

**Beneficial Effects of Germinated Brown Rice on Cardiovascular Risk Factors  
in LDL Receptor Knockout Mice**

by

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## ABSTRACT

Based on accumulating evidence, adequate intake of whole grains is associated with reduced cardiovascular disease CVD risk. Germinated brown rice (GBR) has been used in East Asian countries as an alternative grain. Preliminary studies suggest GBR has potential health benefits, including reducing CVD risk, but the mechanism remains unclear. The hypothesis of the project is that long-term consumption of GBR would reduce atherogenic risk factors in low-density lipoprotein receptor knockout (LDLr-KO) mice. To test the hypothesis, three groups of male LDLr-KO mice were fed with one of the following diets for 24 weeks: (a) commercial mouse chow, used as the control diet; (b) chow was replaced with 60% (w/w) Chinese white rice (CWR); and (c) chow was replaced with 60% (w/w) GBR. All diets were supplemented with 0.06% (w/w) dietary cholesterol to accelerate atherogenesis. Blood samples, hearts, livers and feces were collected and used for biochemical and histological analyses. The results demonstrated that no significant difference was detected in body weights, plasma or fecal lipid profiles and antioxidant enzyme activities among groups. However, GBR consumption significantly decreased atherosclerotic lesion ( $P = 0.003$ ) in the aortic roots as compared with that in the CWR group, but there was no significant difference as compared with that in the control group ( $P = 0.4$ ). In addition, GBR significantly decreased monocyte adhesion to the aorta in LDLr-KO mice as compared to that in the CWR group ( $P=0.0001$ ), but not with the control group. These data suggested that GBR may be beneficial for the prevention of vascular inflammation and atherogenesis in LDLr-KO mice. Additional studies in animal models and humans may further investigate the mechanisms of the beneficial effects of GBR on vascular inflammation and atherogenesis.

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## **DEDICATION**

For the four pillars in my life: GOD, parents, husband and kids.

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## LIST OF ABBREVIATIONS

AIN-93G	American Institute of Nutrition
ASG	Acylated steryl glycoside
ANOVA	Analysis of variance
BR	Brown rice
CaCl <sub>2</sub>	Calcium chloride
CAT	Catalase
CHD	Coronary heart disease
CNTRL	Control
CVD	Cardiovascular diseases
CWR	Chinese white rice
DF	Dietary fiber
FA	Ferulic acid
FDA	U.S. Food and Drug Administration
GABA	Gamma-amino-butyric acid
GAD	Glutamate decarboxylase
GAE/gdw	Gallic acid equivalent/dry weight
GBR	Geminated brown rice
GPx	Glutathione peroxidase
GR	Glutathione reductase

H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HBSS	Hank's balance salt solution
HDL	High density lipoprotein
HF	High fat
HFD	High fat diet
HF-GB	High fat with germinated brown rice
HF-GW	High fat with germinated white rice
HF-NB	High fat with normal brown rice
HF-NW	High fat with normal white rice
HMDS	Hexamethyldisilane
LDL	Low density lipoprotein
LDLr-KO	LDL Receptor Knockout mice
N <sub>2</sub>	Nitrogen gas
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NaHCO <sub>3</sub>	Sodium bicarbonate
NC	Normal control
ND	Normal diet
PR	Polished rice
ROS	Reactive oxygen species
SE	Standard error

SG	High cholesterol diet with simvastatin
SOD	Superoxide dismutase
TC	Total cholesterol
TG	Triglyceride
TMCS	Trimethylchlorosilane
VLDL	Very low density lipoprotein
WG	Whole grains
WR	White rice

## **1. Introduction**

Cardiovascular diseases (CVD) may contribute to one-third of morbidities and mortalities in Canada, and their prevalence is expected to increase in the next decade most likely because of increasingly sedentary lifestyles (Genest et al., 2009). According to Public Health Agency of Canada, in 2007, 1.3 million Canadians aged  $\geq 12$  years had heart disease, while 317,500 Canadians were living with stroke (Public Health Agency of Canada, 2009). CVD are chronic diseases resulting from the interaction of health behaviors, genetics and environmental factors. CVD can be reduced by quitting cigarette smoking, performing regular physical activity, stress management, healthy nutrition and weight control, as well as by early recognition and treatment of high blood pressure, diabetes and hypercholesterolemia (Pearson, 2011).

Hypercholesterolemia is a classical risk factor for CVD (Glass & Witztum, 2001). Cholesterol lowering is the most common management for atherosclerotic CVD (Kane et al., 1990). High cholesterol levels in the plasma can be decreased by suppression of cholesterol absorption within the intestine, thereby preventing atherosclerosis development (Moghadasian et al., 1997). Lowering plasma cholesterol can be achieved by different types of foods, including grains (Wolff et al., 2011). Consumption of whole grains was shown to reduce high cholesterol concentrations in the plasma (Chou et al., 2009; Davis, 2014; Lichtenstein et al., 1994).

Accumulating evidence suggested that intake of whole grains is a protective factor against CVD risk; the exact mechanisms however, are still not clearly understood (de Munter et al., 2007; Jacobs & Gallaher, 2004; Liese et al., 2003; Sahyoun et al., 2006; Steffen et al., 2003). Whole grain products are rich sources of fiber, minerals, small amounts of vitamin E (Liese et al., 2003), B vitamins and phytochemicals (Davis, 2014). Further, potential anti-carcinogenic

and cardio protective properties of those components can lead to beneficial impacts on health, for example, decreased inflammation and improved immune system (Chen et al., 2016). Whole grains include barley, oats, rice, wheat and maize (Hu, 2003).

Intake of brown rice (BR) decreases cholesterol and triglyceride levels and body weight gain, because it is rich in vitamins, fibers, fatty acids and minerals (Quagliariello et al., 2016). Furthermore, BR consumption has beneficial health effects on diabetes, chronic inflammation, cancer and heart disease because of its bioactive compounds, including tocopherols, tocotrienols,  $\gamma$ -oryzanol, phytic acid and other phenolic compounds (Jun et al., 2012). Germination of BR further increases its nutritional and functional values. Over the past few decades, there has been an increasing demand for natural foods containing highly active compounds with antioxidant potential. As a result, products such as germinated brown rice (GBR) gradually became popular in East Asia for health improvement. In addition, GBR is a good example of a functional food because of its ability to reduce the risk of a number of diseases and improve health (Cornejo et al., 2015).

GBR is created by a biological process promoted by soaking brown rice in water, under specific temperature and certain humidity conditions, for several hours (Chen et al., 2016). GBR is popular because it contains considerably more gamma-amino-butyric acid (GABA) than brown rice does (Liu et al., 2013). Also, GBR is a good source of dietary fiber, inositol, ferulic acid, phytic acid, tocotrienols, magnesium, potassium, zinc and  $\gamma$ -oryzanol (Latifah et al., 2010). GBR was reported to have health benefits including weight reduction, anti-hypertension, anti-depression, hypocholesterolemic and anti-diabetic actions, improved learning and memory and anti-cancer effects (Cho & Lim, 2016).

There have been few studies on effectiveness of GBR against risk factors for atherosclerosis (Choi et al., 2006; Ho et al., 2012; Hsu et al., 2008; Lee et al., 2007; Lo et al., 2016). Therefore, the purpose of this study was to investigate the effectiveness of GBR on atherosclerosis risk factors in LDLr-KO mice. Plasma and fecal lipid profiles were analyzed to assess effects of GBR on lipid metabolism. Moreover, activities of endogenous antioxidant enzymes, such as glutathione peroxidase, catalase and superoxide dismutase, were determined in plasma samples. Also, histological examination of the aortic roots and atherosclerotic lesion area calculations were performed.

White rice (WR), the most widely consumed type of rice, was used as a control treatment diet. This is because WR is a commonly used refined grain in the daily diet, served either as a main rice dish, side dish or ingredient in various mixed dishes. It is also sometimes ground into WR flour to make products, such as rice crackers and pasta.

## **2. Literature review**

### **2.1 Cardiovascular diseases**

CVD refers to a group of disorders of the heart and blood vessels, including atherosclerotic coronary heart disease (CHD), cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis and pulmonary embolism (Genest et al., 2009; WHO, 2015). CVD is one of the major causes of illness and death in the world (Stocker, 2004). In Canada, in the past several decades, the number of deaths resulting from CHD was decreased by nearly 40% (Genest et al., 2009). Risk factors contributing to CHD can be divided into two classes: modifiable and non-modifiable risk factors. Modifiable risk factors for CHD include smoking, physical inactivity, alcohol abuse, unhealthy diet, diabetes mellitus, obesity, high blood pressure and hypercholesterolemia. Non-modifiable risk factors include age, gender and genetics (Buttar et al., 2005).

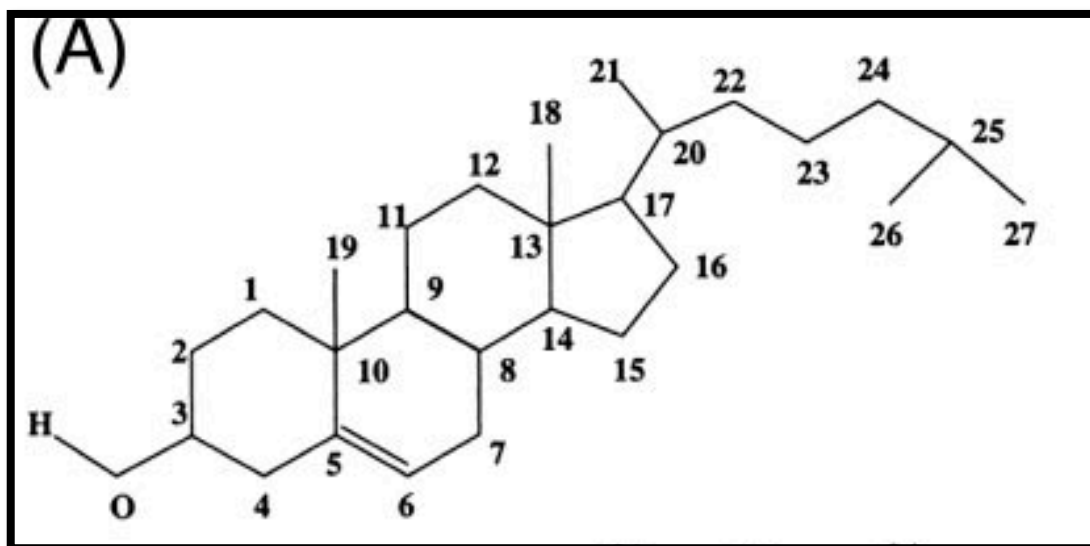
### **2.2 Atherosclerosis**

Atherosclerosis is the primary underlying pathology of CHD and the most prevalent cause of death and illness in the Western world. Atherosclerotic CHD develops when cholesterol-rich plaque builds up in the arterial walls, narrowing arteries and hindering blood flow (Soltero-Perez, 2002). Elevated circulating cholesterol concentrations were identified as a primary risk factor for atherosclerosis. A positive relationship of atherosclerosis with elevated low density lipoprotein (LDL) cholesterol levels and its inverse association with high density lipoprotein (HDL) cholesterol concentrations were detected in CHD patients ( Mascarenhas-Melo et al., 2013).



## 2.3 Cholesterol

Cholesterol is a type of sterol present in plasma or cells, with crucial functions in cellular membrane, bile acids and vitamin D metabolism. In addition, cholesterol is a precursor of sterol hormones (Bellows & Moore, 2012). It contains 27 carbon atoms, 46 hydrogen and one hydroxyl group and, also, is an unsaturated lipid with 1 double bond. **Figure 1** shows the chemical structure of cholesterol. Cholesterol in the body is derived from both endogenous and dietary sources. Approximately 30% of this cholesterol comes from diet and the other 70% is produced *in vivo*. **Table 1** summarizes the major sources of dietary cholesterol (Kapourchali et al., 2016). A high blood cholesterol level is a significant risk factor for CHD. Some genetic disorders, such as abnormalities in LDL receptors can increase blood cholesterol levels, a condition commonly known as familial hypercholesterolemia (Varbo et al., 2014). As a result, Health Canada, (2011) recommended that dietary cholesterol intake not to exceed 300 mg/day for healthy people and 200 mg/day for cardiovascular patients (Health Canada, 2011a). Cholesterol is hydrophobic, therefore, is transported in the blood by incorporation into lipoproteins. The four major lipoproteins in humans are chylomicrons, high density lipoprotein (HDL), low density lipoprotein (LDL) and very low density lipoprotein (VLDL) (Varbo et al., 2014).



**Figure 1: Cholesterol structure**

Adopted with permission from Miao et al., (2002)

**Table 1: Common sources of dietary cholesterol**

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

	Lamb, shank, lean, cooked	70 mg
	Caribou, cooked	109 mg
<b>Other</b>	Deer, roasted	112 mg
	Bison, roasted	82 mg
	Goose, meat only, roasted	96 mg

Adopted with permission from Kapourchali et al., (2016)

### **2.3.1 Chylomicrons**

Chylomicrons are triglyceride-rich lipoproteins formed after a meal in the small intestine. Therefore, people who are fasting may not have chylomicrons in their serum. The central lipid core of chylomicrons primarily consists of triglycerides and monoglycerides (85%–92%), cholesterol (1%–3%), phospholipids (6%–12%) and traces of fatty acids (Julve et al, 2016). The main function of chylomicrons is to transport triglycerides and other fats to the liver and other tissues in the body.

