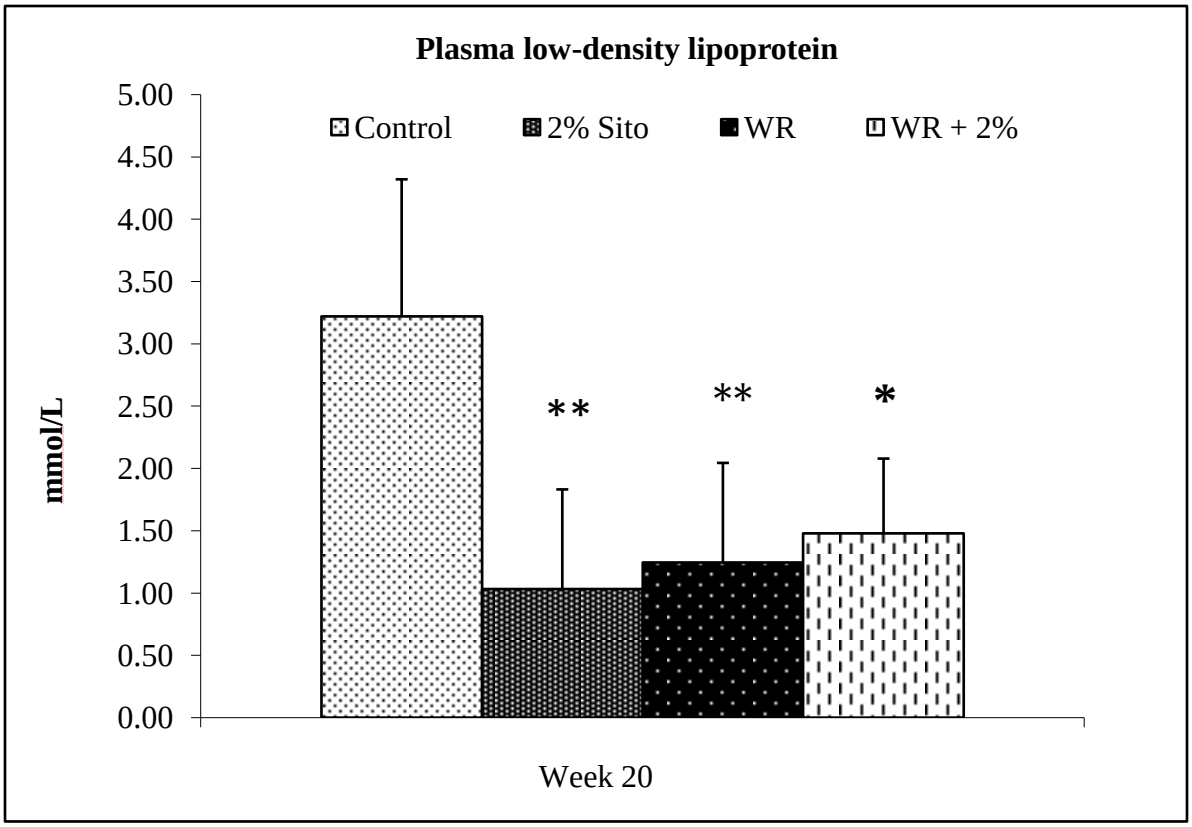
Figure 7. Plasma HDL-cholesterol levels of LDL-r-KO mice after 20 weeks of experimental diet treatment.



Data are presented as mean ± standard deviation. ***P < 0.0001 as compared to the control group.

Control; 2% Sito= phytosterol; WR= wild rice.

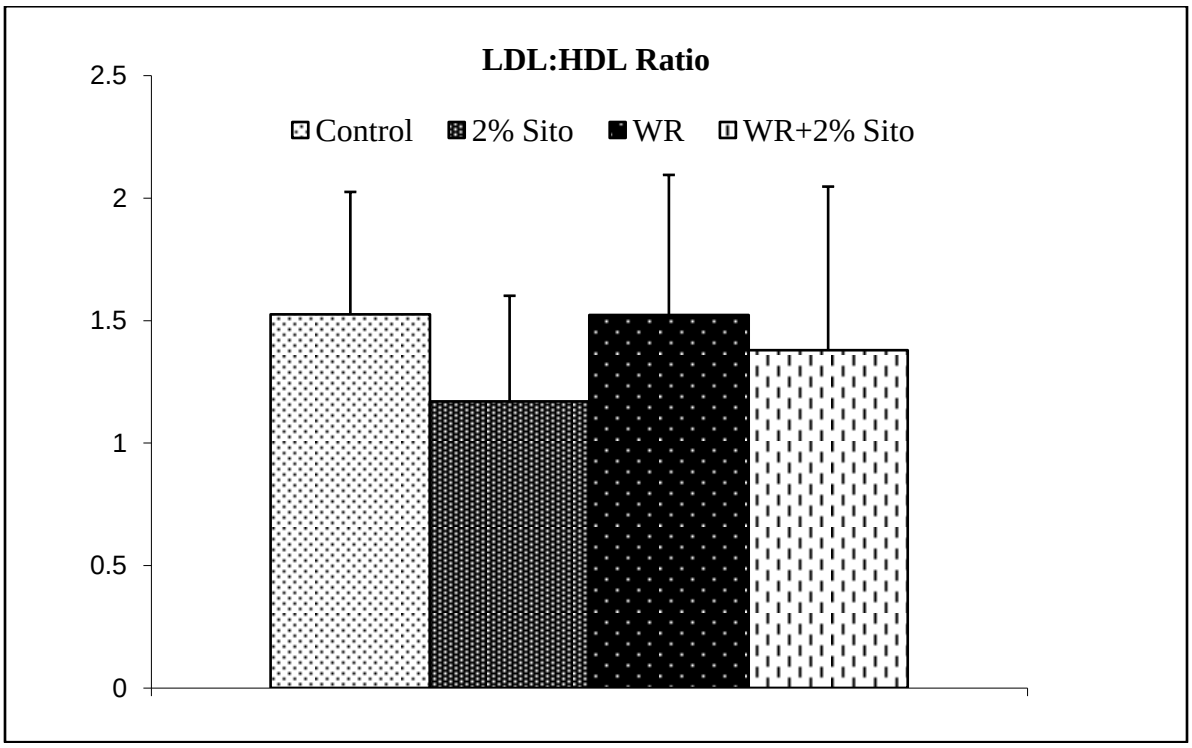
Figure 8. Plasma LDL-cholesterol levels of LDL-r-KO mice after 20 weeks of experimental diet treatment.



