THE EFFECTS OF MANITOBAN WILD RICE ON CARDIOVASCULAR RISK FACTORS IN LOW DENSITY LIPOPROTEIN RECEPTOR KNOCK-OUT (LDLr-KO) MICE

By

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ABSTRACT

Wild rice is recognized as a whole grain by U.S. FDA and it is rich in polyphenol compounds. This study aimed to investigate the potential cardiovascular benefits of wild rice in LDLr-KO mice. Seventy-two male and female mice were randomly assigned to 3 different diet groups, control diet (a), white diet (b), and wild diet (c) for 24 weeks. Dietary cholesterol 0.06 % (w/w) was added in all diets, and digestible carbohydrate source was replaced with: (a) commercial carbohydrate source (sucrose and corn starch); (b) white rice; (c) wild rice. Incorporation of wild rice in the diet distinctly increases total phenolic content of the diet. Plasma total cholesterol, VLDL, and LDL cholesterol levels declined significantly in wild diet group. The hypocholesterolemic properties of wild rice were mainly attributed to the increased excretion of fecal cholesterol. Our data suggest that substituting refined grains with wild rice may potentially lower cardiovascular risks.

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LIST OF ABBREVIATIONS

ABCA1	ATP binding cassette A1
ABCG5	ATP binding cassette G5
ABCG8	ATP binding cassette G8
ACAT	Acyl CoA: cholesterol acyltransferase
CAT	Catalase
CVD	Cardiovascular disease
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
FDA	Food and Drug Administration
GAE	Gallic acid equivalent
HDL	High density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl-co-enzyme A
H_2O_2	Hydrogen peroxide
LDL	Low density lipoprotein
LDLr-KO	Low density lipoprotein receptor knock-out
MDA	Malondialdehyde
ORAC	Oxygen radical absorbance capacity
Ox-LDL	Oxidized low density lipoprotein
SCFA	short chain fatty acids
SD	Standard deviation
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TC	Total cholesterol
TG	Triglyceride
TPC	Total phenolic content
VLDL	Very low density lipoprotein
WG	Whole grain

1. Introduction

Atherosclerotic cardiovascular disease is a leading cause of morbidity and mortality in Canada, representing 32.1 % of all death in 2004. According to the Public Health Agency of Canada, PHAC (2009), cardiovascular disease (CVD) caused one in eight deaths among Canadian ages 35 to 44, one in five deaths among Canadian ages 45 to 54, one in four among Canadian ages 55 to 64, and nearly two of every five among those ages 85 and above. Diet and lifestyle changes are known to affect the incidence CVD. Lifestyle modifications are suggested to decrease the risk of premature CVD by 82 % (Stampfer, Hu, Manson, Rimm, & Willett, 2000), and reduction of 60 % CVD risk can be achieved by improvement of dietary patterns (Kris-Etherton, Etherton, Carlson, & Gardner, 2002). Epidemiological studies have reported that whole grains consumption is inversely associated with incidence of CVD (Sahyoun, Jacques, Zhang, Juan, & McKeown, 2006; Steffen, Jacobs, Stevens, Shahar, Carithers, & Folsom, 2003a). Besides, antioxidant rich foods have been consistently shown to reduce risk of atherosclerotic CVD through their free radical scavenging ability to lower oxidative stress in vitro and in vivo studies (Kaliora, Dedoussis, & Schmidt, 2006). Current findings support the hypothesis that oxidative stress plays an important role in the initiation and progression of atherosclerosis (Hulthe & Fagerberg, 2002; Stocker & Keaney Jr, 2004). Oxidized LDL is believed to generate a vicious cycle of inflammation oxidation within the intima and thus initiate atherosclerosis through foam cell formation (Stocker & Keaney Jr, 2004).

This indicates that it is crucial to regulate oxidative stress and inhibit the inflammatory responses to prevent development of atherosclerosis.

Manitoban wild rice (*Zizania palustris*) is recognized as a whole grain by U.S. Food and Drug Administration (FDA). Nutritional quality of wild rice is comparable with other cereals characterised by high contents of starch and protein, low fat content, and a good source of dietary fibre (Anderson, 1976). In addition, several polyphenol compounds were detected in wild rice, such as ferulic acid, sinapic acid, ρ-coumaric acid, vanillic acid, ρ-OH-benzoic acid, syringic acid, 8-8' disinapic acid, and 8-O-4' diferulic acid (Qiu, Liu, & Beta, 2010). Furthermore, cardio-protective effects of Chinese wild rice (*Zizania latifolia* (Griseb) Turcz) were demonstrated in Zhang et al. (2009) study. Chinese wild rice which is native to China, Japan and Vietnam, had shown to suppress the elevation of triglycerides, total cholesterol, and oxidative stress in rats fed with a high saturated fat and cholesterol diet. However, little is known about the cardiovascular benefits of Manitoban wild rice (*Zizania palustris* L.).

Therefore, the purpose of this study was to investigate the effectiveness of Manitoban wild rice enriched diet on reducing cardiovascular risk in LDLr-KO mice. Plasma, hepatic and fecal lipid profiles were determined to assess the effect of wild rice on lipid metabolism. Antioxidant property was analyzed through examination of plasma antioxidant and peroxidation level, hepatic malondialdehyde level, as well as activities of endogenous antioxidant enzymes such as catalase and superoxide dismutase.

White rice was chosen to be incorporated into another set of treatment diets to be compared with the effectiveness of Manitoban wild rice to reduce cardiovascular risk factors. This is because white rice is a commonly used refined grain in daily diet, either being served as a main rice dish, side dish, an ingredient in various mixed dishes, or even ground into white rice flour for making rice product, such as rice cracker and pasta. Wild rice shares the similar cooking applications as white rice. Therefore, in this study we used white rice as a "control" for wild rice.

2. Review of Literature

2.1. Cardiovascular disease

Cardiovascular disease (CVD) is defined as disease and injuries of the cardiovascular system, which include the heart and the blood vessels system. CVD is the primary cause of death in Canada and United States. In Canada, CVD is accounted for one-third of mortality in adult Canadian men and women (Genest et al., 2009). In 2004, CVD were a main mortality cause of 3 age groups in Canada: 45 to 54 year old (20.2 %), 55 to 64 year old (23.9 %), and over 85 year old (42.4 %) (PHAC, 2009). In United States, nearly 33 % of death is caused by CVD among population aged 75 years or less (Roger et al., 2011). CVD is a chronic disease mainly caused by interactions among genetic predisposition, health behaviours and the environment. The major modifiable risk factors of CVD are known to be smoking, alcohol, high cholesterol, high blood pressure, sedentary lifestyle, unhealthy eating, overweight, diabetes and stress (Wielgosz, 2003). Nine in ten

Canadians have at least one risk factor for CVD. Thereby, early recognition and treatments of these conditions are crucial to reduce the risk of developing CVD.

2.2. Atherosclerosis

Atherosclerosis is the underlying pathology of the majority of clinical cardiovascular events. It is characterized by the build-up of fatty streak along the wall of medium and large sized arteries, which gradually results in luminal narrowing and eventually leads to impairment of blood flow (Hansson, 2005). The initial, progression, and mature stages of atherosclerotic lesions are manifested as stated in Stocker & Keaney (2004): (I) thickening of the intima in lesion-prone arterial sites; (II) accumulation of lipid filled macrophages, known as foam cells; (III) advancement of foam cell formation and macrophage necrosis; (IV) significant build-up of lipid core; (V) fibrous thickening; (VI) crystallization of fibrous cap with ulceration. These vulnerable plaques with abundant amount of active macrophages and T cells are highly prone to rupture, especially when exposed to the relatively high pressure of blood flow (Libby, Ridker, & Maseri, 2002).

Lately, epidemiological studies had reflected an increasing support in the hypothesis of oxidative modification of atherosclerotic lesions development. LDL oxidation is a key early stage in the initiation of atherosclerosis and oxidized LDL (ox-LDL) further contributes to the progression of atherosclerosis event (Stocker & Keaney Jr, 2004; Young & McEneny, 2001). LDL undergoes oxidative modification when exposed to localized vascular cells, such as smooth muscle cells, endothelial cells and macrophages

during its retention in the subendothelial space (Epstein, Diaz, Frei, Vita, & Keaney Jr, 1997; Young & McEneny, 2001) Upon oxidation, net negative charge of LDL is enhanced through modification of apolipoprotein B- 100 lysine groups (Haberland, Fogelman, & Edwards, 1982; Steinbrecher, Parthasarathy, Leake, Witztum, & Steinberg, 1984). These in turn increase ox-LDL susceptibility to macrophages uptake (Brown & Goldstein, 1983). Ox-LDL also promotes monocyte chemotaxis, facilitates macrophages internalization of LDL, increases LDL oxidation, formation of foam cells (Ross, 1993), and thus causes endothelial dysfunction and injury (Berliner & Heinecke, 1996).

Oxidative modification hypothesis has shown the importance of oxidative events and redox reactions in the development of vascular disease. It also includes LDL as the central element that substantially impacts the progression of atherosclerosis. Thus, lowering oxidative stress and LDL cholesterol may effectively attenuate the development of atherosclerosis.

2.3. Whole grains

American Association of Cereal Chemists International and the FDA define whole grains (WG) as “intact, ground, cracked or flaked fruit of the grain whose principal components, the starchy endosperm, germ and bran, are present in the same relative proportions as they exist in the intact grain”. WG are excellent sources of dietary fibre, vitamins, minerals, lignans, phytosterols, and several other phytochemicals (Okarter & Liu, 2010b). The synergistic effects of these functional components have been suggested

to contribute to health benefits associated with WG. The major grains include wheat, rice, and corn, whereas oats, rye, barley, sorghum and millet are categorized as minor grains (Slavin & Slavin, 2004). These grains can be either whole or refined products. Refined grains undergo refining process to remove germ and bran, causing loss of substantial amounts of vitamins, minerals, dietary fibre and phytonutrients (Slavin, Martini, Jacobs, & Marquart, 1999). Moreover, majority of the beneficial compounds are found in the bran and germ fraction of WG, which are fully removed in the refining process (Adom, Sorrells, & Liu, 2005). Thus, it is highly recommended to replace white flour with whole wheat flour in baking, or consume brown rice or wild rice instead of white rice to obtain the known benefits of WG.