### **2.3.2 High-density lipoprotein**

Increasing HDL-cholesterol levels was found to be negatively associated with cardiovascular risk. Therefore, HDL is known as "good cholesterol" (Remaley, 2015). HDL is a primary extracellular acceptor, thus promoting efflux of cholesterol via membrane transporters (AbuMweis & Jones, 2008). The major role of HDL is to transport cholesterol from the peripheral tissues to the liver. This is the main reason HDL cholesterol levels are inversely correlated with the risk of atherosclerosis (American Heart Association, 2016). The normal HDL cholesterol level in healthy people is 1.0 mmol/L for men and 1.3 mmol/L for women. Low HDL levels increase the risk of CHD in adults (Health Canada, 2013a).

### **2.3.3 Low-density lipoprotein**

Low-density lipoprotein (LDL) carries cholesterol from the liver to various tissues. In the bloodstream, LDL is formed from very low density lipoproteins (VLDL) (Gitlin et al., 1958; Fernández-Higuero et al., 2014). LDL levels should be less than 3.4mmol/L (Health Canada,

2013a). Higher levels of LDL cholesterol may result in atherosclerotic plaques in the arterial walls, leading to CHD ( Chung & Vafai, 2014).

#### **2.4. Whole grains**

U.S. Food and Drug Administration (FDA) and Health Canada define whole grains (WG) as "cereal grains that consist of the intact, ground, cracked or flaked kernel, which includes the bran, the germ and the innermost part of the kernel (the endosperm)." Major sources of WG are wheat, oatmeal, corn, rice, barley, rye and buckwheat. These grains can be either whole or refined products (FDA, 2006; Health Canada, 2013b). **Table 2** summarizes the daily-recommended intake of WG. Medical studies showed that increasing daily intake of WG reduced the risk of diabetes mellitus, obesity, high blood pressure, colorectal cancer and CVD (Johnsen et al., 2015). WG kernels are divided into three parts, the bran, the germ and the endosperm. However, in refined grains, the germ and bran are removed. Thus, refined grains have fewer nutrients because the germ and the bran contain dietary fiber, vitamins, minerals and phytonutrients, while the endosperm contain most of the starch, which provides energy (Willem van der Kamp et al., 2014; Steffen et al., 2003). Therefore, it is highly recommended that whole grain products be consumed.

**Table 2: Recommended numbers of servings for grain products per day**

	Age (in years)	Grain products	
		Male	Female
<b>Children</b>	2–3	3	3
	4–8	4	4
	9–13	6	6
<b>Teens</b>	14–18	7	6
<b>Adults</b>	19–50	8	6-7
	51+	7	6

Adopted with permission from Health Canada, (2013b)

### 2.4.1. Whole grains and their phytochemicals

Phytochemicals are bioactive non-nutrient plant compounds present in fruits, vegetables, whole grains and other plant foods. The most essential groups of phytochemicals in WG are phenolic compounds including ferulic acid, syringic acid, caffeic acid, vanillic acid, p-coumaric acid, anthocyanidins, sinapic acid, flavonols, quinines and flavones ( Liu, 2007), also carotenoids, vitamin E,  $\gamma$ -oryzanols, dietary fiber and  $\beta$ -glucan (Okarter & Liu, 2010). Adequate intakes of WG are associated with a decreased risk of major chronic diseases like CVD, diabetes, obesity and cancers (Jonnalagadda et al., 2011). Of these phytochemicals, ferulic acid (FA) is found in most grains in its bound form, accounting for 70%–90% of the phenolic acid. This bound form resists digestion in the upper gastrointestinal tract and has limited bioavailability (Turner et al., 2015). Moreover, FA was reported to decrease oxidative stress (Yogeeta et al., 2006). However, food processing, such as milling, pasteurization, fermentation and thermal processing, help to release the bound phenolic acids and, hence, increase bio-accessibility of these phytochemicals (Ragaei et al., 2014).

Furthermore, WG is a good source of dietary fiber (DF). DF is the edible part of plants and is resistant to digestion and absorption in humans. Also, DF undergoes partial or whole fermentation in the large intestine (Trowell, 1976). Consumption of adequate amounts of DF is strongly recommended to reduce the risks of CVD, high blood pressure, high blood glucose, obesity and certain gastrointestinal disorders (Anderson et al., 2009). The most important components of DF are cellulose, hemicelluloses, lignin and  $\beta$ -glucan (Okarter & Liu, 2010).

In addition, WG contains vitamin E ( $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -tocopherols and tocotrienols), mostly in the germ, which is removed during the refining process (Jensen et al., 2004). Also, each particular grain contains different concentrations of vitamin E (Do et al., 2015). Vitamin E



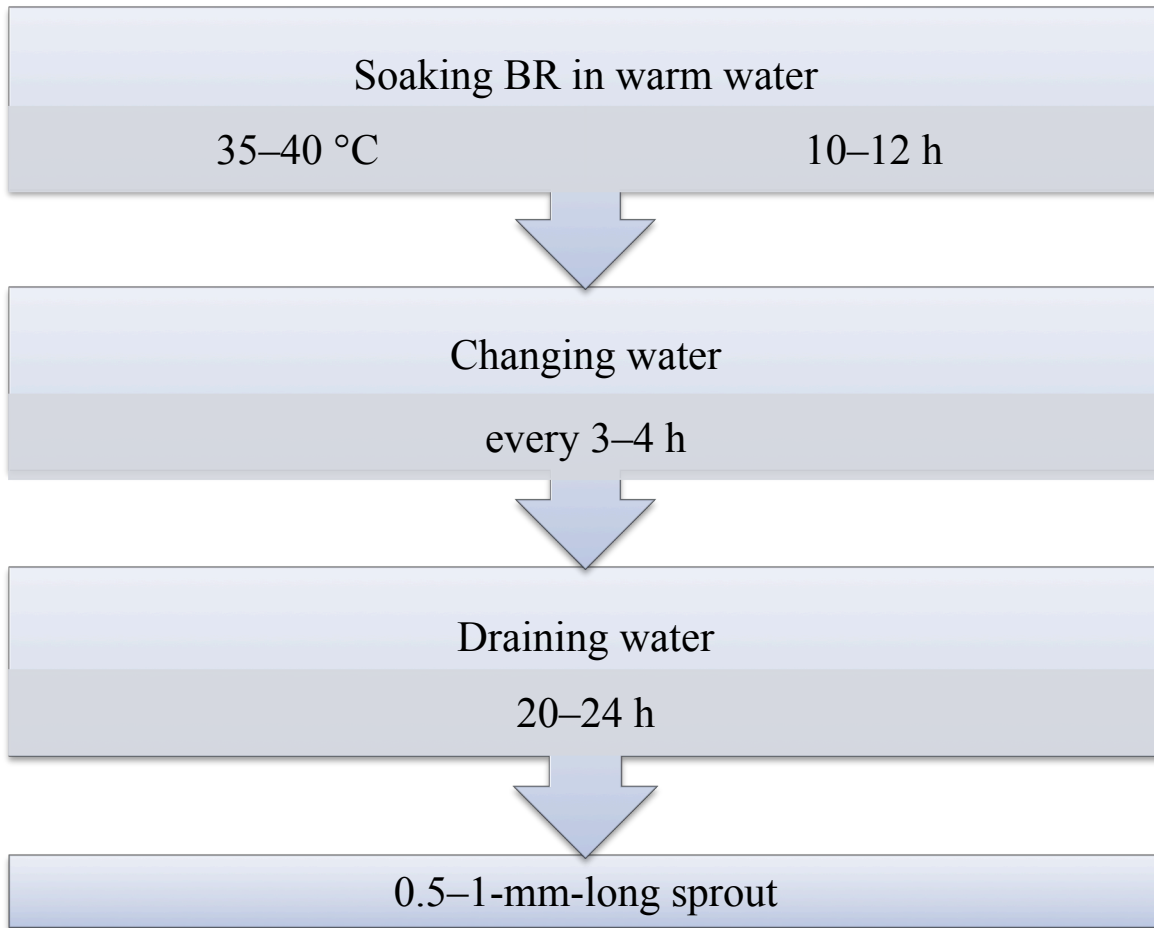
acts as a robust, fat-soluble antioxidant in the human body and has important properties, including anticancer, hypocholesterolemic, cardio-protective, neuro-protective and gastro-protective activities (Yu et al., 2016).

WG also contains phytosterols and  $\gamma$ -oryzanol, with different types and amounts depending on the type of WG (Trautwein et al., 2003). Phytosterols are also found in some vegetables and fruits, vegetable oils, seeds and nuts ( Moghadasian & Frohlich, 1999). Phytosterols decrease cholesterol absorption because their structures are similar to that of cholesterol, enabling them to compete with cholesterol for micelle formation (Calpe-Berdiel et al., 2009).  $\gamma$ -oryzanol, abundant in rice bran oil, is a ferulic acid esterified sterol (Sawadikiat & Hongsprabhas, 2014).

## **2.5. Germinated brown rice**

Germination is a biological procedure caused by stimulation of the remaining enzymes (Cho & Lim, 2016). BR germination involves soaking the rice in water to develop sprouts and enhance nutrient levels. This process also improves the texture of the BR, when cooked (Xu et al., 2012). GBR was marketed beginning in 1995 (Cornejo et al., 2015). In the 1970s, Japanese people consumed BR in abundance because of its richness in nutrients, but it was commonly prepared in a pressure cooker, giving it poor texture and little flavor. Therefore, GBR offered a solution, because it can be cooked in a normal rice cooker with improved organoleptic properties. BR can be germinated by soaking it in warm water (35–40 °C) for 10–12 hours. To prevent spoilage, the water should be changing every 3–4 hours and the temperature should be maintained. After this soaking, the rice is kept under moist conditions for 20–24 hours. At this point, a 0.5–1-mm-long sprout will grow. **Figure 2** illustrates the processing of GBR. The

moisture is important for germination of BR and there are two methods providing sufficient moisture. One is simply soaking the BR in water and the other is a temporary immersion, followed by atmospheric germination. The process of atmospheric germination can produce longer roots and sprouts because it facilitates a higher degree of germination than the simple water soaking method (Lu et al., 2010).



**Figure 2: Flow chart illustrating GBR processing**

### 2.5.1 Nutritional composition of GBR

During germination, the nutritional contents of brown rice are completely changed. GBR contains GABA, dietary fiber, inositol, ferulic acid, phytic acid, tocotrienols, magnesium, potassium, zinc,  $\gamma$ -oryzanol and prolyl endopeptidase inhibitor (Latifah et al., 2010). In addition, GBR contained more total ferulic acid (126%), total dietary fiber (145%), soluble dietary fiber (120%) and insoluble dietary fiber (150%) than BR (Ohtsubo et al., 2005), as well as fewer calories, sugar and fat than BR (Trachoo et al., 2006). **Table 3** shows that several bioactive compound concentrations in GBR were significantly higher than those in either BR or WR (Imam et al., 2013). Furthermore, GBR has higher protein content than BR, which potentially attributes to the synthesis of enzymes during germination, and the production of amino acids during protein synthesis (Uwaegbute et al., 2000).

GBR is a good source of GABA. GABA, a free amino acid widespread in nature, is the main inhibitory neurotransmitter in the mammalian central nervous system (Komatsuzaki et al., 2007). GABA is produced primarily by decarboxylation of L-glutamic acid, catalyzed by the enzyme glutamate decarboxylase (GAD) (Mayer et al., 1990). Low GABA levels are associated with anxiety, depression, hyperactivity, epilepsy, Parkinson's disease, Alzheimer's disease, Huntington's chorea, stiff man syndrome and various other motor diseases (Awad et al., 2007). Furthermore, GABA has beneficial effects on human health, including blood pressure lowering (Saikusa et al., 1994) and inhibition on cancer cell proliferation (Komatsuzaki et al., 2007).

GBR also contains potassium, magnesium and zinc. Potassium is the most important intracellular ion in the body. A lack of potassium is uncommon because it is present in most foods, including all meats, some types of fish, fruits, vegetables, legumes, dairy products and cereals. Potassium decreases heart failure and is crucial for skeletal and smooth muscle

contraction (Lanham-New et al., 2012). Moreover, magnesium is the second most abundant intracellular ions, after potassium. Half the total magnesium in the body is in the bones and the other half in soft tissue, with only 1% in the blood. A lack of magnesium is associated to obesity, diabetes mellitus, CVD, high blood pressure and cancer (Crosby et al., 2013). In addition, zinc is an essential trace metal for humans. Whole grain products are good sources of zinc, as are shellfish, red meat, beans and nuts. Zinc is involved in improving immune function and bone health. It also was reported to decrease cancer and oxidative stress (Huang et al., 2015).

Furthermore,  $\gamma$ -oryzanol is one of the most important nutritional compound in GBR (Jayadeep & Malleshi, 2011). Free ferulic acids are released during its digestion and can induce the biological activity of  $\gamma$ -oryzanol (Mancuso & Santangelo, 2014). The health benefits of  $\gamma$ -oryzanol were demonstrated. For example, it decreases high cholesterol levels by inhibiting cholesterol absorption (Lichtenstein et al., 1994). It also has antioxidant, anticancer (Hirose et al., 1994), anti-diabetic, and anti-inflammatory properties (Sohail et al., 2016).