Data are presented as mean ± standard deviation. *P < 0.05; **P < 0.001 as compared to the control group.

Control; 2% Sito= phytosterol; WR= wild rice.

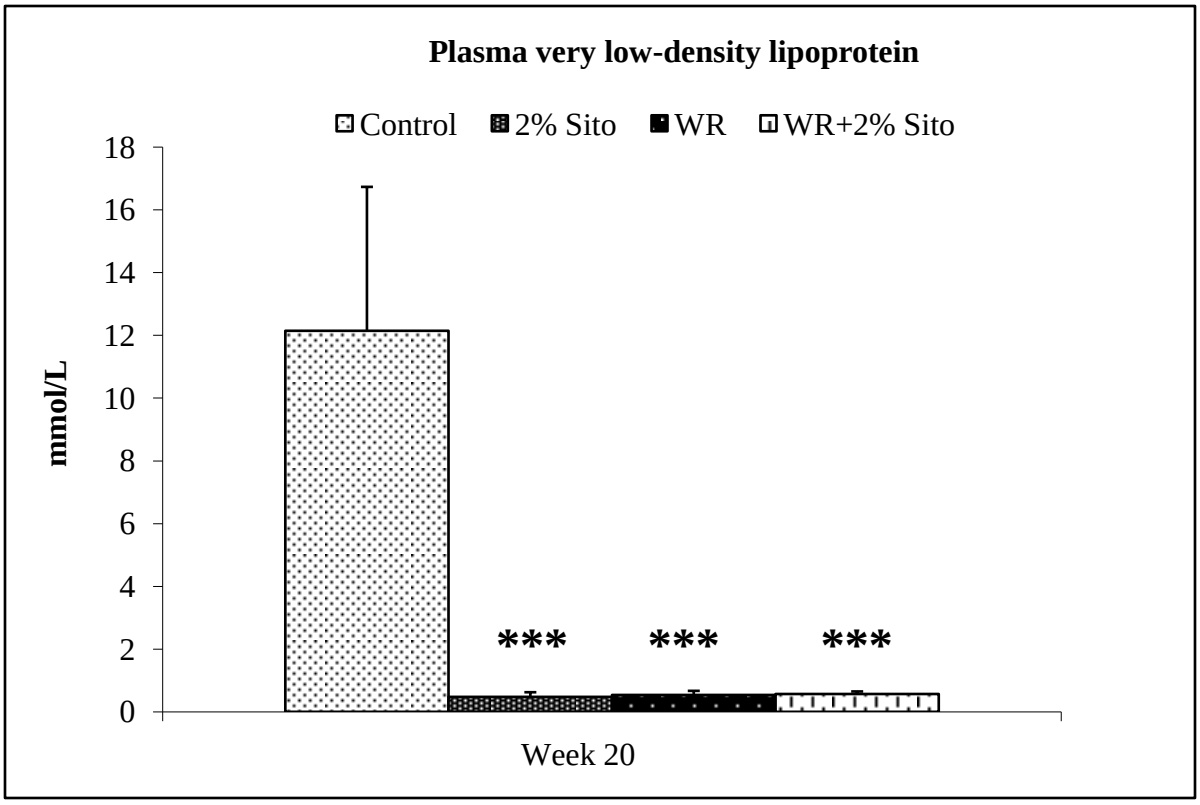
Figure 9. Comparison of LDL:HDL cholesterol ratio between groups of LDL-r-KO mice after 20 weeks of experimental diet treatment.



Data are presented as mean ± standard deviation.

Control; 2% Sito= phytosterol; WR= wild rice.

Figure 10. Plasma VLDL-cholesterol levels of LDL-r-KO mice after 20 weeks of experimental diet treatment.



Data are presented as mean ± standard deviation. ***P < 0.0001 as compared to the control group.

Control; 2% Sito= phytosterol; WR= wild rice.

7.6. Total fecal lipid concentrations

Fecal samples were collected for lipid extraction, and fecal lipid percentages were calculated. As shown in **Figure 11**, 2% phytosterols and wild rice in combination with 2% phytosterols treated mice had significantly higher fecal lipid content than the control and wild rice diet groups. 2% phytosterols and wild rice in combination with 2% phytosterols had higher fecal lipid percentages (P= 0.0001) as compared to control and wild rice diet groups.

7.7. Fecal cholesterol concentrations

Fecal cholesterol concentrations are presented in **Table 12**. 2% phytosterols and wild rice in combination with 2% phytosterols group had higher fecal total cholesterol levels (P=0.0001 and P=0.002 respectively).