2.3.1. Whole grains and their phytochemicals

WG are rich in various classes of phenolic compounds, including ferulic acid, syringic acid, caffeic acid, vanillic acid, p-coumaric acid, anthocyanidins, sinapic acid, flavonols, quinines, flavones, and amino phenolic compounds (Okarter & Liu, 2010b). Among all, ferulic acid is abundantly found in most grains (Klepacka & Fornal, 2006; Sosulski, Krygier, & Hogge, 1982). Ferulic acid is primarily found in bound form, approximately 93 % of total concentrations, than free and soluble-conjugated forms in wheat, oat, corn and rice (Adom & Liu, 2002). Pre-treatment of grains product in food industry including, milling, heating, pasteurisation, and fermentation helps to release the bound phenolic acids and hence increase the bio-accessibility of these phytochemicals (Dewanto, Wu, & Liu, 2002). In addition, ferulic acid has shown to

reduce oxidative stress (Yogeeta et al., 2006; Zhang, Liu, Liu, & Zhang, 2001) and cholesterol level in *in vivo* studies (Kim, Jeong, Lee, Park, & Choi, 2003; Wilson, Nicolosi, Woolfrey, & Kritchevsky, 2007; Yeh, Lee, Hsieh, & Hwang, 2009).

Whole grains are a good source of dietary fibre. Dietary fibre is defined by American Association for Clinical Chemistry (AACC) as the plant edible part that is resistant to digestion and absorption in human, and is subjected to partial or complete fermentation in the large intestine. Major components of dietary fibre consist of cellulose, hemicelluloses, lignin, and β- glucan (Okarter & Liu, 2010b). Dietary fibre has been recognized as an important food component to maintain a healthy dietary pattern. Consumption of sufficient amount of dietary fibre has been strongly associated with reduced risk of obesity (Slavin, 2005), CVD (Mozaffarian et al., 2003), type II diabetes (Panahi, Ezatagha, Temelli, Vasanthan, & Vuksan, 2007), and colorectal cancer (Bingham et al., 2003).

WG also contain vitamin E (α-, β-, γ-, and δ- tocopherols and -tocotrienols), mostly present in the germ, that is removed during the refining process (Slavin et al., 1999). Concentrations of vitamin E compounds varied in different structures with diverse proportion in each particular grain (Panfili, Fratianni, & Irano, 2003). Major protective role of vitamin E in the body is to act as an essential intracellular antioxidant that defends against oxidative damage of cell membranes (Liu, 2007).

In addition, phytosterols and γ-oryzanol are found in WG with the type and amount varying by the type of WG. Both phytosterol and γ-oryzanol are known to lower cholesterol level. Plant sterols and stanols can be found in whole grains, oilseeds, nuts, unrefined vegetable oils and legumes. Trautwein et al. (2003) suggested that similarity of structure with cholesterol allows phytosterol to compete with cholesterol for micellar binding, resulting in decrease of cholesterol absorption. γ-Oryzanol is a ferulic acid esterified of sterol compound, abundantly found in rice bran oil (Berger et al., 2005). γ-Oryzanol also demonstrated positively impact on cholesterol and oxidative stress levels in animal studies (Qureshi, Salser, Parmar, & Emeson, 2001; Wilson et al., 2007).

2.3.2. Whole grains and cardiovascular disease

Health benefits of WG are well established. FDA had approved the health claim stated that "Diets rich in whole-grain foods and other plant foods, and low in total fat, saturated fat and cholesterol may reduce the risk for heart disease and certain cancers". In addition, Canada's Food Guide also encourage consumers to substitute at least 50 % of daily grain products with a variety of WG, such as barley, brown rice, oat, wild rice and quinoa. WG contained a wide range of functional compounds with known health effects, such as dietary fibre, antioxidants, phytosterols, tocotrienols, unsaturated fatty acids, vitamins and minerals. Mechanisms underlying the disease reduction of WG are strongly proposed to be a synergy of all protective components, rather than each or the sum of few individual functional parts.

Numerous epidemiological studies had presented an inverse association between WG ingestion and risk of CVD. Large-scale studies, such as the Health Professionals Follow-Up noted about 15 % reduction in coronary heart disease (CHD) with 25 g of WG/d (Jensen et al., 2004); Atherosclerosis Risk in Communities Study observed a reduction of 28 % in coronary artery disease (CAD) with intake of 3 servings of WG/d (Steffen, Jacobs, Stevens, Shahar, Carithers, & Folsom, 2003b); whereas the Nurse's Health Study had shown 36 % reduction in ischemic stroke risk with 1.3 servings of WG/d (Liu et al., 2000). There are several theories that have been proposed to be responsible for WG CVD risk lowering properties, mainly focused on cholesterol-lowering effects of WG (Jones & Engleson, 2010). First, WG are an excellent source of dietary fibre shown to bind and stimulate excretion of bile acid (Sayar, Jannink, & White, 2006). Short chain fatty acids produced through colonic fermentation of dietary fibre are also known to impair hepatic cholesterol metabolism (Wolever, Spadafora, Cunnane, & Pencharz, 1995; Wong, de Souza, Kendall, Emam, & Jenkins, 2006). Next, WG antioxidants appeared to effectively suppress LDL cholesterol oxidation and platelet aggregation by WG polyphenol compounds (Madhujith & Shahidi, 2007). Lastly, phytochemicals such as phytosterol also may contribute to cholesterol lowering effects of WG (Trautwein et al., 2003).

Nonetheless, different variety or species of whole grains contain diversity in their functional components, different types and amounts, specific to pose similar or entirely different mechanisms to exhibit various health benefits in animal and human trials.

2.4. Wild rice

2.4.1. The plant and geography

Wild rice (*Zizania palustris* L.), a large seeded aquatic grass, is indigenous to Canada and North America (Oelke, 2004). It served as a native staple food to the locals for 10,000 years (Aiken, 1988; Steeves, 1952). It is one of the four species of wild rice genus *Zizania*. Wild rice is also known as Canadian rice, Indian rice, water oats, or blackbird oats (Steeves, 1952). Although both wild rice genus *Zizania* and genus *Oryza* which rice (Asian rice) belongs to, share the same tribe (*Oryzeae, Poaceae*); wild rice *Zizania* wholly differs from the wild type of rice (*Oryza*) (Dore, 1969; Xu et al., 2010).

There are four species of wild rice distributed in North America and in eastern Asia, *Z. aquatica*, *Z. palustris*, *Z. texana*, and *Z. latifolia* (E. A. Oelke, 1993). *Z. palustris* annually grows predominantly in the Great Lakes region in shallow lakes and rivers of the United States and Canada (E. A. Oelke & Hot-dicker, 2000). It is historically consumed as a traditional staple for Native Americans and now serves as an economically important specialty commercial crop in recent years (Aiken, 1988; E. A. Oelke, 1993; E. A. Oelke, 2004).

The other annual species, *Z. aquatica* is widely available along the St. Lawrence River, eastern and south eastern United States coastal areas, and in Louisiana (E. A. Oelke, 1993). *Z. texana*, which is perennial with small seeds, grows in a small area in Texas (E. A. Oelke, 2004). Both *Z. aquatica* and *Z. texana* are not harvested for food due

to their thin and small seeds production (E. A. Oelke, 1993; Xu et al., 2010). Lastly, *Z. latifolia*, perennial widely grown in south-eastern Asia, was an important ancient grain in China (Guo, Li, Peng, & Ke, 2007; Guo et al., 2007; Zhai, Lu, Zhang, Sun, & Lorenz, 2001). It is often infected by fungus *Ustilago esculenta P. Henn*, and produces nutritious shoot that has been cultivated as an edible aquatic vegetable (Guo et al., 2007; E. A. Oelke, 2004).

2.4.2. Manitoba wild rice

Manitoba wild rice is mainly grown in Manitoba's natural lakes, streams and rivers. Abundant supply of sunlight and clean unpolluted water give rise to ideal growing conditions for fuller long grain, flavourful, and better quality wild rice (Manitoba Agriculture and Commodities).

Manitoba wild rice is also known as 'Canadian Lake Wild Rice'. The name is given based on its origin growing nature. In 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.

2.4.3. Harvesting of wild rice

Harvesting wild rice is also known as *knocking the rice*. As the term sounds, wild rice is harvested by knocking the stalks to drop the grains (Mäkelä, Archibold, &

Peltonen-Sainio, 1998). Canoes and traditional forked like poles are used to move through the rice beds to minimize the harm to the rice plant and its root systems (Steeves, 1952). A pair of light-weight wood stick or *knocker* is used as a tool to tap the rice grains. The harvester will first gently draw in some rice stems over the edge of the canoe with one stick, and tap the ripe grains off with another into the bottom of the canoe (Aiken, 1988; Dore, 1969) . Unripe grains that still remain on the stems will be left for future harvesting. These traditional ways of wild rice harvesting are still used in recent time even though new mechanical technologies have been introduced to the harvesters.

2.4.4. Processing of wild rice

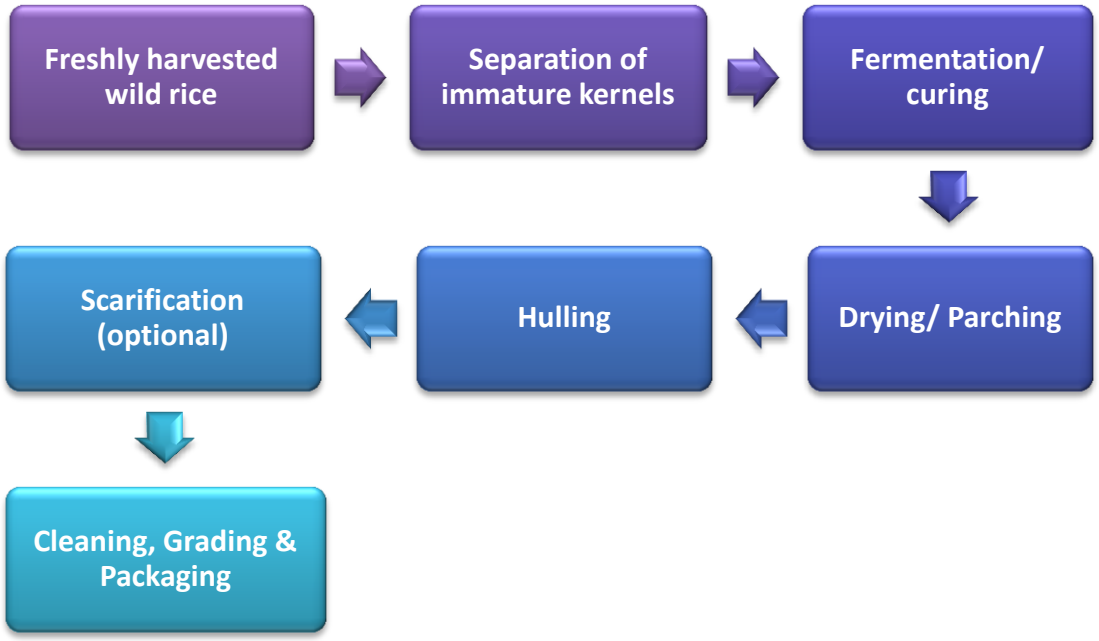
Freshly harvested wild rice contains about 35 % to 45 % of moisture levels (Aiken, 1988; E. Oelke, Porter, Grombacher, & Addis, 1997). Therefore, proper handling is crucial to ensure the quality of wild rice, as well as to avoid grain loss through heating and prevent mould development during long term storage. Unlike other cereal grains, wild rice is harvested at relatively high moisture content to prevent excessive grain loss from shattering during the post-harvesting processing (Oelke & Hot-dicker, 2000).