**Table 3: Concentrations of ASG, GABA, oryzanol and phenolics in germinated brown rice (GBR), compared with in white rice (WR) and brown rice (BR)**

<b>Bioactive compound</b>	<b>GBR</b>	<b>BR</b>	<b>WR</b>
<b>ASG (mg/g)</b>	0.465 ± 0.055	Undetected	Undetected
<b>GABA (mg/g)</b>	0.36 ± 0.04	0.09 ± 0.02	Undetected
<b>Phenolics (GAE/gdw)</b>	20.5 ± 0.01	3.17 ± 1.68	0.60 ± 0.45
<b>Oryzanol (mg/100 g)</b>	30.38–64.22	13.01–22.37	Undetected

ASG = acylated sterol glycoside; GABA = gamma amino butyric acid; GAE/gdw = gallic acid equivalent/dry weight

Adopted with permission from Imam et al., (2013)

### 2.5.2 Potential health benefits of germinated brown rice

GBR and its products are available in China and Japan and have become a common food because of their flavor and nutritional advantages. Many studies reported nutritional value and health benefits of GBR. High levels of GABA,  $\gamma$ -oryzanol, dietary fiber and other antioxidant compounds in GBR reportedly have beneficial physiological effects, such as, anti-hypertension, hypolipidemic as well as decreased risk of cancer, diabetes, CVD, and Alzheimer's disease (Wu et al., 2013). Therefore, GBR is potentially an effective functional food for decreasing CVD risk by preventing LDL oxidation (Chotimarkorn et al., 2008). GBR also has antihypertensive effects. Its ability to lower high blood pressure may be due to its ferulic acid content, which is increased after germination and was reported to decrease blood pressure in an *in vivo* study (Ardiansyah et al., 2008).

As noted, GBR decreases cancer risk. Cancer chemoprevention is one way to limit cancer, by ingesting chemicals that lower the risk of carcinogenesis (Steele, 2003). GBR can potentially decrease mutations because of its production of phenolic compounds during germination (Tian et al, 2004). The bran portion of rice grains mostly contains biologically active compounds exhibiting the various chemo-preventive and antitumor properties attributed to rice (Hudson et al., 2000). Furthermore, GABA, enriched in GBR, was reported to inhibit tumor development in small airway epithelium; therefore, GBR extracts enriched in GABA suppressed leukemia cell proliferation and enhanced cancer cell apoptosis (Oh & Oh, 2004).

Furthermore, GBR contains high concentrations of dietary fiber (DF), therefore its consumption decreases blood glucose levels and the incidence of diabetic vascular complications (Seki et al., 2005). In addition to dietary fiber, GABA produced during germination of BR and

the vitamins, minerals and unknown bioactive lipids in the bran and the germ layer of GBR may also be associated with decreased blood glucose and diabetic complications (Chou et al., 2009).

GBR also decreases risk of CVD. High blood pressure and LDL cholesterol are intermediary biomarkers for assessing CVD risk and GBR has the beneficial effects of lowering both blood pressure and LDL cholesterol. As a result, GBR was implicated in CVD prevention (Kendall et al., 2010). Regular intakes of GBR have beneficial effects on increasing metabolism in the brain, preventing headaches, and decreasing Alzheimer's disease risk (Chanlat et al., 2011). **Table 4** illustrates various physiological effects of GBR, such as blood pressure regulation and decreased obesity and blood cholesterol levels *in vivo* and *in vitro* (Cho & Lim, 2016).



**Table 4: Physiological effects of GBR**

Physiological effects	Subjects	Dietary interventions	Main results	References
Anti-obesity	Obese, hypercholesterolemia-induced male Sprague-Dawley rats	AIN-76 (lard 15%, Ch 0.5%); WR (50% diet), BR (50% diet), or GBR (50% diet), substitution in AIN-76	<p>↓TG, TC, HDL-Ch, total lipids (liver)</p> <p>↑Fecal excretion of Ch, lipids?, TG</p>	(Choi et al., 2006)
Hypotensive	Spontaneously hypertensive rats	AIN-76; BR (50% diet) or GBR (50% diet), substitution in AIN-76	↓FER, SBP, TG	(Choi et al., 2006)
Antidepressant-like	Male ICR mice	AIN-93G; WR or GBR substitution for corn starch in AIN-93G	<p>↓Immobility time (forced swimming test), escape failures (learned helplessness paradigm), 5-HIAA/5-HT (frontal cortex)</p> <p>↑5-HT (frontal cortex)</p>	(Mamiya et al., 2007)
Hypo-cholesterolemic	Hepatoma-bearing male Donryu rats	AIN-93G; WR, BR or GBR substitution for corn starch in AIN-93G	<p>↓TBARS, TC, (VLDL-Ch + LDL-Ch), AI</p> <p>↑HDL-Ch, fecal steroid excretion, hepatic Ch 7a-hydroxylase activity</p>	(Miura et al., 2006)
	Hypercholesterolemic Sprague-Dawley rats	High cholesterol diet; high cholesterol + WR, BR or GBR diet	<p>↓TC, LDL-Ch</p> <p>↑ HDL-Ch</p>	(Roohinejad et al., 2009)
	Hypercholesterolemic male Sprague-Dawley rats with high-cholesterol diet	AIN-76; BR (81.1 g/100 diet), or GBR (83.2 g/100 diet) substitution for corn starch in AIN-76	<p>↓Liver, kidney, lung weights, AI TBARS levels in plasma, liver, heart, kidney, lung, plasma GOT and GPT levels</p> <p>↑Plasma HDL-Ch, fecal Ch excretion, bile acid excretion, hepatic antioxidant enzyme (SOD, CAT, GPx) activities</p>	(Lee et al., 2007)

Antidiabetic	Human subjects with type 2 diabetes	Cooked WR, GBR	↓TC, TG, FBG, fructosamine levels ↑HDL-Ch	(Hsu et al., 2008)
	Healthy human subjects	Cooked WR, BR, GBR	↓GI, IAUC-Glc120	(Ito et al., 2005)
	Male OLETF rats	AIN-93G; WR or GBR substitution for corn starch in AIN-93G	↓Blood glucose levels, HbA1c, plasma PA I-1, TNF- $\alpha$ , adipocytokine concentrations ↑Adiponectin	(Torimitsu al., 2010)
	Streptozotocin-induced diabetic male Wistar rats	AIN-93G; WR or GBR substitution for corn starch in AIN-93G	↓Blood glucose levels, plasma PAI-1, lipid peroxide concentrations	(Hagiwara et al., 2004)
Learning and memory deficits	$\beta$ -Amyloid peptide treated male ICR mice	AIN-93G; WR or GBR substitution for corn starch in AIN-93G	↓Escape latencies (water maze test) ↑Spontaneous alternation behavior (Y-maze test)	(Mamiya et al., 2004)
Anticancer	Azoxymethane-induced colon cancer in male Sprague-Dawley rats	Commercial basal diet + 2.5, 5.0, 10.0 g (rice)/kg (rat weight)	↓Aberrant crypt foci (ACF), $\beta$ -catenin expression, COX-2 expression. ↑Inhibition of colon cancer	(Latifah et al., 2010)

AIN, American Institute of Nutrition; BR, brown rice; Ch, cholesterol; GBR, germinated brown rice; WR, white rice. AI, atherogenic index ( $[\text{total cholesterol}] - [\text{HDL-cholesterol}] / [\text{HDL-cholesterol}]$ ); CAT, catalase; COX-2, cyclooxygenase 2; FBG, fasting blood glucose; FER, food efficiency ratio (body weight gain (g/week)/food intake (g/week)); GI, glycemic index; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; GPx, glutathione peroxidase; HbA1c, hemoglobin A1c; HDL-Ch, high-density lipoprotein cholesterol; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine; IAUC-Glc-120, incremental areas under the glucose curves over 120 min; MDA, malondialdehyde; OLETF, Otsuka Long Evans Tokushima fatty rat; PAI-1, type-1 plasminogen activator inhibitor; SBP, systolic blood pressure, SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; TC, total cholesterol; TG, triglyceride; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; VLDL, very-low-density lipoprotein.

Adopted with permission from Cho & Lim, (2016).

## **2.6. Low-density lipoprotein receptor knockout (LDLr-KO) mice**

Mice are small mammals, easy to handle and maintain in laboratories and their tissues are easily harvested, stored and processed for analysis (deLuna, 2008). Mice do not develop significant atherosclerosis, because they have high levels of HDL and low levels of LDL and VLDL (Daugherty, 2002). Thus, mouse models for atherosclerotic CVD rely on disruption of normal lipoprotein regulation and metabolism, through diet or genetic manipulations, enabling development of extensive and mature atherosclerotic lesions. One of the most widely used dyslipidemic models of atherosclerosis is the LDLr-KO mice (Kowala et al., 2000). The LDLr-KO mouse is a model mimicking familial hypercholesterolemia (Potteaux et al., 2007), and these mice have plasma lipoproteins profiles similar to those in humans with this disorder (Reardon & Getz, 2001). When LDLr-KO mice are fed a high fat diet, they exhibit elevated LDL and VLDL cholesterol levels, because of an absence of hepatic LDL receptors (Daugherty, 2002) that disables hepatic clearance of these circulating lipid particles (Ishibashi et al., 1993). As a result, the dyslipidemic profile of these mice is suitable for studies investigating effects of lipid profile changes on atherosclerotic lesions (Zadelaar et al., 2007).

## **2.7. Antioxidant properties of GBR**

Oxidative stress is involved in pathogenesis and progression of chronic diseases including cancer, diabetes and cardiovascular diseases. Antioxidants from natural products, especially from dietary sources, are known to decrease oxidative stress by inhibiting effects of oxidative free radicals (Mayne, 2003). Several of these antioxidant-rich dietary sources, including GBR, have been studied for their potential roles in management of oxidative stress related chronic diseases. GBR was reported to have high antioxidant capacity, hence decreasing

oxidative stress (Imam et al., 2012). Furthermore, GBR has stronger antioxidant activity than WR or BR (Rashtchizadeh et al., 2008). In addition, the beneficial effects of the numerous phenolic compounds present in GBR are attributed to their antioxidant activities. Moreover, *in vivo* and *in vitro* studies demonstrated that GBR and BR had strong protective effects against oxidative stress induced liver injury, through their antioxidant activities. Also, an *in vivo* study demonstrated that GBR consumption led to decreased oxidative stress, increased plasma antioxidant levels and increased antioxidant enzyme activities in the liver, which may prevent the formation of atherosclerotic plaques (Esa et al., 2013).

### **3. RESEARCH PLAN**

#### **3.1. Study Rationale**

Based on growing evidence, intake of WG, including Chinese germinated brown rice, has a protective effect against CVD risk factors. Such benefits of GBR and other WG are attributed to their bio-functional components. However, little is known about the benefits of GBR on CVD risk factors. Therefore, understanding the anti-atherogenic benefits of GBR might support selection of germinated WG for inclusion in healthy diets and as functional food ingredients in new products. This study was, therefore, performed to examine the effectiveness of Chinese GBR for decreasing cardiovascular risk factors in LDLr-KO mice.

## **4. STUDY HYPOTHESIS AND OBJECTIVES:**

### **4.1. Hypothesis**

Long-term consumption of Chinese GBR will reduce atherogenic risk factors in LDLr-KO mice.

### **4.2. Objectives**

The main goal of this study was to investigate effects of Chinese GBR on cardiovascular risk factors in LDLr-KO mice.

This study was designed to:

1. Investigate the effects of the Chinese GBR on plasma total cholesterol (TC), triglyceride (TG), HDL, LDL and VLDL cholesterol levels.
2. Determine the activities of antioxidant enzymes, glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) in plasma from LDLr-KO mice
3. Determine the effects of Chinese GBR on fecal lipid excretion.
4. Determine the effects of Chinese GBR on atherosclerotic lesion development in aortic roots of LDLr-KO mice.

## 5. MATERIALS AND METHODS

### 5.1. Animals and Diets:

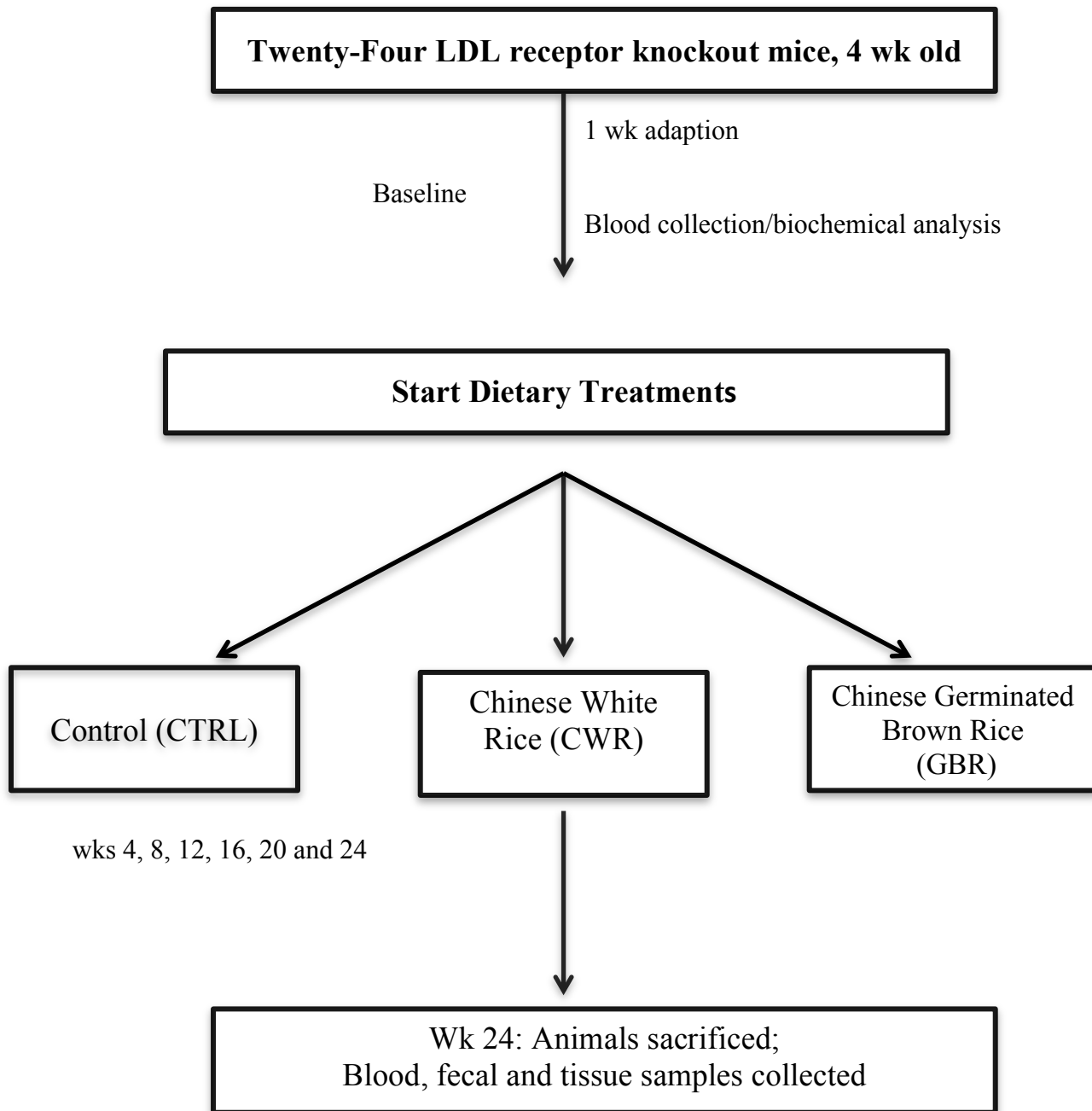
The experimental design for this study is summarized in **Figure 3**. Twenty-four male LDLr-KO mice, 4 weeks old were obtained from Jackson's Laboratory (Bar Harbor, ME, USA). The mice were housed at room temperature (22-24 °C) with a 12:12-h dark-light cycle. LDLr-KO mice are a well-established mouse model for studying LDL oxidation and atherosclerosis (Daugherty, 2002; deLuna, 2008; Reardon & Getz, 2001). After one-week adaption, the mice were randomly divided into three groups (n=8 each group).