7.8. Fecal lathosterol concentrations

Table 12 summarizes the results of fecal lathosterol concentrations in all of the 4 experimental groups at the end of study 2% phytosterols and wild rice group had higher fecal total lathosterol levels than control group. In addition, wild rice in combination with 2% phytosterols diet contained significantly highest fecal lathosterol levels among all groups (P=0.009).

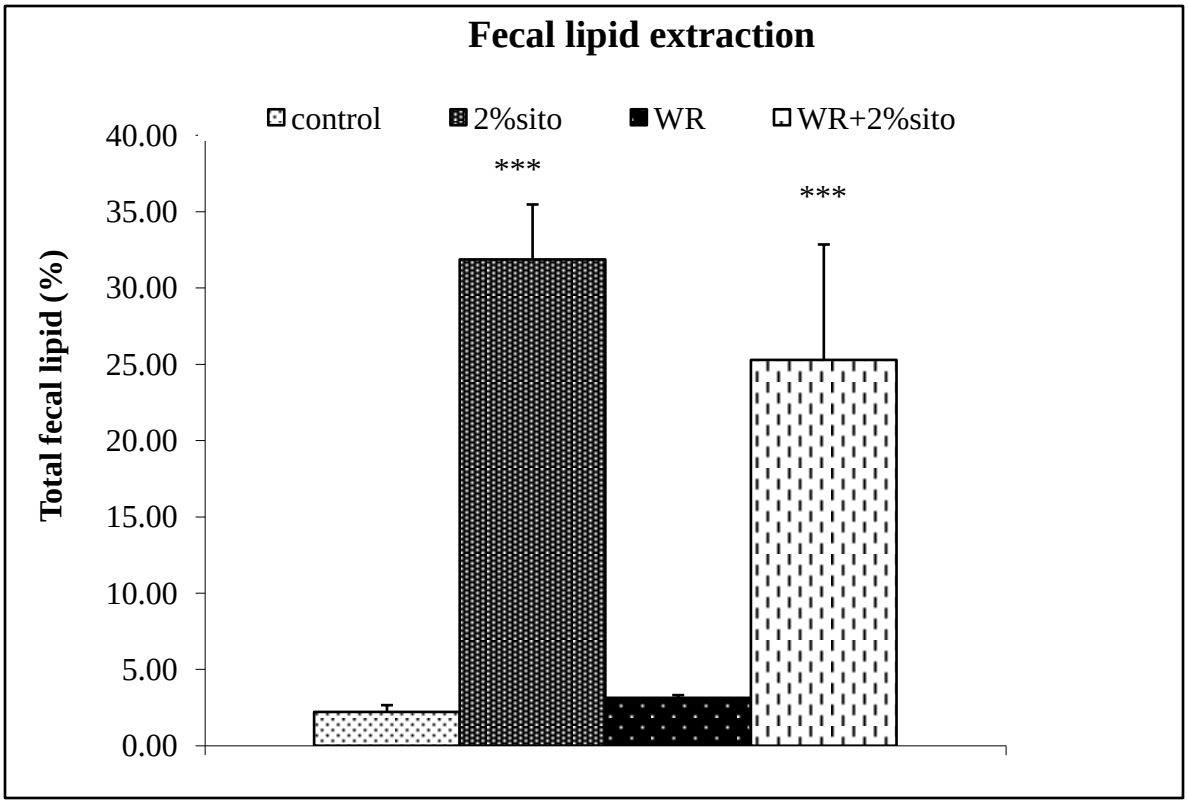
7.9. Fecal campsterol concentrations

Wild rice in combination with 2% phytosterols and 2% phytosterols treated mice had significantly increased excretion of fecal campsterol concentration (P=0.0001 and P=0.0001 respectively) as compared to mice fed control and wild rice diet. Campsterol levels of fecal samples of the experimental animals at week 20 are illustrated in **Table 12**.

7.10. Fecal β-phytosterols concentrations

Fecal β-phytosterols concentration levels are shown in **Table 12**. Wild rice in combination with 2% phytosterols and 2% phytosterols group had significantly increased excretion of fecal β-phytosterols concentration (P=0.0001 and P=0.0001 respectively) as compared to mice fed control and wild rice diet.

Figure 11. Total fecal lipid concentrations of LDLr-KO mice after 20 weeks of experimental diet treatment.



Data are presented as mean ± standard deviation. ***p< 0.0001 compared to control group.

Control; 2% Sito= phytosterol; WR= wild rice.

Table 12. Fecal sterol (mg per gr of feces) in LDL-r-KO mice after 20 weeks of experimental diet treatment

Groups	Control (N=8)	2% Phytosterols (N=8)	Wild rice (N=8)	Wild rice + 2% Phytosterols (N=8)	P value
Cholesterol	0.03 ± 0.01	0.14 ± 0.03**	0.03 ± 0.01	0.11 ± 0.03**	0.025
Lathosterol	0.02 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.10 ± 0.06**	0.0001
Campsterol	0.01 ± 0.01	0.73 ± 0.07***	0.01 ± 0.01	0.54 ± 0.15***	0.0001
β-Phytosterols	0.01 ± 0.01	1.19 ± 0.11***	0.01 ± 0.01	0.90 ± 0.25***	0.0001

Data are presented as mean ± standard deviation.
****P < 0.001; ***P < 0.0001 as compared to the control group.**