The traditional ways of wild rice processing are mainly drying, hulling, and separation of hulls. Firstly, the grains are spread out and dried under the sun or over a fire (Steeves, 1952). Then, hulling is performed by walking or dancing on the dried grains in a container or hole in the ground (Dore, 1969). Lastly, the hulls are separated from

the grains by winnowing. The grains and hull mixtures are gently tossed in the air; the light hulls will be lifted away by the wind caused by the tossing action (Aiken, 1988).

The increased demand and commercialized production of wild rice has gradually brought changes and new innovations into the processing industry. As compared to the ancient ways which required sorely manpower, today's processing plants are mostly equipped with high efficient mechanical tools for greater and better wild rice production. The essential principles of the traditional methods remain unchanged. However, several modifications or additional processing methods have been made with time to increase the yield and enhance the quality of the wild rice. The major processing procedures of a modern wild rice plant are shown in a flow chart in **Figure 1**.

Figure 1. Operation flow chart of wild rice processing (Adapted from Oelke & Hotdicker, 2000)



Separation of immature kernels is an optional first step introduced into the wild rice processing due to the relatively high volume of immature kernels among wild rice harvested by combine in Minnesota (Oelke & Hot-dicker, 2000). Freshly harvested wild rice will be separated into heavy (plump, mature kernels), medium (intermediate maturity) and light (small immature kernels, hulls and chaff) fractions using air-steam separator. The light fraction, which is only about 5 % of the total yield, is not profitably and thus discarded by most processors (Oelke & Hot-dicker, 2000).

After separation, the heavy and medium fractions of kernels will be subjected to fermentation or curing process. Some processors will dry the heavy fractions without any fermentation course. The grains will be lined on an impervious surface under the sun and constantly watered and stirred for about 4 to 7 days (Aiken, 1988; Dore, 1969)(Aiken, 1988; Oelke & Hot-dicker, 2000). Watering helps to maintain its high moisture content; while stirring aids the aerobic condition for respiration and microorganisms activities, as well as eliminating excessive heat and growth of undesirable moulds (Oelke & Hot-dicker, 2000). Curing will lead to color changes of immature kernels, from green to brown; as well as allow immature grains to ripe; partial degradation of hulls, as well as enrichment of flavours (Dore, 1969).

The next step is drying, or referred to as parching. Parching is an essential procedure in which wild rice is roasted to perfection for the rich, dark shade, as well as the development of the finest toasted nutty flavour and aroma in a huge rotary drum parcher. Besides, parching also reduces the moisture content of the grains to

approximately 6 % to 8 % (Oelke & Hot-dicker, 2000). During parching the drum is continuously rotated, which allow individual kernels to come in contact with the hot surface. The temperature in the drum is controlled under 135 °C, and when it reaches 135 °C, the kernel is hardened and the starch is usually completely gelatinized, and ready to be removed (Oelke & Hot-dicker, 2000; Oelke, 2004). The germ of the grains will also be deactivated and prevent the grains from sprouting.

Next, the grains will be de-hulled to remove the tight fitting chaff from the kernel using huller. Then, scarification may apply in some operations depending on the marketed product's requirements. Scarification is a process of polishing outer impermeable layer of the kernel, mainly to obtain a shorter, even or constant cooking time (Aiken, 1988; Morton, Caccetta, Puddey, & Croft, 2000). After that, the grains will be cleaned or separated from the hulls and chaff using vacuum aspirator (Oelke & Hot-dicker, 2000).

The cleaned wild rice will then be graded based on its kernel size or length (Oelke & Hot-dicker, 2000). The Canadian Wild Rice Council defines "wild rice" as processed product resulting from curing, parching, and hulling of *Zizania aquatic* or *Zizania palustris* and in which moisture does not exceed 11 % by weight for the purpose of their grading standards (Oelke & Hot-dicker, 2000). Lastly, wild rice will be packed and stored in a clean, dry environment.

2.4.5. Nutritional composition of wild rice

Since late 20th century, utilization of wild rice has been gaining popularity among consumers. Due to its unique flavour and texture, wild rice is used as an ingredient 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 & Hot-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 FDA. It is universally accepted that regular consumption of whole grains is beneficial for human health, resulting in reduced incidence of CVD. Hull-less wild rice has a long and thin cylindrical shape, and is black in color. It is 7.5 mm to 20.0 mm long and its diameter ranges from 1.5 mm to 4.0 mm (Capen & LeClerc, 1948; Hoover, Sailaja, & Sosulski, 1996). The wild rice kernel has a similar structure as other cereals, consisting of a pericarp, aleurone layer, endosperm, and embryo. The endosperm and aleurone consists of about 90 % of the kernel's weight; while the pericarp and embryo, each accounts for about 5 % of the kernel (Hoover et al., 1996).

The nutritional value of wild rice compares favourably with other cereals, such as oats, wheat, and corn (**Table 1**). It is high in protein, carbohydrate, and minerals, but low in fat. Like other cereals, wild rice grain is a food commodity containing 74 % starch and 13 % protein as the main constituents. It is also a rich source of dietary fibre, consists of 0.6 to 1.1 % of crude fibre, and 5.2 % of total dietary fibre (Anderson, 1976; Dreher,

2001). Crude fibre is the residues of plant material obtained after acid and alkali hydrolysis, which extract only variable portions of the cellulose (0 to 50 %), hemicellulose (85 %), lignin (50 to 90 %), and pectin, gum, and mucilage present in dietary fibre (Marlett & Bokram, 1981; Spiller, 2001). Therefore, food crude fibre is expected to measure about ⅕ to ½ of the dietary fibre value (Marlett & Bokram, 1981). In addition, wild rice is high in vitamins B1, B2, E, as well as some minerals (Anderson, 1976). The mineral composition of wild rice is shown to be comparable with other cereals. It is a good source of calcium, iron, magnesium, potassium, phosphorus, and zinc.

Wild rice grain consists of 12.9 % to 13.4 % of protein, as reported by Terrell & Wiser (1975). The essential amino acids profile of wild rice (**Table 2**) appeared to be similar to that in other cereals (Oelke, 1976). The lysine value of wild rice is about 3.8 % to 4.8 % of its protein content, which is close to that found in oat (3.2 % to 5.2 %), wheat (2.3 % to 4.3 %), and corn at 3.6 % (Pomeranz, Robbins, & Briggle, 1971; Terrell & Wiser, 1975). (Anderson, 1976; Pomeranz et al., 1971)

Fat content of wild rice is less than 1 %. However, the lipids of wild rice comprised mainly linoleic (35 % - 37 %) and α-linolenic acids (20 % - 31 %) acids (Przybylski, Klensporf-Pawlik, Anwar, & Rudzinska, 2009). Low n-6 /n-3 fatty acid ratio in wild rice seems very attractive (Riediger et al., 2008). Other fatty acids include palmitic 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). Furthermore, wild rice lipids contained large amounts of sterols,

ranging from 70 g/ kg to 145 g/ kg. In Przybylski et al. (2009) study, several sterols were found in an unsaponified fraction (**Table 3)** including, campesterol (14 % - 52 %), β-sitosterol (19 % - 33 %), Δ^5-avenasterol (5 % - 12 %), and cycloartenol (5 % - 12 %) (Przybylski et al., 2009). In addition, fatty acids, sterols, tocopherols, and γ-oryzanol in wild rice were comparable with brown rice. γ-Oryzanols, present as the phenolic esters, ranged from 459 mg/ kg to 730 mg/ kg in wild rice lipids (Przybylski et al., 2009). Tocopherols and tocotrienols content of wild rice were about 3682 mg/ kg and 9378 mg/ kg respectively (Przybylski et al., 2009). Research has shown that wild rice lipids contain large amounts of phytonutrients with proven positive health effects.