**Group I:** control group, fed a chow diet. **Group II:** fed the chow diet supplemented with 60% (w/w) Chinese WR. **Group III:** fed the chow diet supplemented with 60% (w/w) Chinese GBR. All diets were also supplemented with 0.06% (w/w) cholesterol to accelerate atherosclerosis. Diet treatments are summarized in **Table 5**. The diets were based on an AIN-93G purified rodent diet.

Chinese WR and GBR were provided by Yuanwang Fuqi Agricultural Products Inc. Nanjing, China. The study period was 24 weeks and, during the study, diets were stored in a cold room (0-4 °C).

### 5.2. Ethics

This study was approved by the Animal Protocol and Care Committee at the University of Manitoba.



**Figure 3: Experimental design of this study**

**Table 5: Summary of the experimental groups and their diets**

<b>Experimental Groups</b>	<b>Diet Treatments</b>
<b>Control (n=8)</b>	Control diet + 0.06 (w/w) dietary cholesterol
<b>Chinese white rice (n=8)</b>	Control diet + 0.06 (w/w) dietary cholesterol + 60% (w/w) CWR
<b>Chinese germinated brown rice (n=8)</b>	Control diet + 0.06 (w/w) dietary cholesterol + 60% (w/w) GBR



## **6. Data collection**

### **6.1. Food intake and body weight**

Body weights of the animals were measured weekly. The 24-hour food intake was estimated every 4 weeks. In each cage, food given and that remaining were weighed to calculate food consumption.

### **6.2. Blood sampling and tissue collection**

At baseline, fasting blood samples were drawn. Subsequently, blood samples were collected every 4 weeks until week 24, when mice were sacrificed. All mice were fasted for 4 hours prior to blood collection. Blood was drawn through the jugular veins of lightly anesthetized animals, using heparinized syringes, by a certified animal technician at the R. O. Burrell Lab, St Boniface Albrechtsen Research Centre. After collection, blood samples were centrifuged at 4 °C (5000 rpm for 15 minutes) to separate plasma. Plasma aliquots were stored at -80 °C until analyses. At week 24, mice were euthanized using carbon dioxide gas and final blood samples were collected via cardiac puncture.

The hearts, livers, kidneys, and abdominal fat tissue samples were collected, weighed and stored at -80 °C until analysis. For future analysis, portions of each tissue were fixed in 10% buffered formalin and sectioned.

### **6.3. Total cholesterol (TC)**

A standard enzymatic kit (Genzyme Diagnostic Chemicals, Ltd., Charlottetown, PE, Canada) was used to measure plasma total cholesterol levels, following manufacturer's instructions. The principle of the enzymatic assay is the simultaneous production of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) with cholest-4-en-3-one, as a result of hydrolysis and oxidation of cholesterol

esters. In the presence of peroxidase, hydrogen peroxide combines with hydroxybenzoic acid and 4-aminoantipyrine to yield a chromogenic product. Briefly, 3  $\mu\text{L}$  of each standards and plasma samples were added to 250  $\mu\text{L}$  of TC reagent and tubes incubated at room temperature for 20 minutes. Next, absorbance at 500 nm was measured. All experimental samples were analyzed in duplicate.

#### **6.4. Triglycerides (TG)**

In this assay, plasma TG are hydrolyzed, phosphorylated and oxidized to produce  $\text{H}_2\text{O}_2$ .  $\text{H}_2\text{O}_2$  is then reacted with p-chlorophenol and 4-aminoantipyrine to produce a red quinoneimine complex, with color intensity proportional to TG concentration in the sample. A standard enzymatic kit (Genzyme Diagnostic Chemicals Ltd.) was used to assess plasma TG concentrations. The manufacturer provided a standard solution (200 mg/dL), used to generate a linear response curve. Aliquots of 5  $\mu\text{L}$  plasma samples and standards plus 300  $\mu\text{L}$  kit reagent were added to each well. After incubation at room temperature for 10 minutes, absorbance values were determined at 540 nm. All experimental samples were analyzed in duplicate and averaged.

#### **6.5. HDL-cholesterol**

A standard enzymatic kit (HDL Ultral Sekisui Diagnostics, LLC) was used to quantify plasma HDL-cholesterol (HDL-C) levels, following the manufacturer's instructions. First, to prepare the standards, the calibrator provided in the kit was equilibrated at room temperature before reconstitution. After it reached room temperature (~ 1 hour), distilled or deionized water was added and the mixture incubated for 20 minutes. Next, it was swirled gently to dissolve

completely and avoid foaming. A 200  $\mu\text{L}$  aliquot of this standard solution was placed into each 1.5 ml centrifuge tube and these were stored at  $-80\text{ }^{\circ}\text{C}$  for up to 4 weeks. To measure HDL-cholesterol, 1.5  $\mu\text{L}$  samples, standards or controls were pipetted into wells of a 96-well plate. Then, 150  $\mu\text{L}$  Reagent 1 was added, and the plate incubated at  $37\text{ }^{\circ}\text{C}$  for 5 minutes in a heating block (IsoTemp 125D). After incubation, 50  $\mu\text{L}$  Reagent 2 was added and the plate incubated again at  $37\text{ }^{\circ}\text{C}$  for 5 minutes. Absorbance values were then read at 630 nm every 10 minutes for 40 minutes, using a BioTek reader. All experimental samples were analyzed in duplicate and averaged.

## **6.6. LDL-cholesterol**

A standard enzymatic kit (LDL Ultral Sekisui Diagnostics, LLC) was used to quantify plasma LDL-cholesterol (LDL-C), following the manufacturer's instructions. To prepare standards, the calibrator provided in the kit was equilibrated at room temperature before reconstitution. After it reached room temperature ( $\sim 1$  hour), distilled or deionized water was added and the mixture incubated for 5 minutes. Then, the contents were fully dissolved by gentle swirling to avoid foaming. Aliquots of 200  $\mu\text{L}$  were placed in 1.5 ml centrifuge tubes and stored at  $-80\text{ }^{\circ}\text{C}$ , unless used that day. To measure LDL-cholesterol, 1.5  $\mu\text{L}$  samples, standards or controls were added in duplicate to a 96-well plate. Then, 150  $\mu\text{L}$  Reagent 1 was added to each well and the plate incubated at  $37\text{ }^{\circ}\text{C}$  for 5 minutes in the incubator. Next, 50  $\mu\text{L}$  Reagent 2 was added to each well and the plate incubated again at  $37\text{ }^{\circ}\text{C}$  for 5 minutes. Absorbance values were then read at 546 nm every 10 minutes for 40 minutes.

## **6.7. VLDL-cholesterol**

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

## **6.8. Glutathione peroxide (GPx) assay**

GPx activities in plasma samples were analyzed using a GPx assay kit (Item no. 703102, Cayman Chemical Company, Ann Arbor, MI, U.S.A.) following the manufacturer's instructions. Production of oxidized glutathione by GPx was accompanied by decreased hydroperoxide, and glutathione was then recycled to its reduced state by glutathione reductase (GR) and nicotinamide adenine dinucleotide phosphate (NADPH). NADPH oxidation was then monitored spectrophotometrically. Aliquots of 120  $\mu$ L assay buffer and 50  $\mu$ L co-substrate mixture were loaded into 20  $\mu$ L of three wells in the micro-plate reader. In addition, 20  $\mu$ L diluted GPx, 100  $\mu$ L assay buffer and 50  $\mu$ L co-substrate mixture (control) were added to other three wells. The reaction was initiated by adding cumene hydroperoxide to all wells. After that, 100  $\mu$ L assay buffer, 50  $\mu$ L co-substrate mixture and 20  $\mu$ L samples were loaded into all wells. The plate was covered and incubated for a few seconds and absorbance was measured at 340 nm.

### **6.9. Catalase (CAT) assay**

CAT activities in plasma samples were analyzed using a catalase assay kit, (Item no. 707002, Cayman Chemical Company), following the manufacturer's instructions. The process is based on the reaction of the enzyme with methanol in the presence of H<sub>2</sub>O<sub>2</sub>. The purple color upon oxidation results from formaldehyde produced, forming a bicyclic heterocycle with 4-amino-3-hydrazino-5-mercapto-1, 2, 4-triazole (Johansson & Borg, 1988). Aliquots of 30 μL methanol and 100 μL assay buffer were added to 20 μL aliquots of standards and samples in a 96-well plate. The reaction was initiated by adding 20 μL diluted hydrogen peroxide to all wells. Then, the plate was covered and incubated on a shaker for 20 minutes at room temperature. After that, 30 μL potassium hydroxide was added to each well to terminate the reaction, followed by 30 μL of the chromogen. The plate was covered and incubated for another 10 minutes. Next, 10 μL potassium periodate was added to each well and plates incubated for 5 minutes. Absorbance was then measured at 540 nm.

### **6.10. Superoxide dismutase (SOD) assay**

SOD activities in plasma samples were analyzed using an SOD assay kit (Item no. 706002, Cayman Chemical Company), following the manufacturer's instructions. This assay utilizes a tetrazolium salt to detect superoxide radicals generated by xanthine oxidase and hypoxanthine. Diluted radical detector (200 μL) was added to 10 μL aliquots of standards and samples in a 96-well plate reader. Then, 20 μL diluted xanthine oxidase was added. The plate was covered and incubated on a shaker for 20 minutes at room temperature. Absorbance was then measured at 440–460 nm.

## 6.11. Fecal lipid extraction

Lipid extraction from fecal samples was performed as described previously (Folch et al., 1957) with slight modification (Surendiran et al., 2013). About 0.5 g fecal samples were weighed and immersed in 5 mL 0.025% calcium chloride (CaCl<sub>2</sub>) overnight. Samples were then vortexed, using a mini vortex (VWR, Mississauga, ON, Canada), for 3 minutes to homogenize the softened fecal samples. To extract the lipids, 25 mL chloroform: methanol (2:1, v/v) was added and samples vortexed for 1 minute. The samples were then centrifuged at 2000 rpm for 10 minutes at 4 °C in a Centrifuge 5804 R (Eppendorf Canada, Mississauga, ON, Canada) to separate the extracted lipid fraction from the sample matrix. The lipid extracts were collected and dried at 55 °C on a heating block (IsoTemp. 125D, Fisher Scientific, Pittsburgh, PA, USA) using an evaporating unit (Model 18780 *REACTI-VAP* evaporating unit, Pierce, Rockford, IL, USA) and nitrogen gas (N<sub>2</sub>). Dried lipids were stored at -20 °C until analysis. The lipids were analyzed for TC, TG (The procedures of TC and TG determinations were similar with that of plasma TC/TG assays as described above) and sterol contents. Gas chromatography was used to identify specific sterol components.

### 6.11.1 Derivatization of fecal sterols

Fecal lipid extract samples were reconstituted in 1 mL chloroform: methanol (2:1, v/v). Then 100 µL of each reconstituted sample was dried at 55 °C on a heating block using an evaporating unit and N<sub>2</sub>, followed by addition of 200 µL 1,1,1,3,3,3-hexamethyldisilane (HMDS), trimethylchlorosilane (TMCS) and pyridine, chlorotrimethylsilane, hexamethyldisilazane, sylon™ HTP, trimethylchlorosilane, trimethylsilyl chloride (HMDS:TMCS:Pyridine, 3:1:9 (Sylon™ HTP)). The mixture was dried and then reconstituted in

300  $\mu$ L hexane, vortexed and left to sit for 15 minutes before centrifuging at 3000 rpm for 1 min at 4 °C. The supernatant was transferred to the newly labeled GC vials. Fecal sterol GC analysis was performed using a previously described method (Wu et al., 2009).