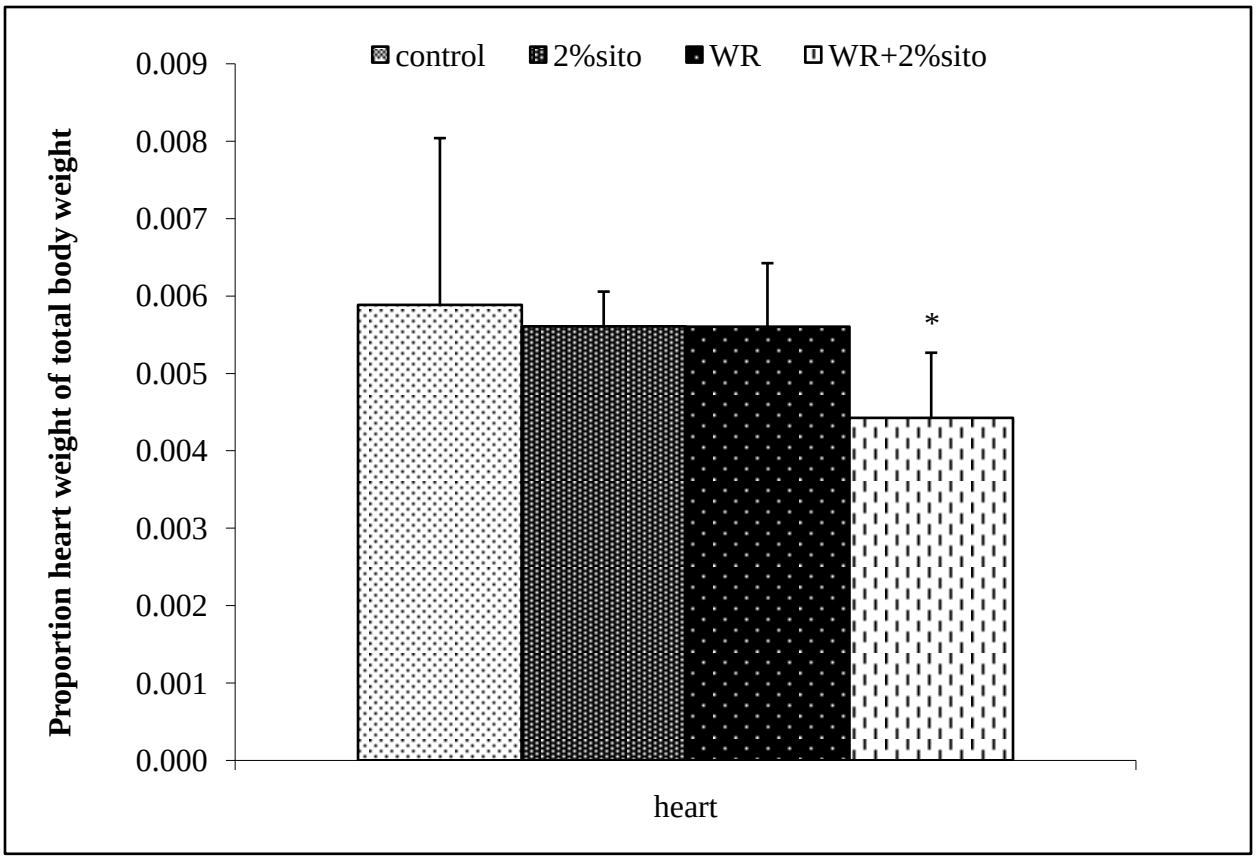
7.11. Heart weight

The proportion of heart weight to total body weight of the experimental mice is demonstrated in **Figure 12.** There was no statistically significant difference among the groups. However, wild rice in combination with 2% phytosterols had lower proportion heart weight (P=0.002) as compared to control.

7.12. Heart histology

Representative photomicrographs of the atherosclerotic lesions in the aortic root of the treated and control groups of mice are illustrated in **Figure 13.** Advanced atherosclerotic lesions in the aortic root of control mice are shown in **Figure 13A**. Corresponding sections from wild rice and 2% plant sterols treated animals stained with hematoxylin and eosin (H&E) show only a limited lesion in the aortic root (**Fig 13B** and **13C**) but wild rice+ 2% plant sterols show free of lesions development in the aortic root (**Fig 13D**). The lesion area in aortic root is presented in **Figure 14**. The measurement of lesion area was significantly different between the groups (P=0.0001). Wild rice, 2% phytosterols and wild rice in combination with 2% phytosterols treated mice had significant decreases (P=0.0001, P=0.0001 and P=0.0001 respectively) in the lesion area measurement in aortic root compared to the control group.