Table 1. Nutritional composition of wild rice, brown rice, white rice, corn, wheat, and oats (Adapted from Anderson, 1976; Dreher, 2001*)

Component	Wild rice	Brown rice	White rice	Corn	Wheat	Oats
Total carbohydrate (%)	72.3-75.3	77.40	80.40	72.20	71.70	68.20
Protein (%)	12.9-13.4	7.50	6.70	8.90	12.30	14.20
Fat (%)	0.5-0.8	1.90	0.40	3.90	1.80	7.40
Crude fibre (%)	0.6-1.1	0.90	0.30	2.00	2.30	1.20
Total dietary fibre (%)*	5.2	3.90	1.30-2.80	11.00	12.60	10.30
Vitamin (mg/ 100g)						
Thiamine	0.45	0.34	0.07	0.37	0.52	0.60
Riboflavin	0.63	0.05	0.03	0.12	0.12	0.14
Niacin	6.20	4.70	1.60	2.20	4.30	1.00
Minerals (mg/ 100g)						
Calcium	17-22	32.00	24.00	22.00	46.00	53.00
Iron	4.2	1.60	0.80	2.10	3.40	4.50
Magnesium	80-161		28.00	147.00	144.00	160.00
Potassium	55-344	214.00	92.00	284.00	352.00	370.00
Phosphorus	298-400	221.00	94.00	268.00	405.00	354.00
Zinc	3.3-6.5		1.30	2.10	3.40	3.40

Table 2. Essential amino acids in wild rice, white rice, wheat, and oats (% of protein)

(Adapted from Anderson, 1976; Terrell & Wiser, 1975; Oelke, 1976)

Component (% of protein)	Wild rice	White rice	Wheat	Oats
Isoleucine	4.4	4.6	3.5	4.0
Leucine	7.4	8.0	6.8	8.3
Lysine	4.6	3.5	2.4	4.2
Methionine & cysteine	3.3	2.9	2.2	3.1
Phenylalanine & tyrosine	9.1	10.1	8.0	9.9
Threonine	3.2	3.5	2.4	3.2
Valine	7.0	6.5	4.4	5.8

Table 3. Fat composition of wild rice, brown rice, white rice, wheat, and oats (% of total fatty acid) (Adapted from Anderson, 1976; Przybylsk et al., 2009)

Component (% of total fatty acid)	Wild rice	Brown rice	White rice	Wheat	Oats
Palmitic acid (16:0)	14.5	20.4	33.8	24.5	16.2
Stearic acid (18:0)	1.1	1.6	2.7	1.0	1.8
Oleic acid (18:1)	15.9	41.3	43.3	11.5	41.2
Linoleic acid (18:2)	37.7	34.5	18.0	56.3	38.8
Linolenic acid (18:3)	30.0	1.0	0.6	3.7	1.9

2.4.6. Antioxidant properties of wild rice

Earlier studies have focused mainly on the ability of wild rice in reducing lipid oxidation in food, especially meat products. Incorporation of wild rice in meat products had been shown to inhibit lipid oxidation and development of rancidity during frozen storage. The value of thiobarbituric acid reactive substances (TBARS) was shown to be lower in ground beef with addition of wild rice (Minerich, Addis, Epley, & Bingham, 1991; Wu et al., 1994). In addition, Wu, et al. (1994) identified the occurrence of phytic acid as one of the natural antioxidant compounds found in wild rice grain, which contribute to its antioxidant activity in food. These findings have increased the interest of food industry to incorporate wild rice in a variety of food products to prevent rancidity, prolong the food shelf life, as well as targeting consumers which highly prefer natural antioxidant food additives as their healthier food choices.

In 2009 & 2010, Qiu, Liu, & Beta further studied the specific antioxidant compounds available in commercial wild rice and its antioxidant activities. Total phenolic content of 9 different wild rice samples were reported to vary from 419 $\pm$ 14 mg GAE/ kg to 588 $\pm$ 27 mg GAE/ kg, about 9 – 13 times higher than that in white rice (46 $\pm$ 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) found in wild rice, followed by sinapic acid which ranged from 55.13 mg/ kg – 96.94 mg/ kg. Several other phenolic acids were also identified, such as ρ-coumaric, vanillic, syringic, ρ-hydroxybenzoic acids, 8-8' disinapic acid, and 8-O-4' diferulic acid.

2.5. Low density lipoprotein receptor knock-out (LDLr-KO) mice

Mice are one of the favorably used animal models in studying disease. Mice are small, easy to handle or maintained, and the tissues can be easily harvested, stored and processed for analysis (deLuna, 2009). Mice do not develop significant atherosclerosis as a result of high levels of high density lipoprotein (HDL) and low level of low density lipoprotein (LDL) and very low density lipoprotein (VLDL) (Getz & Reardon, 2006; Zadelaar, 2007). Thus, most of the atherosclerotic mice models used in research are mainly depend on disruption of normal lipoprotein regulation and metabolism through dietary or genetic manipulations, which allow extensive and mature lesion development. LDLr-KO mice are one of the most extensive used dyslipidemia models of atherosclerosis (Kowala, Recce, Beyer, Gu, & Valentine, 2000). They exhibit elevated levels of LDL and VLDL cholesterol when fed a high fat diet due to the absence of hepatic LDL receptors, which disables hepatic clearance of these circulating lipid particles (Ishibashi et al., 1993). When LDLr-KO mice were fed on regular chow, modest elevation (nearly 5 mmol/ L) of plasma cholesterol was observed, as compared to 2 mmol/ L in wild type C57BL/6 mice. When high fat cholesterol diet was fed, plasma cholesterol level of LDLr-KO mice were elevated (>25 mmol/ L) and atherosclerosis were developed rapidly (Knowles & Maeda, 2000). The plasma lipoproteins profile is similar to human, and in part mimics familial hypercholesterolemia conditions (Getz & Reardon, 2006). LDL receptor deficient mice generally have lower cholesterol levels and susceptibility to develop atherosclerotic lesions compared to Apolipoprotein E deficient mice (deLuna, 2008).

This mild or modest dyslipidemic profile is desired for studies investigating the effects of lipid profile changes on atherosclerotic lesions (Zadelaar et al., 2007).

3. Study rationale

Increased whole grain intake is constantly demonstrated to have beneficial impacts on cardiovascular risk. The protective roles of several dietary antioxidants such as polyphenol compounds have also been shown. Chinese wild rice was found to pose several beneficial effects on lipid profile and antioxidant systems in rats. Currently, Manitoban wild rice also appears to have high level of antioxidant activities. However, little is known about the health beneficial effects of Manitoban wild rice. Therefore, this study was carried out to generate evidence of the effectiveness of Manitoban wild rice on reducing cardiovascular risk factors in experimental animals with dyslipidemia and atherosclerosis.

4. Study hypotheses and objectives

4.1. Hypotheses

Incorporation of Manitoba wild rice in animal diet will beneficially impact the cardiovascular risk reduction, mainly through improving lipid profile and lowering lipid peroxidation with increased endogenous antioxidant activities in an atherosclerosis mouse model.

4.2. Objectives

The purpose of this study was to investigate the cardiovascular benefits of Manitoban wild rice in LDLr-KO mice. The specific objectives include:

1. To determine the effects of wild rice on plasma total cholesterol, triglyceride, VLDL, LDL, and HDL cholesterol levels;
2. To determine the effects of wild rice on hepatic and fecal lipid contents, to assess the effects of wild rice on plasma and hepatic lipid peroxidation markers;
3. To assess the effects of wild rice on erythrocyte catalase and superoxide dismutase activities;
4. To investigate the effects of wild rice consumption on plasma total phenolic contents
5. To investigate whether there is a sex-dependent variation in response to wild rice consumption in mice

5. Materials & Methods

5.1. Experimental Animals

Seventy-two, 4 week old male and female LDL receptor knock-out (LDLr-KO) mice were purchased from Jackson Laboratory (Bar Harbor, ME, U.S.A). During one week quarantine period in Animal Facility at St. Boniface Hospital Research Centre (Winnipeg, MB, Canada), mice were fed with standard mouse chow and water. The mice were housed in a temperature-controlled room at 22 °C to 24 °C, with a 12:12-hour light-dark cycle. After one week of acclimation period, blood samples were taken through jugular vein under light anaesthesia after 12 hours fasting and baseline plasma total cholesterol was measured. Mice were randomly grouped into three different diet groups with similar mean plasma total cholesterol concentrations among the groups.

LDLr-KO mice are one of the most extensive used dyslipidemia models of atherosclerosis (Kowala, Recce, Beyer, Gu, & Valentine, 2000). This mild or modest dyslipidemic profile is desired for studies investigating the effects of lipid profile changes on atherosclerotic lesions (Zadelaar et al., 2007). Sixteen weeks of semisynthetic diet with 4.3 % fat and 0 % or 0.02 % cholesterol was proven to promote hypercholesterolemia and atherosclerotic lesion at the aortic root (Teupser, Persky, & Breslow, 2003). Moreover, raising dietary cholesterol to 0.15 %, 0.3 %, and 0.5 % lead to significant increase in plasma cholesterol, en face lesions, aortic root and brachiocephalic arteries lesion. However, no differences were observed in plasma cholesterol and lesion area under these three dietary cholesterol levels. Therefore,

Teupser et al. (2003) summarized that LDLr-KO mice had highest sensitivity towards dietary cholesterol at 0.02 % to 0.15 % (w/w) doses.

5.2. Ethics

Animal protocols and schedules, which include animal housing, care, diets, animal experimental procedures, data collection and personnel, were approved by the Animal Care Committee on the use of animals in Research at the University of Manitoba, Winnipeg, MB, Canada.

5.3. Experimental design

Male and female LDLr-KO mice were randomly separated into three different diet groups (n=12) including, control, white rice or wild rice diets. The experimental course was 24 weeks. Two mice were housed together in the same cage due to their social nature. Some of the male mice were caged individually to avoid injuries and stress due to fighting issues. After 24 weeks of feeding, mice were euthanized using carbon dioxide as previously performed (Riediger et al., 2009). Final blood samples were collected through cardiac puncture; tissues were weighed and stored at -80 °C, part of the tissues were fixed in formalin and sectioned for histological examinations.

5.4. Experimental diets

The treatment diets were designed as outlined in **Table 4**. 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. 0.06 % (w/w) dietary cholesterol was added in all treatment diets, and the digestible carbohydrate source was replaced with: a) commercial carbohydrate source used in rodent diet, such as sucrose and corn starch (control diet); b) white rice (white rice diet); c) wild rice (wild rice diet). Locally grown wild rice (Far North Wild Rice) was purchased from Flin Flon, MB, Canada; while Rooster brand white rice was bought from The Real Canadian Superstore, Winnipeg, MB, Canada. These rice samples were sent to Covance Laboratory (Madison, WI, U.S.A) for macronutrients determination before incorporation into each diet. All three 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.