### **6.12. Histological examination**

At sacrifice, specimens of the basal part of each heart, containing the aortic root, were collected and fixed in 10% buffered formalin. The fixed aorta and liver tissue samples were dehydrated with graded alcohol concentrations, 70%, 80%, 95% and 100% (each for 1 hour). After drying, samples were washed twice for 1 hour with 100% xylene to remove the alcohol and were allowed to air dry. The dried tissue samples were placed in TRUFLOW™ tissue cassettes (Fisher Scientific, Ottawa, ON, Canada) and embedded by submerging in 100% pre-melted paraffin wax for 3 hours at 70 °C using a Barnstead electro-thermal paraffin section mounting bath (Thermo Scientific, Conroe, TX, USA). The samples were then removed from the cassettes and molded into tissue blocks using metal mold and fresh pre-melted paraffin wax, then placed on ice to harden the tissue blocks. To visualize tissues in detail, at the single cell level, tissue blocks were sectioned at 5  $\mu$ m with a Shandon Finesse 325 microtome (Thermo Scientific Inc, Philadelphia, PA, USA). Selected sections were placed in a water bath (60 °C) and floated onto microscope slides. For each mouse, six sections were cut from different levels of the aortic sinus and sections were also prepared from the liver tissue. The slides were air dried overnight to allow binding of the paraffin wax to slides and ensure good staining. To stain the sections, dried slides were incubated at 65 °C on a heating block for 20 minute to melt the paraffin, followed by incubation in 100% xylene for 10 minutes to remove paraffin, then in 95% and 100% alcohol (each for 10 minutes) to remove xylene. Thereafter, slides were washed about 5 times with water and stained with hematoxylin (a nuclear stain) for 10 minutes, washed with water, and then

incubated in 2% sodium bicarbonate ( $\text{NaHCO}_3$ ) and washed with water again. The slides were then stained with alcoholic eosin for 30 seconds and washed with water, then submerged in 100% alcohol, 95% alcohol and 100% xylene (each for 5 minutes). Finally, they were mounted with coverslips, using Permount and dried overnight. The six sections, spanning the whole aortic root length, were used for morphological and morphometrical analysis of atherosclerotic lesions (Moghadasian et al., 1999), using light microscopy and an Image ProPlus digitizing system, respectively. Atherosclerotic lesions were identified and quantified using the imaging software. The lesion area can be determined at specific distances from the aortic sinus at 40–50- $\mu\text{m}$  intervals, depending on the thickness of each section. A plot of these area measurements versus distance gives a profile of the atherosclerotic lesion. The area under the curve represents an estimate of the total atherosclerotic lesions.

### **6.13. Monocyte adhesion to mouse aorta**

Monocyte adhesion was assessed as previously described (Zhao et al., 2014). Perivascular adipose tissue was isolated from each aorta and submerged in ice-cold Hank's balance salt solution (HBSS). Within 1 hour after harvesting, each aorta was opened lengthwise with fine ophthalmological scissors and fixed, with 27-gauge needles, on the bottom of a 35-mm culture dish containing 2 mL ice-cold HBSS. Free dye was removed from cells by centrifugation. Fluorescently labeled monocytes were added to each plate containing an aortic strip. Then, aortic strips with mouse monocytes were incubated on a rotating mixer at room temperature for 30 minutes. Non-adhered monocytes were removed with two washes of HBSS. Adhered monocytes on the strips were fixed in HBSS with 2% glutaraldehyde. Monocytes adhered to the intima of each aorta were counted under a fluorescence microscope, using a 10 $\times$  objective. From each



aortic strip, monocytes in at least five fields were counted and these counts were averaged (Zhao et al., 2014).

#### **6.14. Statistical Analysis**

Data were analyzed using one-way analysis of variance (ANOVA) with the SAS program (SAS Institute, JMP Statistical discovery, 2016). Means from different groups were compared using Tukey-Kramer multiple comparisons test and were considered significantly different at  $P < 0.05$ . Results were expressed as means  $\pm$  standard error (SE).

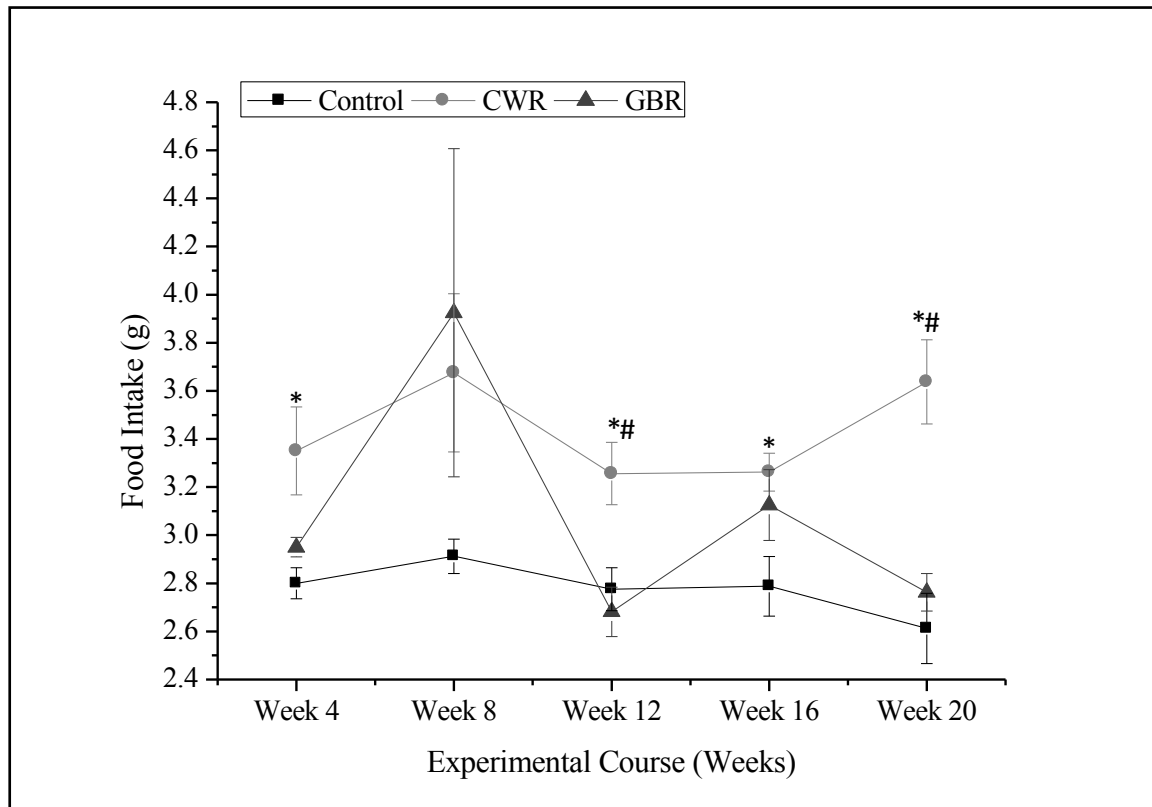
### **7. Results**

#### **7.1. Food intake, body weights and tissue weights**

Mean food intake is summarized in **Figure 4**. The mice fed with GBR had higher food intakes at weeks 4, 12 and 20 as compared to mice in the control group.

The mean body weight ranges were comparable among all groups, as shown in **Figure 5**. The final body weights of mice were not significantly different among groups ( $P = 0.3733$ ).

At end of the study, all tissues, including liver, kidneys, heart and abdominal fat, were weighed. **Table 6** shows these organ weights. Similar to the body weights, these values were not significantly different among the groups.



**Figure 4: Food intakes of LDLr-KO mice after 20 weeks of experimental diet treatments.**

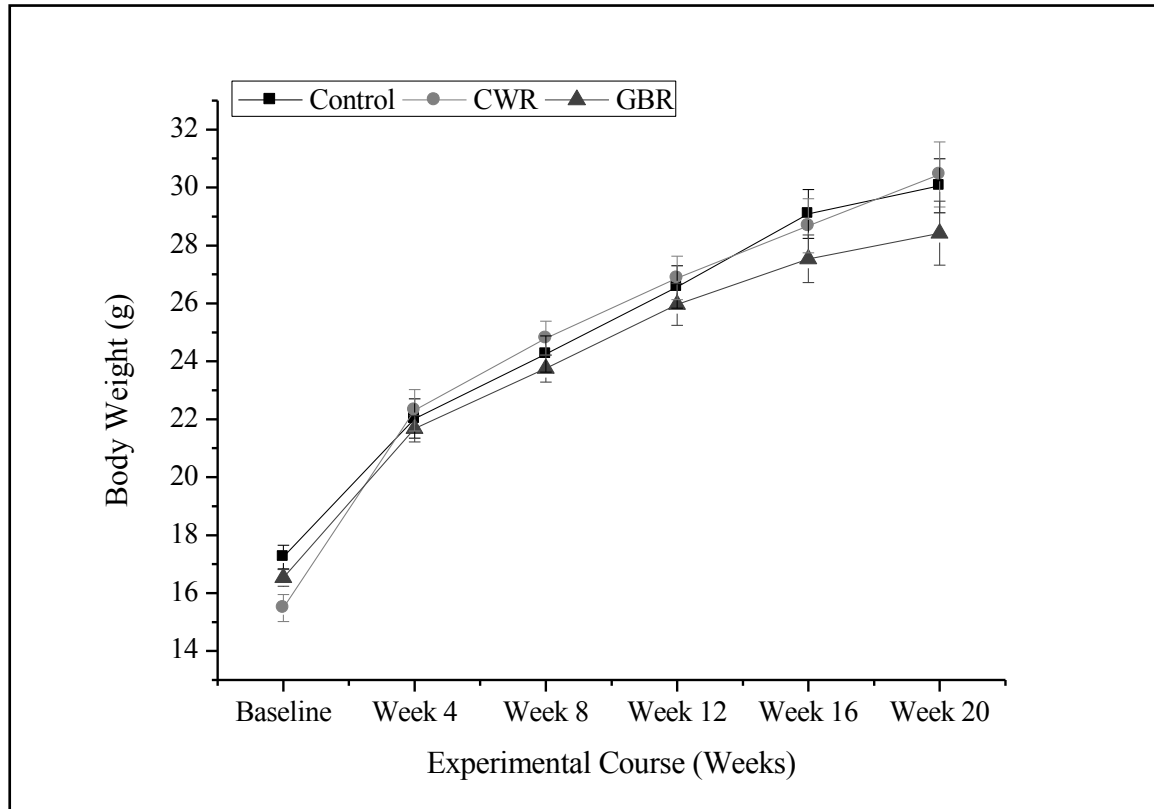
**The mice fed with GBR had higher food intakes at weeks 4, 12 and 20 as compared to mice in the control group.**

**Control; CWR = Chinese white rice; GBR = germinated brown rice**

**Data are means  $\pm$  SE.**

**$P < 0.05$  was used to determine significant differences in means among groups.**

**\*  $P < 0.05$ ; #  $P < 0.001$**



**Figure 5: Mean body weights of LDLr-KO mice after 20 weeks of experimental diet treatments. All groups showed consistent weight gains.**

**Control; CWR = Chinese white rice; GBR = germinated brown rice**

**Data are means  $\pm$  SE.**

**$P < 0.05$  was used to determine significant differences in means among groups.**

**Table 6: Organ weights (g) of LDLr-KO mice after 20 weeks of experimental diet treatments.**

<b>Organ</b>	<b>Control</b>	<b>CWR</b>	<b>GBR</b>	<b>P Value</b>
<b>Liver (g)</b>	1.53±0.07	1.52±0.12	1.41±0.07	0.4600
<b>Kidney (g)</b>	0.40±0.016	0.41±0.014	0.38±0.013	0.1866
<b>Heart (g)</b>	0.17±0.008	0.17±0.010	0.15±0.006	0.0660
<b>Abdominal Fat (g)</b>	0.80±0.12	0.89±0.14	0.70±0.08	0.8478

**Data are means ± SE.**

**$P < 0.05$  was used to determine significant differences in means among groups.**

## **7.2. Total cholesterol concentrations**

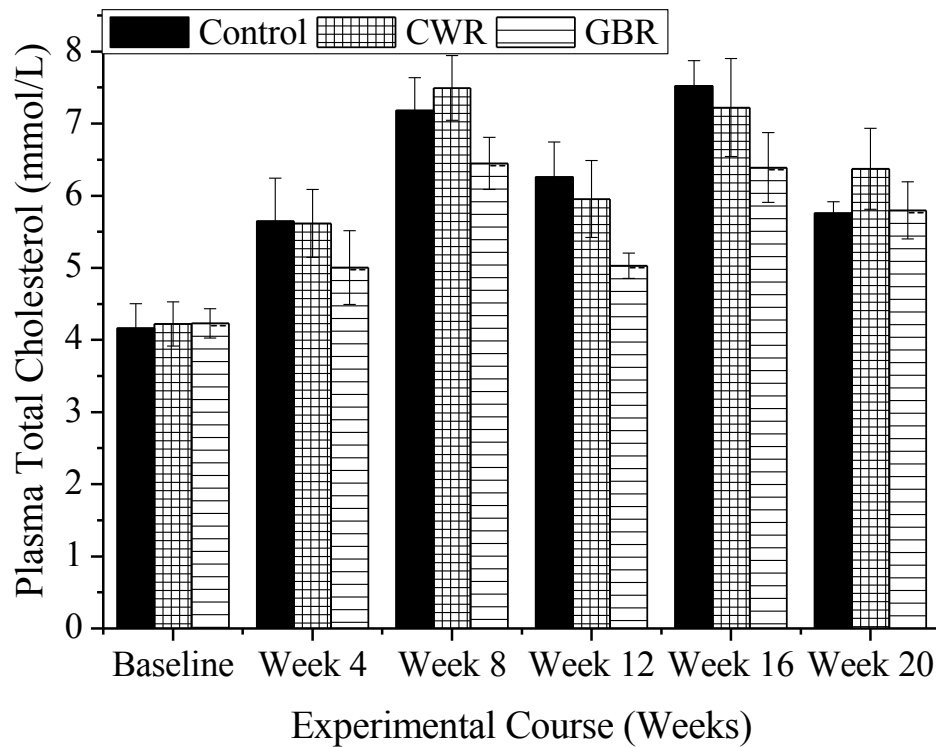
**Figure 6** shows the TC concentrations at baseline and at 4, 8, 12, 16 and 20 wk. Total cholesterol concentrations were comparable among all groups, throughout the study.