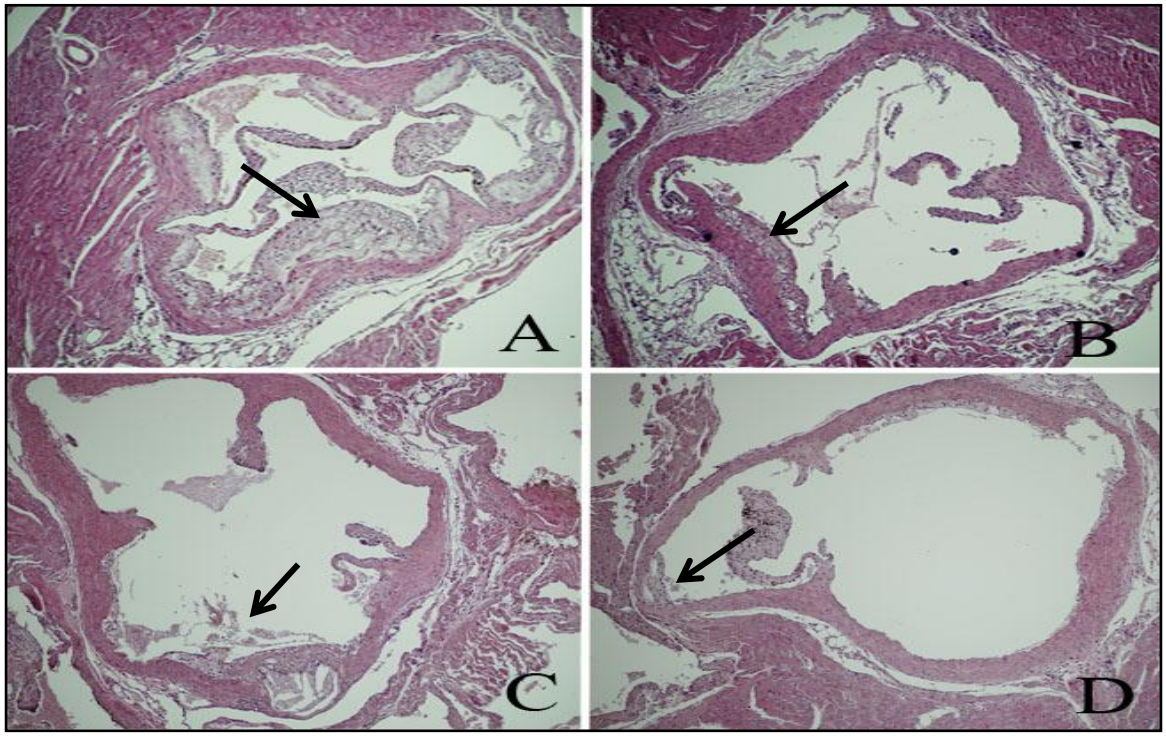
Figure 12. Proportion heart weight of total body weight of LDLr-KO mice after 20 weeks of experimental diet treatment.



Data are presented as mean ± standard deviation. *p< 0.05 as compared to control group.

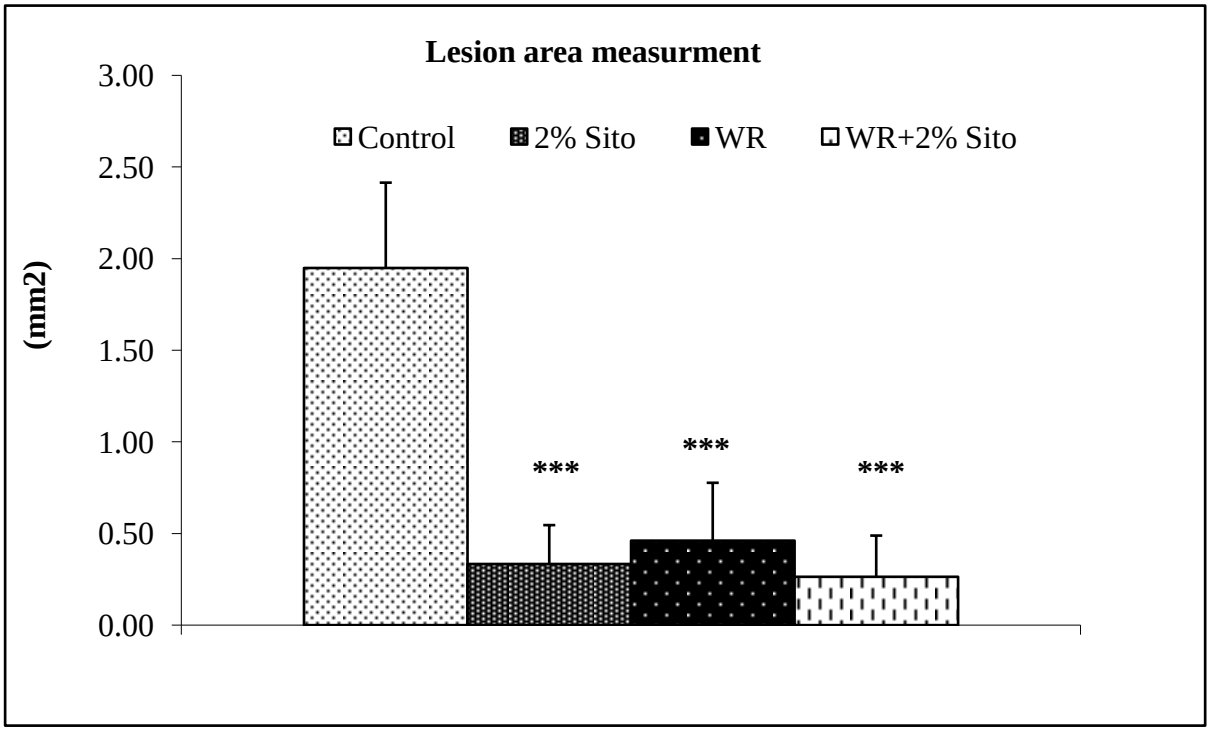
Control; 2% Sito= phytosterol; WR= wild rice.

Figure 13. Histopathology of aortic root of LDL-r-KO mice. Control group (Panel A); wild rice group (Panel B); 2% phytosterols group (Panel C); WDR+2%PS group (Panel D).



Arrows show atherosclerotic lesions

Figure 14. Atherosclerotic lesion area (mm^2) of LDLr-KO mice after 20 weeks of experimental diet treatment.



Data are presented as mean ± standard deviation. ***p< 0.0001 compared to control group.

Control; 2% Sito= phytosterol; WR= wild rice.