Table 4. Experimental diet composition (g/ kg diet)

Ingredients	Control	White Rice	Wild Rice
Casein	200.0	142.6	117.5
L-Cystine	3.0	3.0	3.0
Sucrose	100.0	0.0	0.0
Cornstarch	396.9	0.0	0.0
Dyetrose	132.0	56.8	81.9
Rooster Brand Rice	0.0	629.5	0.0
Wild Rice	0.0	0.0	629.5
Soybean Oil	70.0	70.0	70.0
t-Butylhydroquinone	0.01	0.01	0.01
Cellulose	50.0	50.0	50.0
Mineral Mix	35.0	35.0	35.0
Vitamin Mix	10.0	10.0	10.0
Choline Bitartrate	2.5	2.5	2.5
Cholesterol	0.6	0.6	0.6

Table 5.Macro-nutrient composition of the experimental diets (%)

Components (%)	Control	White Rice	Wild Rice
Total carbohydrate	64.47	56.90	57.96
Sucrose	11.74	1.74	1.74
Cornstarch	39.53	-	-
Dyetrose	13.20	5.68	8.19
Rice	-	49.48 (White)	48.03 (Wild)
Total protein	20.46	19.55	19.23
Total fat	7.00	7.00	7.00

Table 6. Energy composition of the experimental diets (%)

Components (%)	Control	White Rice	Wild Rice
Energy- carbohydrate	63.04	55.11	56.19
Energy- protein	19.52	18.96	18.63
Energy- fat	16.76	17.01	16.98
Total energy (kcal/kg)	3757.89	3703.99	3709.52

5.5. Data collection

5.5.1. Total phenolic compound extraction from experimental diets

Total phenolic compound extractions were performed with slight modification as described by Qiu, Liu, & Beta (2010). Pellets of treatment diets were ground into fine flour using electronic coffee grinder. 40 ml of 80 % acidified methanol (0.1 % hydrochloric acid) was added into 2 g of ground samples, vortexed to mix. After 1 hour incubation in a shaker at room temperature, samples were centrifuged at 4000 rpm for 5 minutes. Supernatants were dried under nitrogen gas at 35 °C and re-dissolved in 5 ml of 50 % methanol (Qiu et al., 2010).

5.5.2. Determination of total phenolic contents

Total phenolic contents (TPC) of the experimental diets and plasma samples were analyzed using modified Folin-Ciocalteau method (Qiu et al., 2010; Singleton & Rossi Jr, 1965). Folin-Clocalteau colorimetric assay is based on chemical reduction of reagent, a mixture of tungsten and molybdenum oxides to form a blue color mixture. The absorbance of the blue complex formed is proportional to the concentration of phenol content (Waterhouse, 2002). Briefly, 200 µl of crude extract was mixed with 1.5 ml of 10- fold diluted Folin-Ciocalteau reagent, followed by 1.5 ml of sodium carbonate (60 g/L). The absorbance of each sample was read at 725 nm using spectrophotometer after 1 hour reaction period in the dark. Gallic acid was used as a standard, and results were expressed as mg of gallic acid equivalents (GAE) per kg of rice on dry weight basis. For plasma samples, results were expressed as mg of GAE per ml of plasma.

5.5.3. Total Dietary fibre from the experimental diets

Samples from all three experimental diets were sent to the Central Testing Laboratory Ltd. (Winnipeg, MB, Canada) for total dietary fibre determination. Tests were performed according to AOAC Method 991.43, 985.29, using an enzymatic gravimetric procedure (Lee, Prosky, & De Vries, 1992; Li, Andrews, & Pehrsson, 2002). Briefly, duplicate samples were milled into flour (S1 and S2). Then, each sample was suspended in phosphate buffer and digested sequentially with α-amylase at 95 °C - 100 °C for 30 minutes, protease at 60 °C for 30 minutes, and amyloglucosidase at 60 °C for 30 minutes. Next, mixed solution was filtered and the residue (R1) was rinsed with diluted alcohol, followed by acetone. The filtrates were mixed with 4 fold of volume of 95 % ethanol to allow precipitation. After 1 hour, precipitates were filtered (R2). Both residues obtained from previous filtration were combined (R1 + R2). One of the combined residues (from S1) was then dried overnight at 105 °C in oven and subjected to protein analysis. Another combined residue (from S2) was ashed at 525 °C for 5 hours. Total dietary fibre was quantified as: [mean combined residues (from S1 + from S2) minus ash and crude protein] divided by mean sample weight (S1 + S2).

5.5.4. Food intake and body weight

Twenty-four hours food intake was estimated every 4 weeks and measurements of food intake were taken at identical times. The food given and food remaining in each cage were weighed to calculate the total food consumption. The mice bedding in the cage were checked to remove any hidden small pellets of diets before food intake

measurements were taken to avoid under or over-estimation of food consumption. Body weights of the animals were measured weekly.

5.5.5. Blood collection

Blood samples were taken at baseline and every subsequent 4 weeks period. All mice were fasted for 12 hours prior to blood collection. A maximum of 0.6 % of mice circulating blood volume was collected (approximately 150 µl) at each blood collection performed by certified animal technician at the St. Boniface Hospital Research Centre (SBRC) animal facility. Blood were drawn through jugular vein of lightly anesthetised animals using heparinized syringes. Syringes were pre-heparinized to avoid blood coagulation. At week 24, final blood samples were taken via cardiac puncture during sacrifice. The blood samples were kept on ice and centrifuged at 5000 rpm for 15 minutes (Centrifuge 5804 R; Eppendorf) at 4°C to obtain plasma samples. Aliquots of plasma were stored at -80°C for analyses.

5.5.6. Plasma lipid assays

Plasma total cholesterol (TC) and triglyceride (TG) concentrations were measured using standard enzymatic kits (Diagnostic Chemicals Limited, Charlottetown, PE, Canada) based on the manufacturer's instructions. The principle underline the enzymatic assay is the simultaneous production of hydrogen peroxide (H_2O_2) with cholest-4-en-3-one as a result of hydrolysis and oxidation of cholesterol esters. In the presence of peroxidase, hydrogen peroxide reacts with 4-aminoantipyrine and phenol to

yield a chromogen. The intensity of the color produced is proportional to the cholesterol concentration of the sample (Allain, Poon, Chan, Richmond, & Fu, 1974). Briefly, 250 µl of TC reagent was added to 3 µl of each standards and plasma samples pre-pipetted on a microplate reader. The readings were taken at 500 nm after 20 minutes of incubation at room temperature using microplate reader (autoreader EL311; Bio-tek instruments).

For TG assay, plasma triglycerides are hydrolyzed, phosphorylated and oxidized to yield H_2O_2. H_2O_2 coupled with p-chlorophenol and 4-aminoantipyrine to give rise to a red quinoneimine complex, which intensity of color is proportional to concentration of triglycerides in the sample (McGowan, Artiss, Strandbergh, & Zak, 1983). The procedures of plasma triglyceride determination were similar with that of plasma cholesterol assay, except 300 µl of reagent was added to each 5 µl of standards and plasma samples. All samples were measured in duplicate to obtain mean values.

Plasma very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) cholesterol were quantified using High Performance Gel Chromatography (HPGC), followed by online post-column enzymatic measurement as previously described (Okazaki et al., 2005; Usui, Hara, Hosaki, & Okazaki, 2002; Zhao et al., 2009). 10 µl of plasma samples were injected into the system with a flow rate of 1.0 ml/minute. These plasma lipoprotein particles were first eluted through a superose gel column that was pre-calibrated and then reacted with the enzymatic reagent in a post-column reactor. When the lipoprotein particles transverse through the column, they were separated according to their size, with the larger particle (VLDL) eluted first,

followed by the medium (LDL) and smaller (HDL) particles. Therefore, the fractions of plasma lipoprotein were defined by their retention time (VLDL, 17-18 min; LDL, 23-24 min; HDL, 33-34 min) and the concentration of each lipoprotein was quantified by measuring the area under each lipoprotein fraction's curve.

5.5.7. Tissue lipid extraction

Lipid extraction of liver tissue was performed according to a method described by Folch et al. (1957). Lipid weight and percentage of lipid to total tissue weight were calculated. Approximately 0.15 g of liver tissues was homogenized in 5 ml of 0.025 % of calcium chloride ($CaCl_2$) for 3 minutes. Then, 25 ml of chloroform: methanol (2:1, v/v) was added to each sample and vortexed for 1 minute to extract the lipids. The mixtures of samples were centrifuged at 5000rpm for 10 minutes at room temperature. 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 liver sample weight to calculate lipid weight as percentage of total liver weight.

5.5.8. Fecal lipid extraction

Lipid extraction of fecal samples was performed with slight modification from liver tissues (Folch, Lees, & Sloane-Stanley, 1957). About 0.5 g of fecal samples were weighed and soaked overnight in 5 ml of 0.025 % $CaCl_2$. The mixtures were then vortexed for 3 minutes to homogenize the softened fecal samples. Then, 25 ml of chloroform: methanol (2:1, v/v) was added to each sample and vortexed for 1 minute to

extract the lipid. Thereafter, the followed steps were identical to those previously described in section 5.5.7.

5.5.9. Hepatic and fecal cholesterol and triglyceride analyses

Cholesterol and triglyceride concentrations in the liver and fecal samples were analyzed using standard enzymatic kits (Diagnostic Chemicals Limited). First, dried hepatic and fecal lipid samples were resuspended in 1 ml of 100 % ethanol, vortexed to mix. Then, 250 µl of ethanol dissolved lipid sample was pipetted into new glass vial and dried under nitrogen gas at 50 °C. After that, 250 µl of 1 % Triton X-100 in chloroform was added into each sample, vortexed to mix and dry. Lastly, 250 µl of deionized water was added followed by vortexing and centrifuging at 5000 rpm for 10 minutes. The clear solution obtained was stored at -20 °C for TC and TG analysis as previously described in section 5.5.6.

5.5.10. Histological examination

Specimens from liver tissues were taken at sacrifice and fixed in 10 % formalin. Samples were cut and stained with oil red O as well as hematoxylin and eosin for light microscopy examinations (Moghadasian, McManus, Godin, Rodrigues, & Frohlich, 1999).

5.5.11. Total phenolic compound extraction from plasma samples

For plasma samples, total phenolic compounds were extracted according to Serafini et al., 1998. First, 1 ml of 1.0 mol/L hydrochloric acid was added into 500 µl of plasma samples, vigorously vortexed for 1 minute and then incubated for 30 minutes at 37 °C. After that, 1 ml of 2.0 mol/L sodium hydroxide in 75 % methanol was added, vortexed for 3 minutes and incubated for 30 minutes at 37 °C. Next, 1 ml of 0.75 mol/L metaphosphoric acid was added, vortexed for 3 minutes, and centrifuged at 1500 g for 10 minutes. The supernatant was kept on ice in the dark. Lastly, 1 ml of 1:1 (v/v) acetone: water was added into the residue and centrifuged at 2,700 g for 10 minutes. Both supernatants were combined for total phenolic content determination (Serafini, Maiani, & Ferro-Luzzi, 1998).