## **7.3. Total triglyceride concentrations**

**Figure 7** shows TG levels at baseline and during the experimental course. Plasma TG concentrations were comparable among all groups, throughout the study.

## **7.4. Plasma lipoprotein profiles**

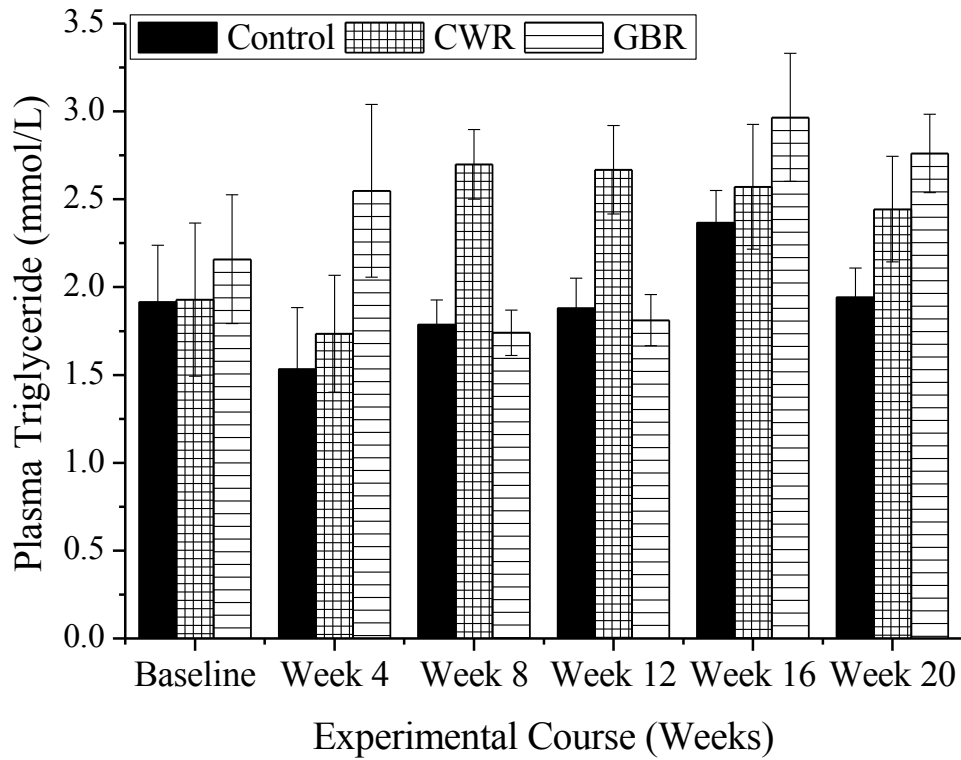
Mean HDL-, LDL- and VLDL-cholesterol concentrations at wk 20 are shown in **Figure 8**. Overall, there were no significant differences among the groups.



**Figure 6: Plasma total cholesterol concentrations.**

**Data are means  $\pm$  SE.**

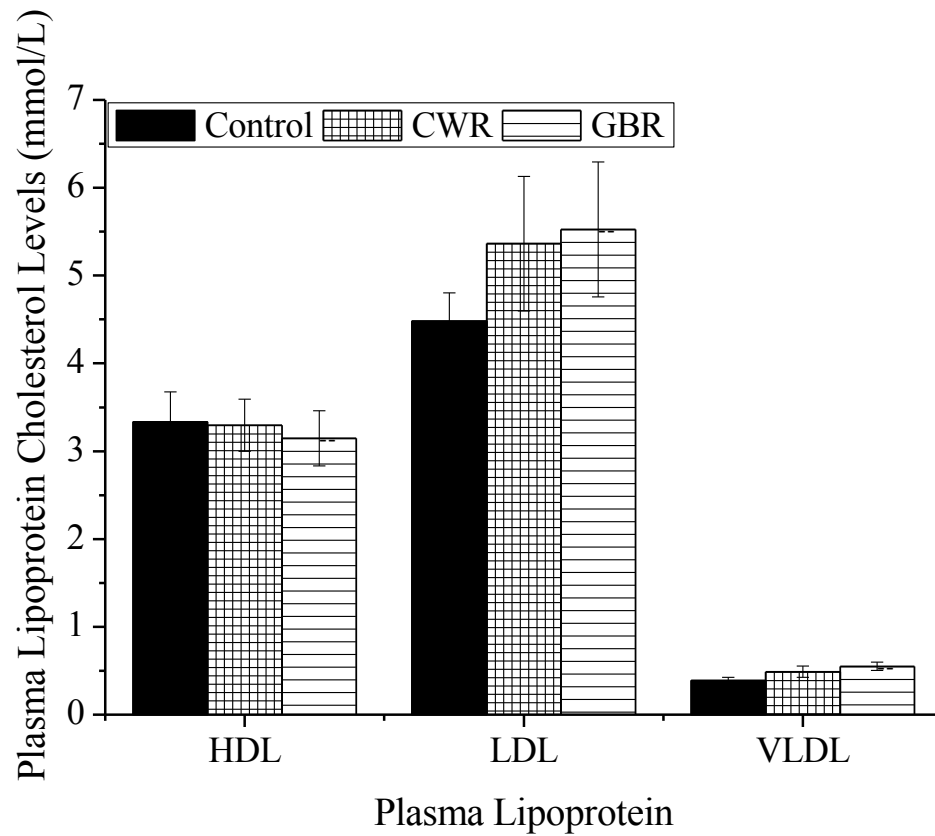
**$P < 0.05$  was used to determine significant differences in means among groups.**



**Figure 7: Plasma total triglyceride concentrations.**

**Data are means  $\pm$  SE.**

**$P < 0.05$  was used to determine significant differences in means among groups.**



**Figure 8: Plasma lipoprotein profiles.**

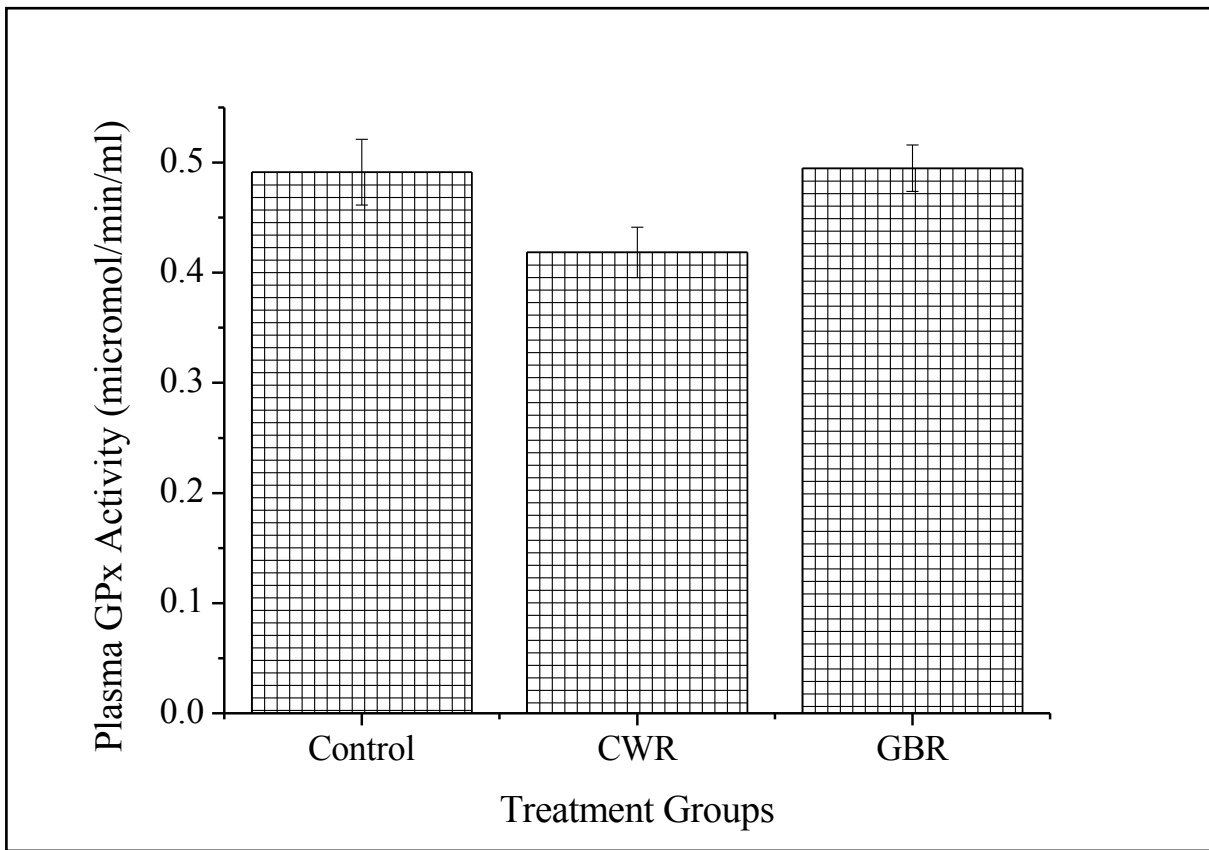
**Data are means  $\pm$  SE.**

**$P < 0.05$  was used to determine significant differences in means among groups.**



### **7.5. Antioxidant enzyme activities**

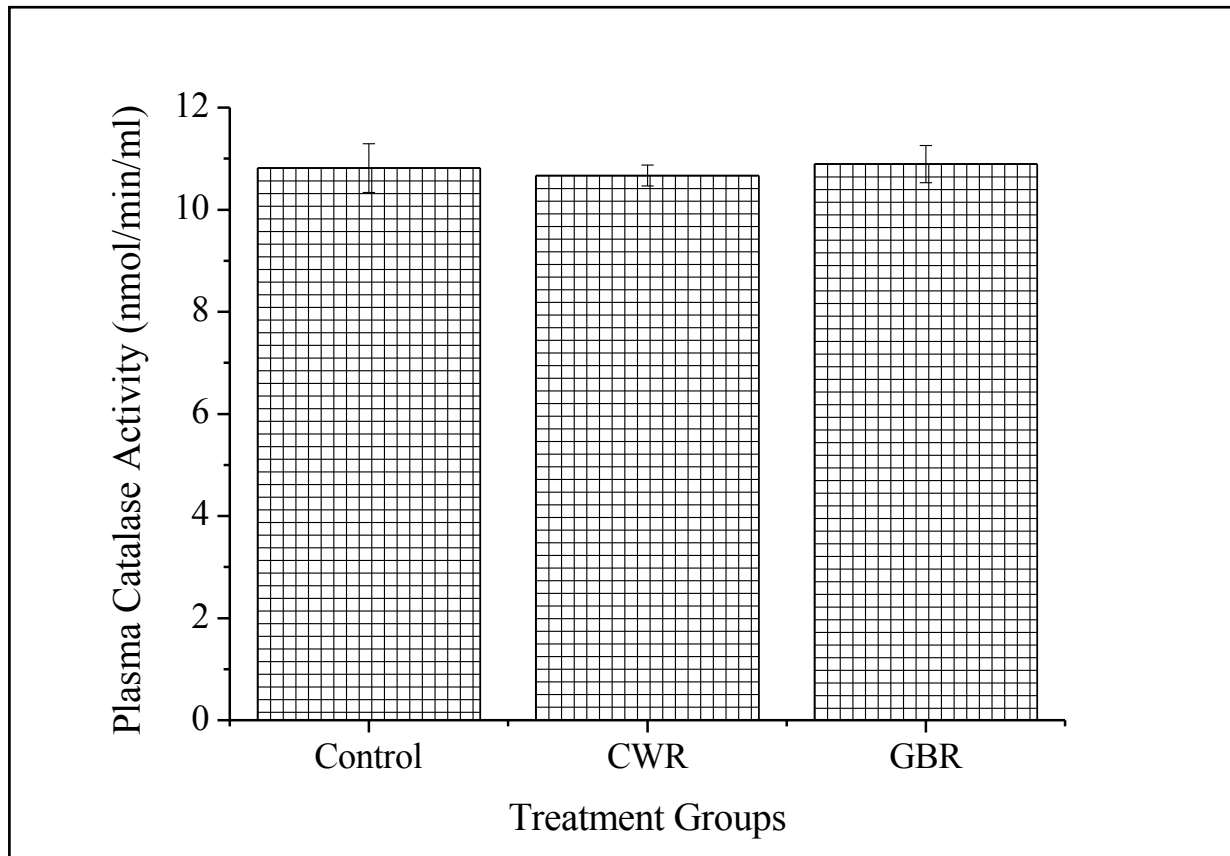
GPx, CAT and SOD activities were comparable among all groups. GPx activities are shown in **Figure 9**, CAT in **Figure 10**, and SOD in **Figure 11**. There were no statistically significant differences in antioxidant enzyme activities among the groups.