8. Discussion

8.1. Effects of dietary wild rice and plant sterols administration

The primary objective of the present study was to investigate whether dietary Manitoban wild rice in combination with plant sterols has additive effects in the reduction of atherosclerosis risk in hyperlipidemia mice model. Herein, we provide the evidence that both dietary wild rice and phytosterols are two dietary interventions that slow atherosclerosis progression in LDLr-KO mice. Consumption of wild rice in combination with phytosterols significantly reduced total plasma concentrations of cholesterol (TC), low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) in LDL-r-KO mice; this was associated with an increased fecal excretion of cholesterol. Moreover, both of these dietary treatment decreased LDL/HDL-cholesterol ratio.

8.2. Food intake, body weight and abdominal fat

Food consumption among the four groups had no significant differences. Similar observations were made by Han et al., (2012). The average food intake among the four groups of rats had no significant differences. The mean body weight and abdominal fat mass of mice in current study did not significantly difference between diet groups, given that similar amounts of diets were consumed. In agreement with our previous study, body weight and abdominal fat mass of mice fed wild rice had no significantly difference between diet groups (Surendiran et al., 2013). Similar observations were made in a double-blind randomized controlled trial of men and women aged 25-75 with hyperlipidemia who consumed three margarines (two experimental ones based on

rapeseed or tall oil sterols and one control) for 4 weeks and showed no change in body weight (Heggen et al., 2010). However, Zhang et al., (2009) showed that replacing white rice and processed wheat starch with wild rice as a chief carbohydrate source in a high fat cholesterol rats fed high fat cholesterol diet lowered body weight and abdominal fat mass, despite similar dietary energy intake among experimental groups. Recently, Han et al., (2012) reported that WDR lowered body weight and the liver organ weights in rats.

8.3. Plasma and fecal lipid profile

The relationship between serum cholesterol concentrations and the risk of cardiovascular disease has been reported. In the present study, the effects of wild rice in combination with plant sterols on circulating levels of plasma lipids and lipoproteins were determined. Wild rice in combination with plant sterols supplemented group was observed to exhibit plasma cholesterol lowering effects on the experimental animals. Wild rice in combination with plant sterols group significantly reduced plasma total cholesterol, VLDL and LDL cholesterol concentrations compared to control. In agreement with our study, rats fed with high saturated fat and cholesterol diet with incorporation of wild rice for 8 weeks, suppressed the elevation of serum total cholesterol level (Zhang et al., 2009). Similarly, rats exposed to a high-fat and cholesterol with wild rice for 8 weeks had reduced TC and TG concentration, and a raised HDL-C level as compared to city diet (CD) group (Composition of the diet consumed by the residents of modern china) (Han et al., 2012).

Wild rice has been accepted as a whole grain by the American Association of Cereal Chemists International and the FDA because the starchy endosperm, germ, and

bran, are present in the same relative proportions as they exist in the intact grain as defined by the American Association of Cereal Chemists International and the FDA. Conflicting results on association between whole grain and lipid profile have been published in randomized clinical trials. A recent study showed that increased whole grain consumption (60 g and 120 g) for a 16 week period did not show significant impact on plasma cholesterol levels of participants (Brownlee et al., 2010). Similarly, Andersson et al., (2007) failed to report any alterations in serum lipids in a randomized, crossover dietary intervention study with a 6-week consumption of whole grain rich diet.

In contrast, healthy individuals consumed wholemeal wheat foods for 3 weeks, showed a significant reduction of fasting plasma cholesterol, as well as LDL cholesterol (Giacco et al., 2010). Similar observations were made in a randomized, single-blinded cross-over trial using 22 healthy subjects (14 females; 8 males) who consumed products made from Kamut for 8 weeks and showed that consumption of Kamut products had a significant reduction of total cholesterol and low-density lipoprotein cholesterol (Sofi et al., 2013).

Likewise, TC and LDL cholesterol levels were decreased in Chinese adults with hypercholesterolemia after consuming 100 grams of wheat flour-based noodles daily for 6 weeks (Zhang et al., 2012). Risk of cardiovascular disease was reduced in middle-aged people, preferentially with a daily consumption of 3 portions of whole-grain foods for 12 weeks (Tighe et al., 2010).

Several studied have reported cholesterol lowering properties of whole grains (Brownlee et al., 2010; Giacco et al., 2010; Jones and Engleson, 2010). Whole grains

include wheat, barley, rye, oats, millets, brown and wild rice are known to be a rich source of fiber, minerals, vitamins, phenolic compounds, and several other phytochemicals (Slavin, 2004; Jones and Engleson, 2010; Slavin, 2003). The potential protective effect of whole grain has been much discussed and suggested to reduce risk factors of cardiovascular diseases. Among all components of whole grains, dietary fiber has more attention in improving serum lipid concentration (Okarter and Liu, 2010; Giacco et al., 2010; Jones and Engleson, 2010; Slavin, 2003; Andersson et al., 2002; Jensen et al., 2006; Newby et al., 2007). Zizania wild rice is a rich source of dietary fiber, approximately 5.2 % of total dietary fiber, 3.3 % insoluble and 0.8 % of soluble fiber (Bunzel et al., 2002; Dreher, 2001). In the present study, the total dietary fiber amount of the wild rice used was higher than that in the control group (8.3 vs. 6.5 g/ 100 g). The cholesterol lowering effect of wild rice may be due to higher content of dietary fiber compared to control group. The mechanisms behind the hypocholesterolemic effects of dietary fiber have been elucidated. Dietary fiber decreases cholesterol absorption and reabsorption of bile acids in the intestinal lumen. Increased bile acids excretion promotes uptake of cholesterol circulating in the plasma for bile acids synthesis in order to replenish the bile acids pool, thus lowering concentration of plasma cholesterol (Andersson et al., 2002; Sayar et al., 2006; Erkkila and Lichtenstein, 2006; Lundin et al., 2004). Moreover, low glycemic index of dietary fiber is linked to reduced insulin secretion by attenuating the activation of 3-hydroxy-3-methylglutaryl-co-enzyme A (HMG-CoA) reductase, which potentially leads to reduction in hepatic cholesterol synthesis (Erkkila and Lichtenstein, 2006; Juntunen et al., 2003; Mann, 2007). Lastly, short chain fatty acids (SCFA; acetate, butyrate, and propionate) formed during colonic