5.5.12. Plasma TBARS assay

Lipid peroxidation in plasma samples was analyzed using a thiobarbituric acid reactive substances (TBARS) assay kit (Cayman Chemical Company, Ann Arbor, MI, U.S.A.) according to the kit's instructions. The basic principle of this assay is based on the malondialdehyde (MDA)-thiobarbituric acid (TBA) adduct formed by the reaction of both MDA and TBA under low pH and high temperature (Yagi, 1998). Briefly, 12 µl of SDS (sodium dodecyl sulphate) solution was added into 12 µl of each samples and standards, followed by 480 µl of the color reagent. Mixtures of samples were then incubated in boiling water for an hour. Reactions were stopped by placing the samples on ice immediately after incubation for 10 minutes. After that, samples were

centrifuged at 5000 g at 4 °C for 6 minutes. Then, 150 µl of supernatants were loaded into plate reader in duplicate and the absorbance was read at 530 nm.

5.5.13. Hepatic TBARS assay

To prepare liver tissues for TBARS assay, approximately 25 mg of liver tissues were homogenized with 250 µl of RIPA buffer with 1 mM EDTA for 15 seconds over ice. Homogenized tissues were then centrifuged at 5000 g for 10 minutes at 4 °C and supernatants obtained were stored at -80 °C. Similar to TBARS methods described above in section 5.5.12, 12 µl of each supernatant sample was analyzed using a TBARS assay kit purchased from Cayman Chemical Company.

5.5.14. Erythrocyte lysate preparation

Erythrocytes were lysed in four times its volume of ice-cold deionized water. Mixtures were blend and centrifuged at 10,000 g for 15 minutes at 4 °C. Supernatants were kept at -80 °C for catalase and super oxide dismutase analyses.

5.5.15. Catalase assay

Catalase activities of erythrocyte lysate were analyzed using a catalase assay kit, (Item no. 707002, Cayman Chemical Company, Ann Arbor, MI, U.S.A.) following manufacturer's instructions. The method is based on the reaction of the enzyme with methanol in the presence of H_2O_2. The formaldehyde produced forms a bicyclic heterocycle with 4-amino-3-hydrazino-5-mercapto-1, 2, 4-triazole, which give a purple color upon oxidation (Johansson & Borg, 1988). Briefly, 100 µl of assay buffer and 30 µl

of methanol were added into 20 µl of each standards and samples00 in the microplate reader. The reaction was initiated by adding 20 µl of diluted hydrogen peroxide to all wells, and the plate was covered and incubated on a shaker for 20 minutes at room temperature. Then, 30 µl of potassium hydroxide was added to each well to terminate the reaction, followed by 30 µl of chromagen. The plate was covered and incubated for another 10 minutes. Lastly, 10 µl potassium periodate was added to each well and incubated for 5 minutes. Absorbance was taken at 540 nm.

5.5.16. Superoxide dismutase assay

Super oxide dismutase (SOD) activities of erythrocyte lysate were analyzed using an SOD assay kit (Item no. 706002, Cayman Chemical Company, Ann Arbor, MI, U.S.A.) following the manufacturer's instructions. This assay utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. Diluted radical detector (200 µl) was added into 10 µl of each standards and samples in the microplate reader. Then, 20 µl of diluted xanthine oxidase was added. The plate was covered and incubated on shaker for 20 minutes at room temperature. Absorbance was read at 440-460 nm.

5.6. Statistical analysis

Data were analyzed using SPSS 11.5 statistical software for Windows (SPSS Inc., Chicago, IL, U.S.A). Homogeneity of variances between groups was assessed by Levene's test for equality of error variance. Then, two-way analysis of variance (ANOVA), followed by the post-hoc Tukey test were performed to determine the statistically significant differences among the experimental groups, genders, and the group x gender interaction. Comparison between genders within each treatment groups were performed if the gender effect or the group x gender interaction was significant. Results are presented as means +/- standard deviation. The statistical significance value was determined at $p<0.05$.

6. Results

6.1. Total phenolic content of experimental diets

Total phenolic content of three experimental diets are shown in **Figure 2**. Incorporation of wild rice significantly increased the total phenolic contents of the wild rice diet as compared to control and white rice diets. The mean phenolic content of wild rice diet was 0.52 mg of gallic acid equivalent (GAE) per gram of sample, which was 4-fold of that of control (0.12 mg of GAE/g) or white rice diets (0.13 mg of GAE/g). As compared to the control diet, white rice diet contained about 7 % higher total phenolic contents.

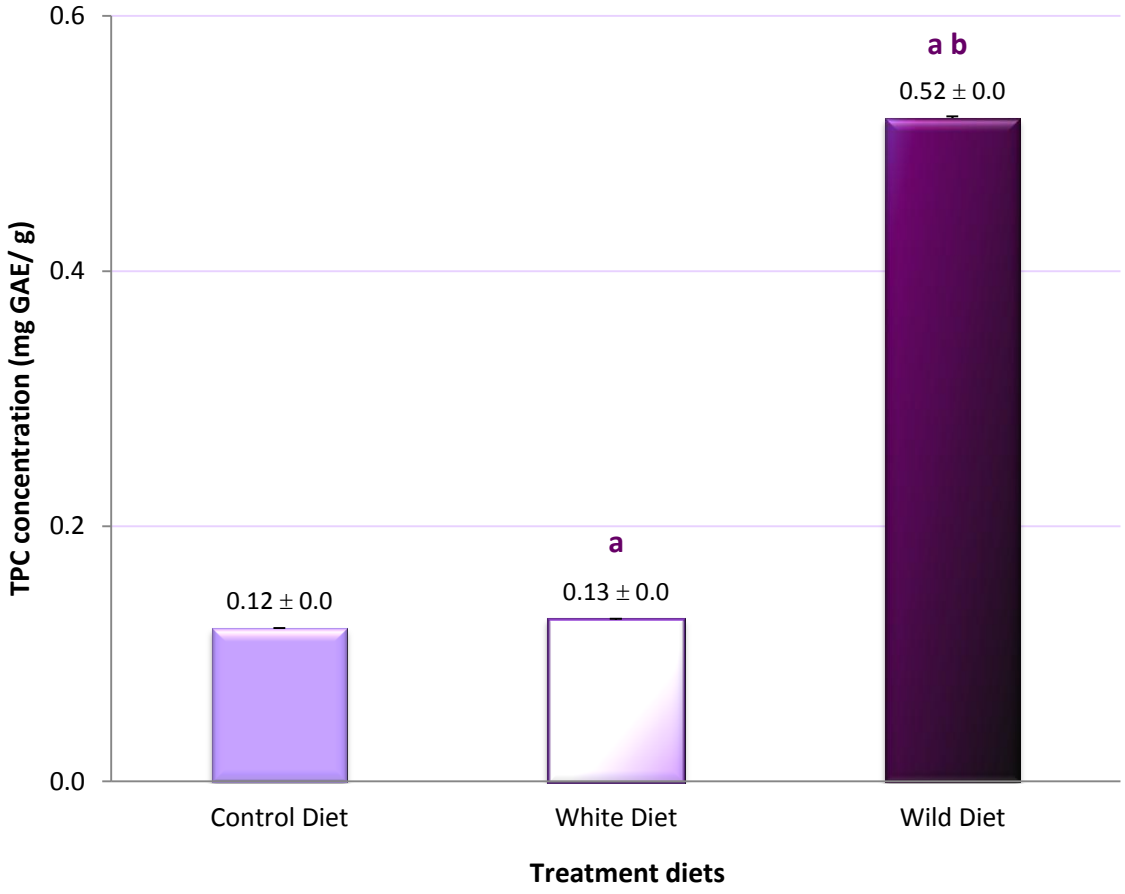
6.2. Total dietary fibre of the experimental diets

Total dietary fibre contents of control, white rice and wild rice diet were 6.5, 5.3, and 8.3 g dietary fibre/ 100 gram of sample, respectively. Wild rice diet with a total of 8.3 g dietary fibre/ 100g of sample showed approximately 22 %, and 36% more fiber contents as compared to the control or white rice diets, respectively.

6.3. Food intake

Overall female mice consumed less food than male mice did. Female mice on control, white, and wild diet had 11 %, 16 %, and 12 % lower food intake as compared to male mice on the same diet. However, no statistically significant differences were observed among the groups in both genders, as presented in **Figure 3**.

Figure 2. Mean total phenolic content (TPC) of experimental diets



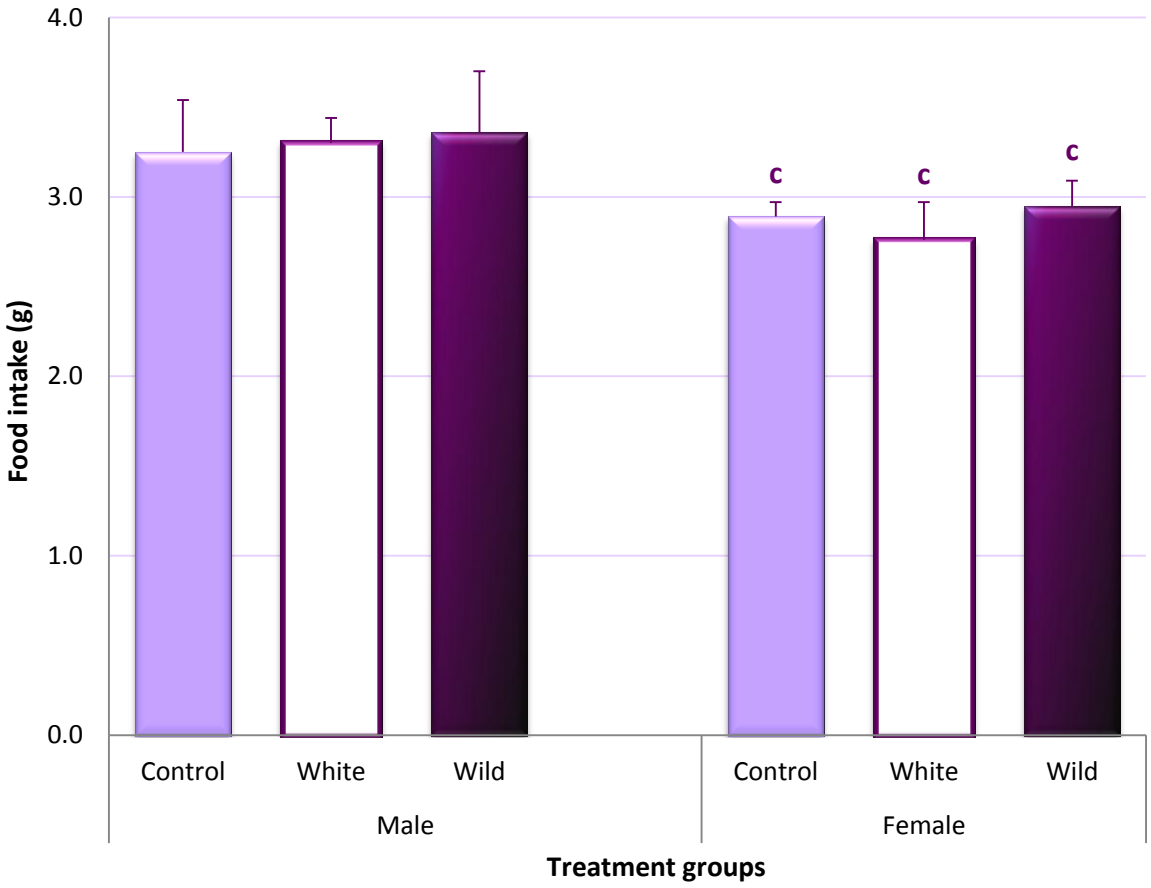
Data are presented as mean ± standard deviation