**Figure 9: Glutathione peroxide activities.**

**Data are means  $\pm$  SE.**

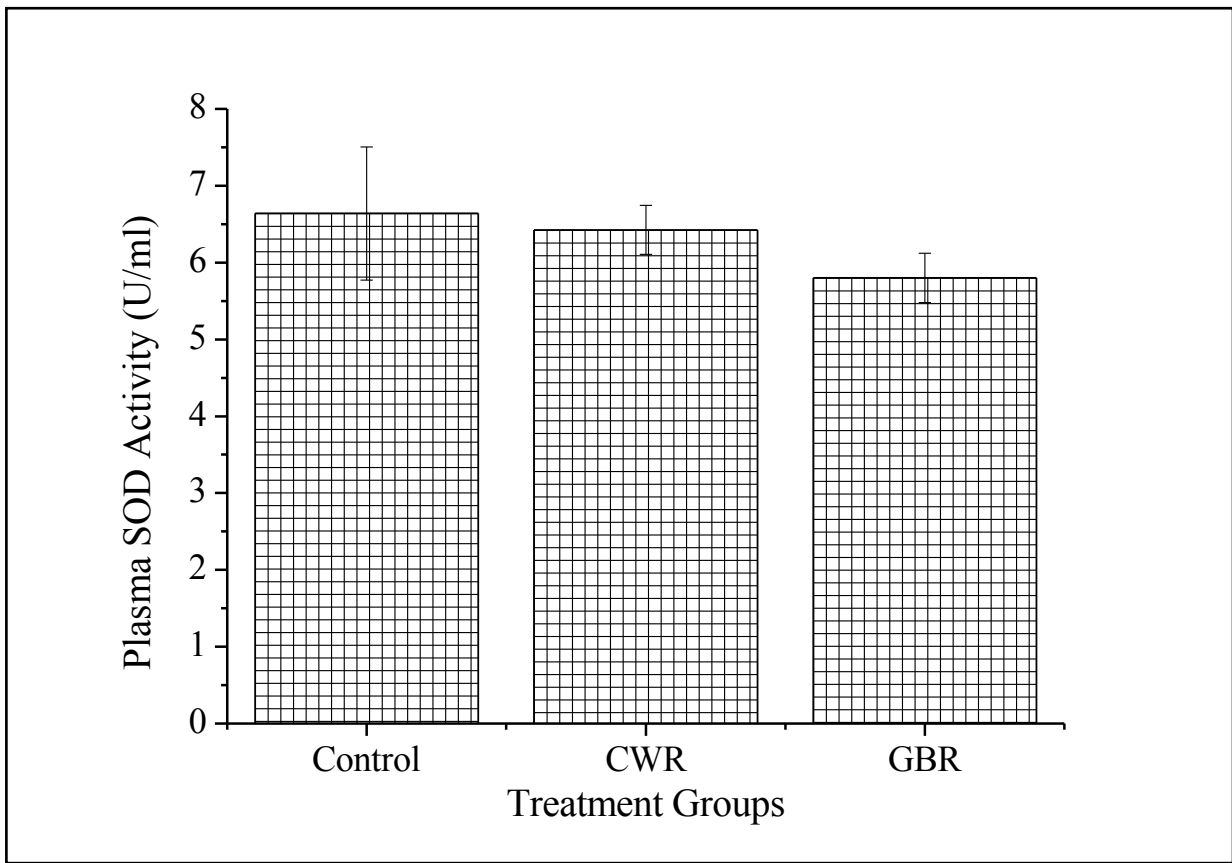
***P* < 0.05 was used to determine significant differences in means among groups.**



**Figure 10: Catalase activities.**

**Data are means  $\pm$  SE.**

**$P < 0.05$  was used to determine significant differences in means among groups.**



**Figure 11: Superoxide dismutase activities.**

**Data are means  $\pm$  SE.**

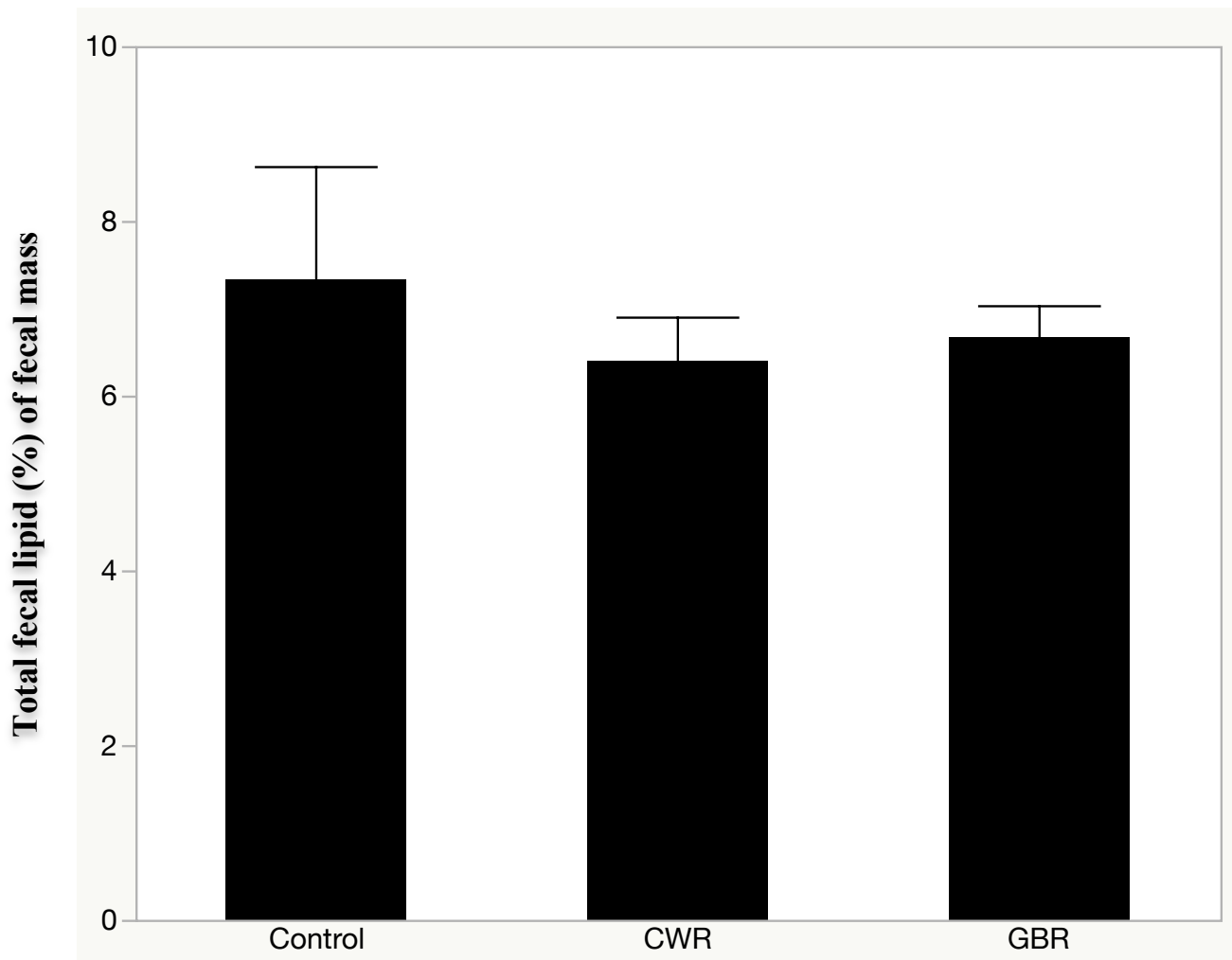
**$P < 0.05$  was used to determine significant differences in means among groups.**

## **7.6. Total fecal lipid concentrations**

Fecal samples were collected for lipid extractions and fecal lipid percentages of fecal mass were calculated. As shown in **Figure 12**, there were no statistically differences among the groups.

### **7.6.1. Fecal sterols**

**Table 7** summarizes the results of fecal sterol analyses, performed at the end of study.



**Figure 12: Total fecal lipid concentrations for LDLr-KO mice after 20 weeks of experimental diet treatments.**

**Data are means  $\pm$  SE.**

**$P < 0.05$  was used to determine significant differences in means among groups.**

**Table 7: Amounts of individual fecal sterols (mg/g feces) from LDLr-KO mice after 20 weeks of experimental diet treatments.**

<b>Groups</b>	<b>Control</b>	<b>CWR</b>	<b>GBR</b>
<b>5<math>\alpha</math>-Cholestane</b>	35.4 $\pm$ 5.1	33.6 $\pm$ 5.2	29.3 $\pm$ 3.5
<b>Cholesterol</b>	28.8 $\pm$ 5.1	33.6 $\pm$ 4.2	30.0 $\pm$ 2.9
<b>Demosterol</b>	8.1 $\pm$ 2.0	6.6 $\pm$ 0.5	8.0 $\pm$ 0.2
<b>Latosterol</b>	5.5 $\pm$ 1.4	4.4 $\pm$ 0.2	4.3 $\pm$ 0.4
<b>Campesterol</b>	34.1 $\pm$ 5.5	29.1 $\pm$ 1.2	34.1 $\pm$ 1.7
<b>Stigmasterol</b>	16.4 $\pm$ 3.3 <sup>a</sup>	6.0 $\pm$ 2.5 <sup>b</sup>	9.3 $\pm$ 1.8 <sup>ab</sup>
<b><math>\beta</math>-Sitosterol</b>	18.1 $\pm$ 3.9	14.5 $\pm$ 1.1	18.6 $\pm$ 1.6

Values are means  $\pm$  SE ( $n=8$ ).

Letters (a, b) within a row indicate significant differences at  $P < 0.05$ , determined by the Tukey–Kramer multiple range test ( $a > b$ ).

### 7.7. Atherosclerotic lesion size

**Figure 13** shows atherosclerotic lesion sizes in the aortic roots. GBR consumption significantly decreased atherosclerotic lesion development in the aortic roots, as compared with that in the CWR group ( $P = 0.0018$ ). On the other hand, CWR significantly increased atherosclerotic lesion development in the aortic roots as compared to the control group ( $P = 0.02$ ).

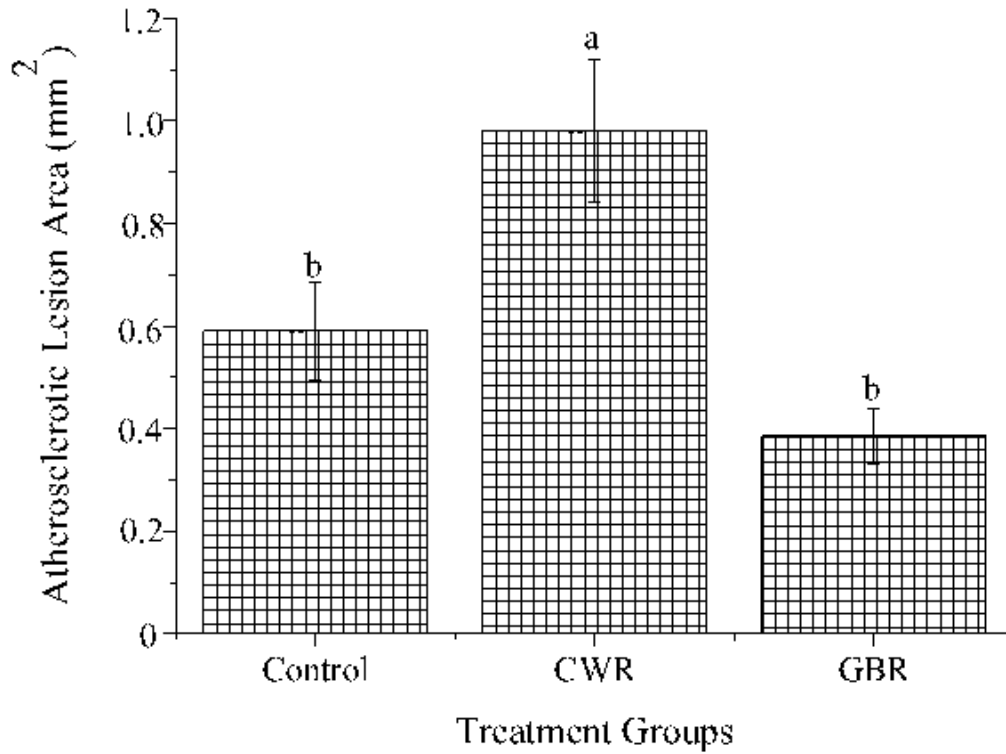
Representative photomicrographs of atherosclerotic lesions in the aortic roots of treated and control mice are shown in **Figure 14**.

Advanced atherosclerotic lesions in the aortic root of a control mouse are shown in **Figure 14A**. Also, a corresponding section from a CWR treated animal is shown in **Figure 14B**. In contrast to A and B, a section from a GBR treated mouse shows only a limited lesion formation in the aortic root **Figure 14 C**.

### 7.8 Monocyte adhesion to aortic intima

The number of monocytes adhered to the surface of aorta from GBR treated LDLr-KO mice were significantly lower than those from CWR mice as shown in **Figure 15** ( $P=0.0001$ ). This finding is in agreement with atherosclerotic lesion size among the experimental groups.