fermentation of dietary fiber have also been reported to lower plasma cholesterol level (Bridges et al., 1992; Wolever et al., 1995; Wong et al., 2006).

In addition, wild rice appeared to carry a rich source of phytosterols. Przybylski et al.,(2009) determined phytosterol composition of seven wild rice samples obtained from different growers across United States and Canada. Wild rice is a rich source of phytosterols, mainly consisting of campesterol, β-phytosterols , and cycloartenol. The total amount of sterol content of wild rice lipid ranged from 70 g to 145 g of sterol/ kg of wild rice lipid (Przybylski et al., 2009). Since 1950's researchers have been studying the cholesterol-lowering properties of plant sterols. A randomized, double-blind, crossover study in eighty mildly hypercholesterolemic volunteers provided by 20 g phytosterols enriched spread into regular daily dietary intake for 4 weeks, successfully reduced total cholesterol (-4.4 %), and LDL cholesterol (-5.6 %); however, no changes were observed in HDL cholesterol and triglycerides levels (Eady et al., 2011). In agreement with Varady et al., (2004), in placebo-controlled, parallel-arm clinical trial 84 subjects received sterols for 8 weeks, showed that sterol supplementation significantly decreased total cholesterol concentrations by 8.2% and lowered LDL-cholesterol (Varady et al., 2004). Similar findings were found in sixty non-hypercholesterolemic healthy subjects received 3 cups of low fat yogurt with 1 g of plant stanol esters for one week (Mensink et al., 2002). Similar findings have been reported in numerous clinical trials (Miettinen et al., 2000; Mussner et al., 2002; Plat et al., 2000). This may support our findings from the present study that plasma total and LDL cholesterol were statistically lowered without any significant changes in HDL cholesterol and triglyceride levels between wild rice in combination with plant sterol and control diet group. Plant sterols have a

very similar molecular structure with cholesterol, which allows it to act as a competitor in micellar binding, resulting in reduction in absorption of intestinal cholesterol and enhanced excretion of unabsorbed cholesterol. Moreover, a number of proteins have been shown to involved in intestinal uptake, which rely on ATP binding cassette transporter A1 (ABCA1) that facilitates the cholesterol efflux from blood to the intestinal lumen (De Smet et al., 2012). Plant sterols also may interfere with Acyl CoA: cholesterol acyltransferase (ACAT) activity to form cholesteryl ester. Free cholesterol and cholesteryl esters are packaged into chylomicrons which lead to reduced cholesterol absorption (Trautwein et al., 2003).

In addition, in 2010, Health Canada's approved the health claim that addition of plant sterols as food ingredients to decrease low-density lipoprotein cholesterol (LDL-C), when consumed at a recommended daily dose of 2 g (Rideout et al., 2012), has a significant impact on lowering serum cholesterol concentration through reducing intestinal cholesterol absorption and increased excretion of fecal cholesterol (Miettinen et al., 2000). In placebo-controlled, crossover feeding trial, 18 adults who received moderate (459 mg) and high (2059 mg) plant sterol consumption daily for 4 weeks, showed increased total fecal cholesterol excretion (Racette et al., 2010). Similar observation was made in a randomized, double-blind, controlled, crossover study, where twenty-one mildly hypercholesterolemic individuals who received phytosterols for 3 weeks, had significantly increased fecal cholesterol (Lin et al., 2011). Similarly, fecal sterol with a slight increase in campesterol and phytosterols fecal extraction were observed in colectomized patients after consuming 2 g stanol for 7–18 d (Miettinen et al., 2000). This corresponds with our study that mice fed wild rice in combination with plant

sterols had significantly higher fecal lipid content compared to control, total cholesterol, lathosterol, campsterol and β-phytosterol compared to control. The major route of sterol elimination from the body is by excretion of unabsorbed cholesterol and bile acids as fecal neutral and acidic sterol (Wang, 2007). Moreover, increased bile acid secretion was found to be inversely correlated with elevated of cholesterol levels and the prevalence of coronary artery disease (Charach et al., 2011; Charach et al., 2012).

In addition, several potent phenolic acids have been identified in wild rice; among them ferulic acid has the highest levels (Qiu et al., 2010). Ferulic acid is reported to have potential health benefits including hypocholesterolemic properties. Golden Syrian hamster fed high cholesterol diet supplemented with 0.5 % ferulic acid had significantly reduced total cholesterol concentrations (Wilson et al., 2007). Moreover, rats fed high cholesterol with 0.2 % ferulic acid, had significantly lower plasma lipid, hepatic cholesterol, hepatic HMG-CoA reductase and ACAT activities and increased excretion of neutral sterol and acidic sterol (Yeh et al., 2009). Similar results were reported in rats that received high cholesterol diet incorporation of minimal amount of ferulic acid (0.013 %); however, no changes were observed in hepatic total cholesterol and triglyceride levels (Kim et al., 2003). Inhibition of HMG-CoA reductase, the rate limiting step in cholesterol biosynthesis, leads to decreased intracellular cholesterol concentration (Trautwein et al., 2003). Esterification of cholesterol by limiting ACAT activities results in limiting assembly of VLDL and leading to reduction of VLDL secretion from the liver (Wang, 2007).