Values are presented as mg of gallic acid equivalent (GAE) per g of sample

[a] $p<0.05$ compared to control group

[b] $p<0.05$ compared to white rice group

Figure 3. Mean daily food intake per mice per group



Data are presented as mean ± standard deviation

(Male & female: control, n=6; white rice, n=6; wild rice, n=6)

[c] $p<0.05$ compared to male fed with the same experimental diet

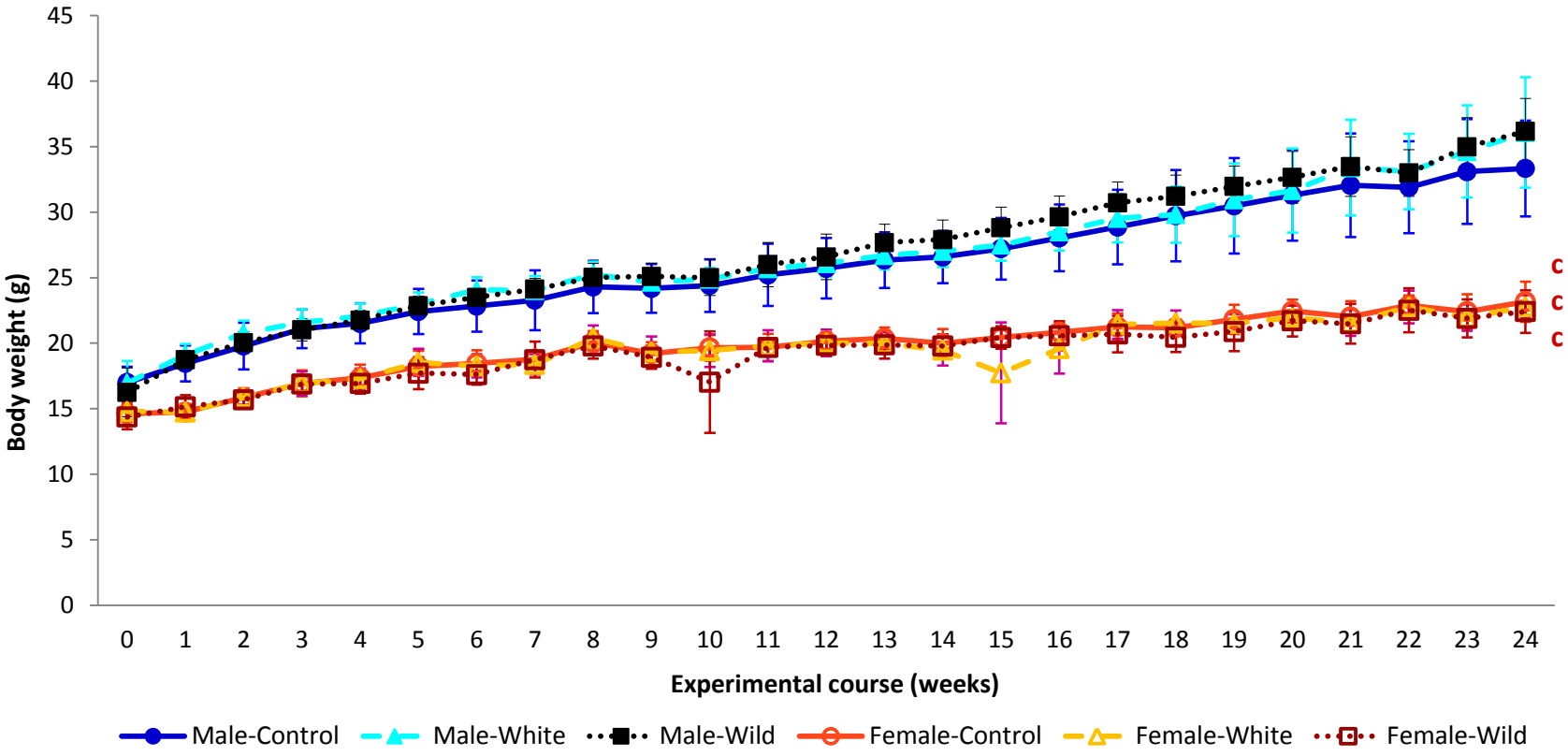
6.4. Body weight

Mean body weight of the mice did not significantly differ among the groups in both genders throughout the study. However, female mice in all groups had significantly lower body weight compared to male mice, as illustrated in **Figure 4**. At week-24, female mice had 31 %, 37 %, and 38 % lower body weight than male mice in control, white rice, and wild rice diet groups, respectively. Besides, a steady weight gain was observed in all diet groups over the experimental course, which may indicate a high degree of diet tolerance.

6.5. Tissue weight

The weight of the body tissues, including abdominal fat, liver, kidney, spleen, and heart of the experimental animals are presented in **Table 7**. Similar to the body weight, differences of body tissues among the groups are not statistically significant in either gender. Female mice in all diet groups had significantly smaller abdominal fat, kidney, and heart versus male mice in the same diet group.

Figure 4. Mean body weight per group



Data are presented as mean ± standard deviation (male & female: control, n=6; white rice, n=6; wild rice, n=6)

[c] $p<0.05$ compared to male fed with the same experimental diet

Table 7. Mean tissue (abdominal fat, liver, spleen, kidney, and heart) weight

Gender	Treatment groups	Abdominal fat	Liver	Spleen	Kidney	Heart
MALE	Control	0.99 ± 0.43	1.11 ± 0.15	0.07 ± 0.00	0.35 ± 0.03	0.14 ± 0.02
	White	1.35 ± 0.66	1.23 ± 0.17	0.08 ± 0.01	0.38 ± 0.01	0.15 ± 0.01
	WILD	1.49 ± 0.30	1.29 ± 0.20	0.07 ± 0.01	0.39 ± 0.04	0.15 ± 0.01
FEMALE	Control	0.01 ± 0.03 [c]	0.89 ± 0.06	0.07 ± 0.02	0.26 ± 0.01 [c]	0.11 ± 0.01 [c]
	White	0.00 ± 0.00 [c] *	0.91 ± 0.06 [c]	0.07 ± 0.01	0.26 ± 0.02 [c]	0.11 ± 0.01 [c]
	WILD	0.00 ± 0.00 [c] *	0.86 ± 0.04 [c]	0.07 ± 0.01	0.26 ± 0.02 [c]	0.11 ± 0.01 [c]

Data are presented as mean ± standard deviation

(Male & female: control, n=6; white rice, n=6; wild rice, n=6)

[c] $p<0.05$ compared to male fed with the same experimental diet

* Amount is too small, below the limit of measurement. Number '0.00' is included for statistical analysis

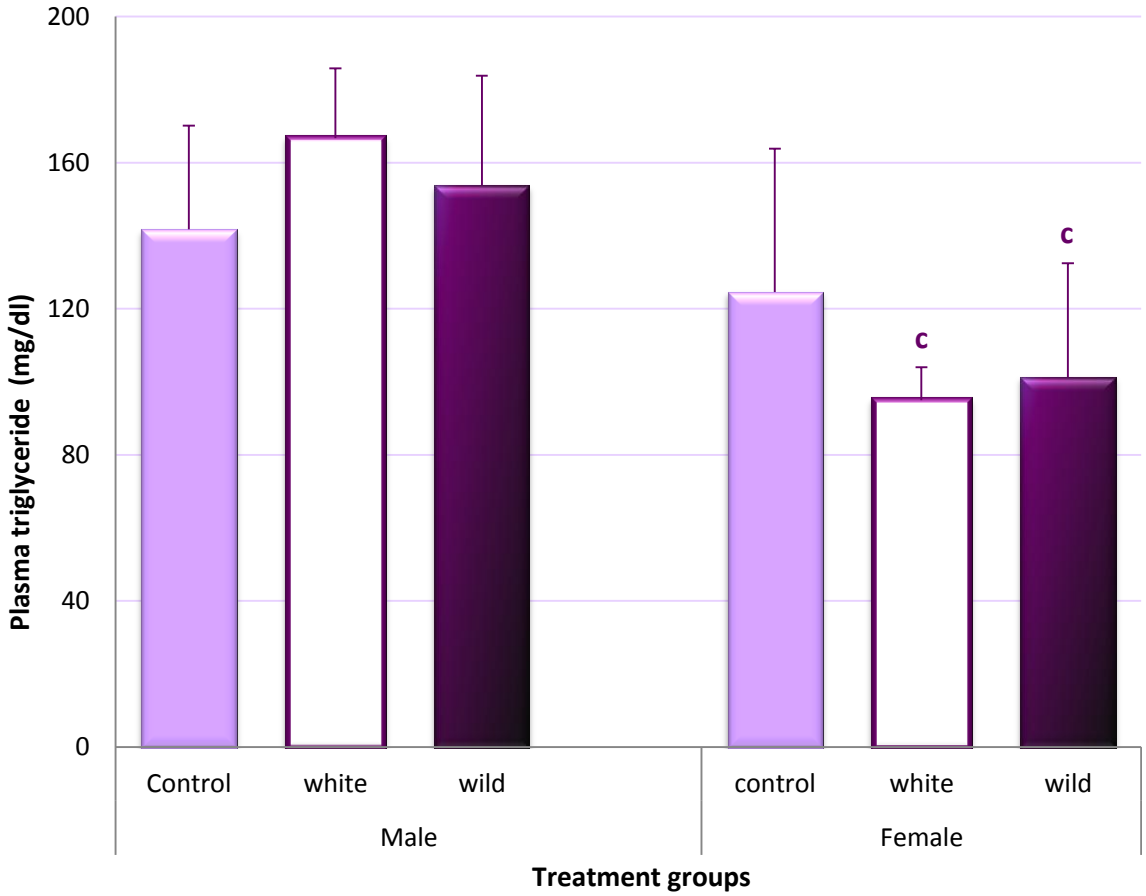
6.6. Plasma triglycerides (TG)

Plasma triglyceride concentrations at week 24 were comparable among all treatment groups in both male and female mice (**Figure 5)**. Female mice fed with white rice and wild rice diet had significantly lower plasma triglyceride concentrations, 43 % and 34 % respectively, as compared to male mice fed with the same treatment diet.