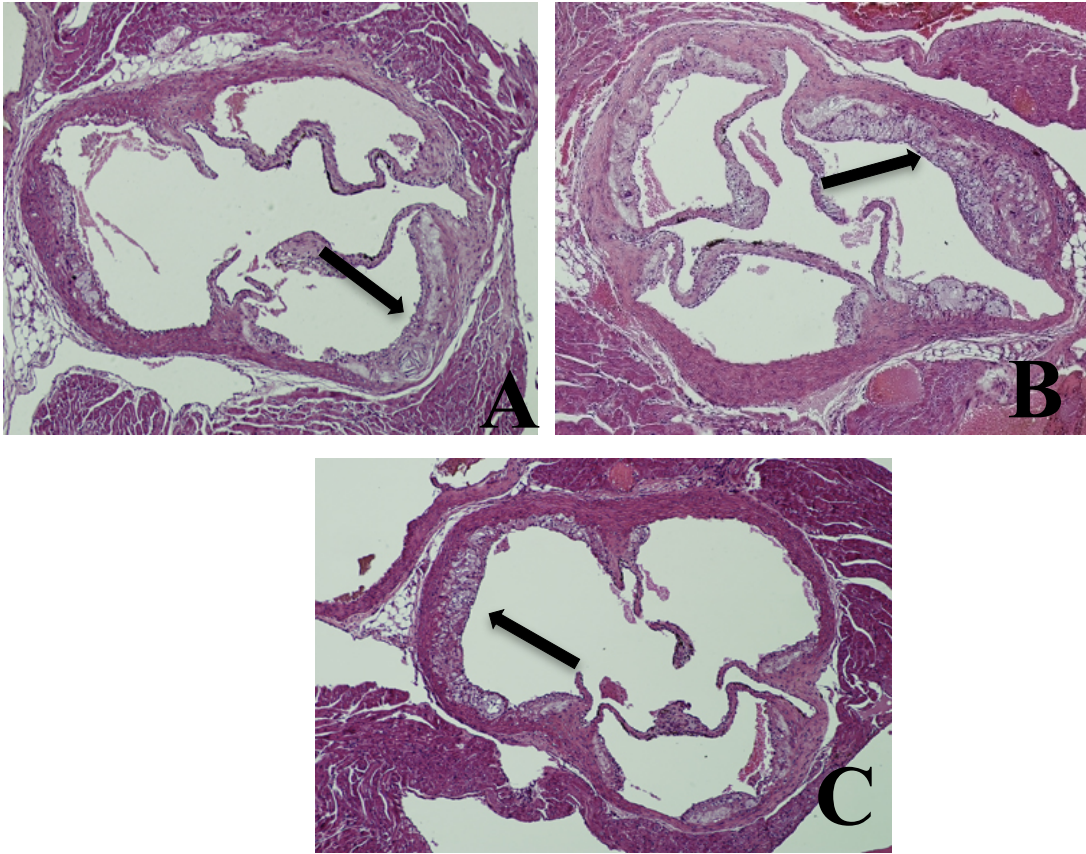




**Figure 13: Atherosclerotic lesion sizes.**

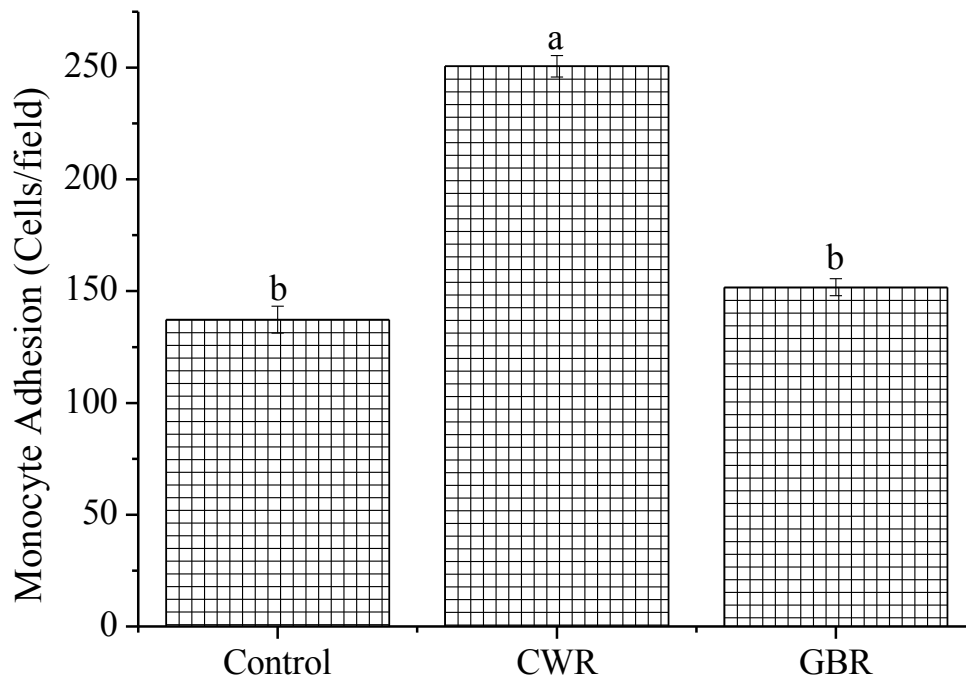
**Values are means  $\pm$  SE ( $n = 8$ ).**

**$P < 0.05$  was used to determine significant differences in means among groups.**



**Figure 14: Histopathology of aortic roots from LDLr-KO mice. Control group (Panel A); Chinese white rice group (Panel B); germinated brown rice group (Panel C).**

**Arrows indicate atherosclerotic lesions.**



**Figure 15: Monocyte adhesion.**

**Values are means  $\pm$  SE ( $n = 8$ ).**

**$P < 0.05$  was used to determine significant differences in means among groups.**

## **8. Discussion**

### **8.1. Effects of dietary GBR administration**

The main objective of this study was to investigate whether dietary GBR would decrease the risk of atherosclerosis in an atherosclerotic mouse model. Our results showed that dietary GBR slowed atherosclerosis progression in LDLr-KO mice. The consumption of GBR significantly decreased atherosclerotic lesion in the aortic roots. Moreover, monocyte adhesion to aorta was significantly lower in LDLr-KO mice fed with GBR than in those fed with CWR, but was not significantly different from those fed the control diet.

### **8.2. Food intake, body weight and abdominal fat**

Food consumption was significantly different among the three groups at some time points of the study. This was inconsistent with results reported by Han et al. (2012). Similarly, Surendiran et al. (2013) did not report changes in food consumption when wild rice (WR) was tested in six groups of mice that fed either control diet, WR diet, or wild rice diet for 24 weeks all showing no significant differences in average food intake among groups. Mean body weight and abdominal fat mass values in our study did not significantly differ among groups. This is consistent with our previous report, that body weight and abdominal fat mass of mice fed wild rice were no different from those of other diet groups ( Surendiran et al., 2013). Similar observations were made by Shirai et al. (2010) with no significant differences in average final body weights among groups. However, our findings were contrary to those of Ho et al. (2012). In that study, mice were fed three diets, normal diet (ND), high fat diet (HFD) or germinated brown rice (GBR) diet. Body weights were significantly higher in the HFD, compared with in the ND group, and were significantly lower in the GBR than in the HFD group. In another study, by

Chung et al. (2014), six groups of mice were fed different diets for seven weeks. These diets were normal control (NC), high fat (HF), HF supplemented with instant normal white rice (HF-NW), HF with normal brown rice (HF-NB), HF with germinated white rice (HF-GW) and HF with germinated brown rice (HF-GB). The HF and NC fed groups had significantly higher body weight and body fat values, while these parameters were markedly decreased in the HF-GB diet group.

### **8.3. Plasma and fecal lipid profiles**

Associations between serum cholesterol concentrations and CVD risk were previously reported. In our study, there were no significant differences among groups in plasma TC, VLDL, LDL cholesterol and TG concentrations. Similarly, in another study, no changes in lipid profiles were detected, after 4 weeks, in groups consuming polished rice (PR), brown rice (BR) and germinated brown rice (GBR) (Shirai et al., 2010). In contrast, Ho et al. (2012) reported significantly decreased plasma TC and significantly elevated HDL-C in the GBR group, compared with the HF and NC diet groups. Also, TC and TG levels were lower in the mice fed HF-GB. Moreover, HDL-C concentrations in these mice were significantly higher than those in fed HF diet alone. Furthermore, our study showed no significant differences in fecal lipid excretion among all groups. However, in a previous report, obese rats were fed with NC, high fat diet (HFD) or high fat supplemented with germinated brown rice (HF+GB) and fecal lipid excretion was significantly elevated by the HF+GB diet (Lim et al., 2016). In addition, Lo et al. (2016) observed a significant increase in fecal TC and TG levels in ovariectomized Sprague–Dawley rats, comparing a group fed NC with one fed NC supplemented with 200.0 g non-germinated and germinated brown rice, for nine weeks.

#### **8.4. Histology and atherosclerosis development**

Atherosclerosis is associated with chronic inflammation and characterized by accumulation of fatty substances and cholesterol in the vessel wall. The deposition of cholesterol-rich lipoprotein in vascular wall is the consequence of vascular inflammation, characterized by monocyte adhesion/migration and the conversion of monocytes to macrophage in vascular wall. Macrophages are able to incorporate cholesterol-rich lipoproteins to form atherosclerotic lesions (Stary et al., 1995; Ross, 1999; Soltero-Perez, 2002). Antioxidant phytochemicals are expected to inhibit LDL oxidation (Stocker, 2004). Decreased LDL oxidation is associated with an attenuated inflammatory state. Altogether, antioxidant agents would be expected to prevent development of advanced atherosclerotic lesions. GBR is rich in phenolic antioxidants, compounds believed to produce health benefits through their antioxidant properties (Burlando & Cornara, 2014). Consequently, atherosclerotic lesions were significantly decreased in animals fed GBR. Our findings are consistent with those in our previous study, where LDLr-KO mice were fed wild rice for 24 weeks and showed significantly attenuated atherosclerotic lesions, compared with those on a control diet (Surendiran et al., 2013). These findings are also consistent with a study performed by Esa et al. (2013). This study used six groups of rabbits, either on a NC diet or the following experimental diets: NC with high cholesterol (PC), high cholesterol diet with white rice powder (WR), high cholesterol diet with brown rice powder (BR), high cholesterol diet with GBR or high cholesterol diet with simvastatin (SG). Rabbits fed the GBR diet had attenuated plaque formation, compared with those fed the BR or WR diets. Decreased lesion areas in the aortic root of GBR fed mice may be related to the presence of oryzanol, tocopherols and tocotrienols in the outer layer of GBR (Esa, et al., 2011), as well as the large amounts of GABA (Kayahara et al., 2001). Moreover, high

levels of non-enzymatic antioxidants (vitamin E including tocopherols and tocotrienols) in GBR should also help to decrease oxidative stress in the blood (Esa et al., 2013).

Atherosclerotic plaque formation is a long, very complex process. Atherosclerosis development occurs at the site of endothelium with injury or highly stressed. Although it is clinically manifested in later adults, the initial signs of plaque formation can be detectable in the much younger age (Chatzizisis et al., 2007). Although the endothelium consists of only one layer of cells, it plays an important role in atherosclerosis development. The endothelium produces a wide range of factors influencing vascular tone, cellular adhesion, vessel wall injury, thromboresistance and smooth muscle cell proliferation (Pamukcu et al., 2010). Adhesion and migration of monocytes occurs at the beginning of atherosclerotic plaque formation. These cells later differentiate into tissue macrophages, leading to development and stabilization of local inflammation and their transformation into foam cells. Previous studies indicated that monocyte adhesion was enhanced under atherosclerotic conditions (Pamukcu et al., 2010; Tropea et al., 1996). Our study demonstrated increased adhesion of normal monocytes to the aorta in LDLr-KO mice fed with CWR, suggesting that CWR enhances vascular inflammation at the surface of endothelium in aorta of LDLr-KO mice. Monocyte adhesion was significantly decreased in LDLr-KO mice fed the diet supplemented with GBR, compared with in those fed CWR. This implied that monocyte adhesion to the aorta in LDLr-KO mice was attenuated by dietary administration of GBR.

### **8.5. Strengths and limitations**

LDLr-KO mice were chosen as a dyslipidemia and atherosclerosis model for our study (Getz, 2005). The plasma lipoprotein profiles of these mice mimic familial hypercholesterolemia in humans (Zadelaar et al., 2007). Atherosclerosis is a chronic condition, reported to worsen with

time. Therefore, we performed this study over a relatively long period, 24 weeks, a time length that should be sufficient to observe effects of both time and diet on any risk factors measured.

Our study, like many others, has potential limitation. There were no wild-type C57BL/6 mice to serve as controls, which limits us to detect the difference between normal animals and CWR-fed and GBR-fed high cholesterol-fed LDLr-KO mice or other cardiovascular risk factors (Zadelaar et al., 2007).

### **8.6. Recommendations for future research**

Future studies to understand the effects of GBR on cardiovascular risk factors and its underlying mechanisms of actions are needed. One potential area of investigation would address the degree of absorption, metabolism and distribution of dietary phenolic compounds from GBR and their impacts on atherosclerosis prevention. Additional studies are also required to understand effects of germination on the characteristics of the metabolic changes in bioactive components during germination. Monocyte adhesion is an early event in vascular inflammation, and it is regulated by a large number of cellular proteins and signaling pathways. Identification of inflammatory molecules and interactions between blood and vascular cells will help to clarification the anti-atherosclerotic effect of GBR.

### **8.7. Conclusions**

Our study showed that dietary GBR slowed atherosclerosis progression in LDLr-KO mice. Consumption of GBR did not improve plasma lipid profiles, fecal cholesterol excretion or antioxidant enzyme activities in LDLr-KO mice. However, histological examination demonstrated that GBR attenuated atherosclerotic lesions in the aortic root compared to CWR diets.



The anti-atherosclerotic effect of GBR potentially attributed from the functional components of GBR, which may inhibit inflammatory events in vascular wall.

In conclusion, long-term consumption of GBR reduced atherosclerotic development in the aortic root, compared with CWR diets, which is associated with attenuated vascular inflammation in GBR-fed mice.

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**Title:** The Role of Dietary Cholesterol in Lipoprotein Metabolism and Related Metabolic Abnormalities: A Mini-review

**Author:** Fatemeh Ramezani Kapourchali, Gangadaran Surendiran, Amy Goulet, et al

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