8.4. Histology and development of atherosclerosis

Accumulations of fatty substances and cholesterol are thought to be a major cause of injury to the endothelium and underlying smooth muscle cells, recruiting macrophages and resulting in an early stage of atherosclerosis, namely, foam cell formation (Stary et al., 1995; Ross, 1999). Antioxidant phytochemicals are expected to inhibit LDL oxidation (Stocker, 1999). Reduced levels of LDL oxidation are associated with reduced levels of inflammatory state. Altogether, these events resulted in prevention of advanced atherosclerotic lesions. Wild rice is rich in phenolic antioxidants, and therefore, the phenolic antioxidants are thought to produce health benefits through their antioxidant properties (Qiu et al., 2010; Slavin, 2003). Consequently, atherosclerotic lesions were significantly reduced in wild rice, 2% phytosterols and wild rice in combination with 2% phytosterols. In agreement with our previous study, mice fed wild rice for 24 weeks had significant decreases in atherosclerotic lesions (Surendiran et al., 2013). More than 50% reduction in the average lesion area in the aortic sinuses of phytosterol-fed apo E-KO mice, as compared with those in the control group (Moghadasian et al., 1997). Similar observations were observed in apoE-deficient mice after 20 weeks of phytosterols supplementation (Moghadasian et al., 1999). Reducing lesion area in aortic root of plant sterol treated mice may be related to the inhibition of intestinal cholesterol absorption. The reduction of plasma cholesterol in wild rice treated animals most likely resulted in the absence of cholesterol clefts in the attenuated atherosclerotic lesions of the treated animals. Besides, the antioxidant in wild rice are expected to inhibit LDL oxidation. This slowing in the progression of atherosclerosis was reflected in the absence of advanced lesions in the aortic root.

8.5.Strengths and limitations

We selected LDLr-KO mice as a dyslipidemia and atherosclerosis model for this study (Getz and Reardon, 2006). These models develop atherosclerosis remarkably in the same manner of familial hypercholesterolemia developing in humans (Zadelaar et al., 2007). This would suggest better reflection of the impact of our protocol if it is to be applied to human subjects. Moreover, study design and length are among strengths of our study. We carried out the study for a relatively long period, 20 weeks, because atherosclerosis is a chronic condition that has been reported to increase with time.

The present study, like many others, may have several limitations. We used only male LDL-r-KO mice animals, so there was missing of wild type C57BL/6 mice to use as a control in cardiovascular risk factors (Zadelaar et al., 2007). We did not have a sufficient sample size to measure LDL oxidation which is known to promote atherogenesis through foam cell formation and inflammatory responses. Therefore, we were unable to draw conclusions regarding the mechanism of action involved in lower cholesterol responses in wild rice groups imparted by the results.

8.6.Recommendations for future research

Additional studies are needed to understand the mechanism of action of wild rice on the prevention of atherosclerosis in LDLr-KO mice. One potential area includes the investigation of the dose-response study of the effectiveness of wild rice on the prevention of atherosclerosis. Besides other functional components of wild rice, such as phytosterols, soluble fibers, resistant starch, and others need to be examined to identify

potential health benefits. Determination of HMGCR, PPPARα, CYP7A1 and the production of nitric oxide ($^{\bullet}$No) / expression analysis of eNOS activities may be an initial step to know the mechanism of cholesterol lowering properties of wild rice. Next, the activities of HMG-CoA reductase, can be analyzed to provide the rate-limiting enzyme in biosynthesis of cholesterol as well as the activities of bile acid synthesis and cholesterol catabolism enzymes such as cholesterol 7-α hydroxylase and sterol 25 hydroxylase.

8.7.Conclusion

The presented study showed that dietary wild rice and phytosterols are two dietary interventions that slow atherosclerosis progression in LDL-r-KO mice. Consumption of wild rice in combination with phytosterols significantly reduced total plasma concentrations of cholesterol (TC), low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) in LDL-r-KO mice; this was associated with an increased fecal excretion of cholesterol

The hypocholesterolemic properties of phytosterols are mainly attributed to the increased rate of fecal cholesterol excretion. Moreover, this reduced atherosclerosis in LDLr-KO treated mice. Histological examination showed mature atherosclerotic lesions in the aortic root in the control group while, wild rice and phytosterols treated mice showed only early lesions containing mainly superficial foam cells. Reducing lesion area in aortic root of plant sterol treated mice may be due to the inhibition of intestinal cholesterol absorption. The reduction of plasma cholesterol in wild rice treated animals most likely resulted in the absence of cholesterol clefts in the attenuated atherosclerotic lesions of the treated animals. Besides the potential health benefits of Manitoban wild

rice are probably due to the synergy of its functional components and phytochemical compounds. This slowing in the progression of atherosclerosis was reflected in the absence of advanced lesions in the aortic root of treated animals.

Long term consumption of Manitoban wild rice or plant sterols have desirable protective roles on improving plasma lipid profile in LDLr-KO mice. Replacing white rice or other refined grain products with Manitoban wild rice (*Zizania palustris*) may potentially lower cardiovascular risk through its hypocholesterolemic effect.

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