6.7. Plasma total cholesterol (TC) levels

Female mice fed with the white rice or wild rice diets had significantly lower plasma TC concentrations compared to female mice fed the control diet. Plasma TC level of female white rice and wild rice groups were 24 % and 40 % lower relative to that in the female control group. On the other hand, male mice fed with wild rice diet shown to have the lowest plasma TC concentration, 20 % and 24 % significantly lower versus male in control and white rice diet group. However, plasma TC level of male mice was all statistically higher than that in female mice. As compared to male mice, plasma TC values of female mice were 21 %, 44 %, and 41 % lower in control, white rice, and wild rice diet group respectively. Results of the plasma TC concentrations were presented in **Figure 6.**

Figure 5. Plasma triglycerides concentrations

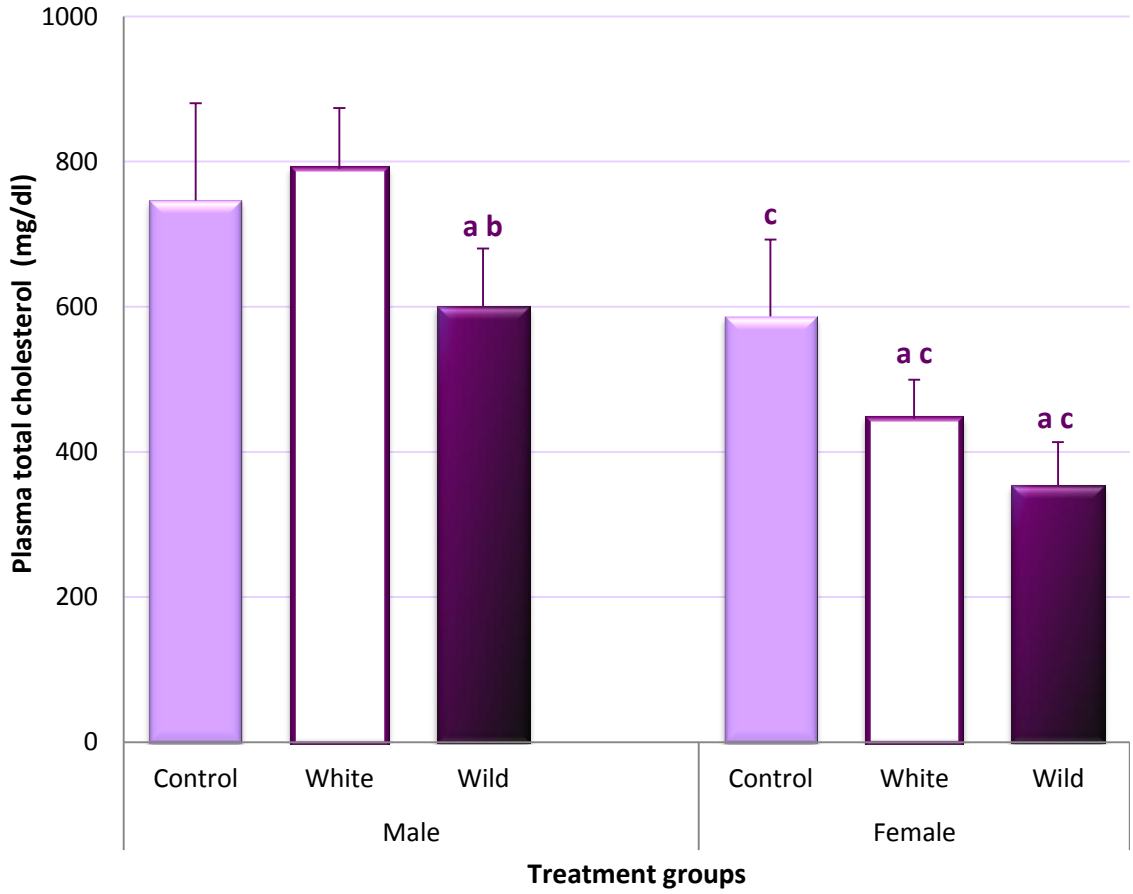


Data are presented as mean ± standard deviation

(Male & female: control, n=6; white rice, n=6; wild rice, n=6)

[c] $p<0.05$ compared to male fed with the same experimental diet

Figure 6. Plasma total cholesterol concentrations



Data are presented as mean ± standard deviation

(Male & female: control, n=6; white rice, n=6; wild rice, n=6)

[a] $p<0.05$ compared to control diet in the same gender group

[b] $p<0.05$ compared to white rice diet in the same gender group

[c] $p<0.05$ compared to male fed with the same experimental diet

6.8. Plasma lipoprotein profile

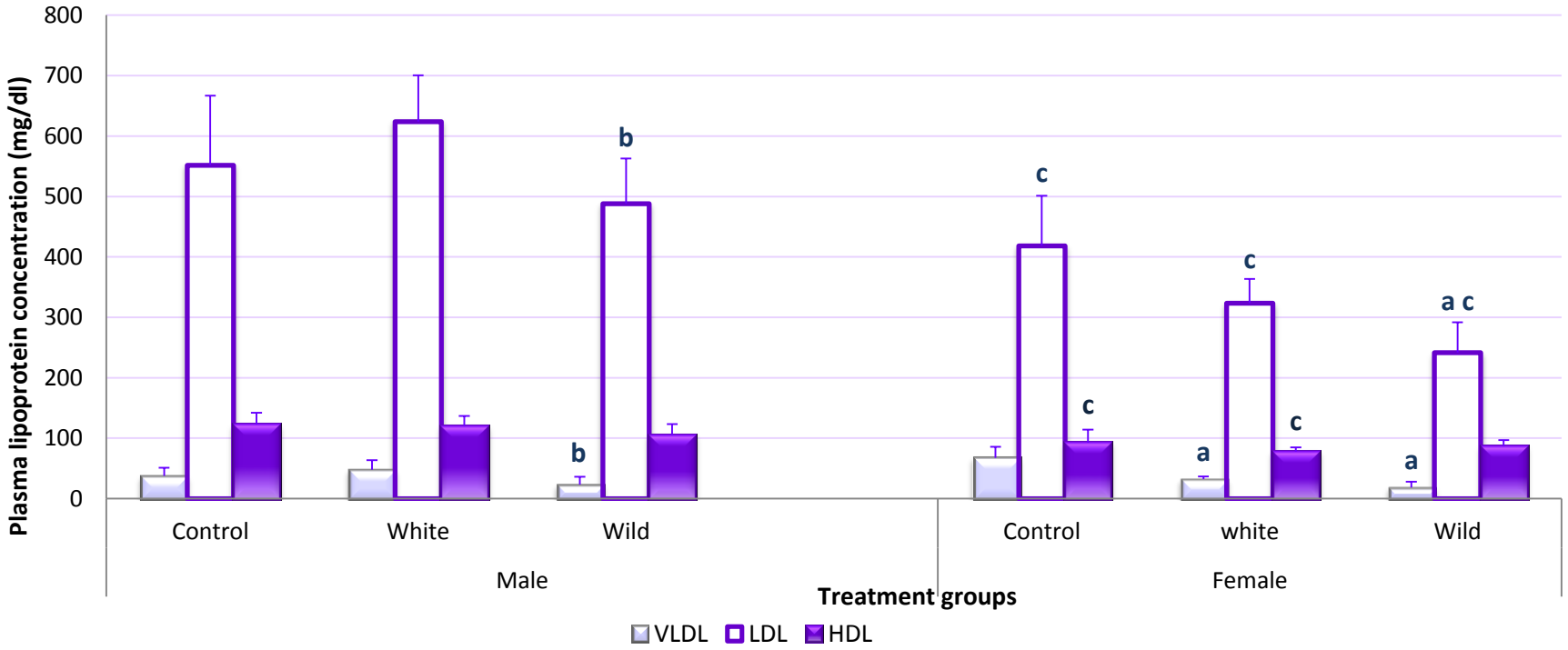
Mean VLDL, LDL, and HDL-cholesterol concentrations of the experimental animals were determined and illustrated in **Figure 7**. Representative HPGC chromatograms of male and female mice were captured and demonstrated in **Figure 8** and **Figure 9,** respectively. Total area percentage under HPGC chromatogram curve of each class of lipoprotein particles was measured and presented in **Table 8**.

VLDL and LDL-cholesterol levels of male mice fed with wild rice diet were statistically differed from those of male mice fed with white rice diet. Male wild rice group had about 51 % and 22 % lower VLDL and LDL-cholesterol, respectively compared to male white rice group. No differences were found among HDL-cholesterol values between groups.

On the other hand, female white rice and wild rice groups had significantly lower VLDL-cholesterol concentrations relative to the female control group. VLDL-cholesterol concentrations of female white rice and wild rice groups were 52 % and 73 % lower, respectively, than those in female control group. In addition, LDL-cholesterol levels of female wild rice diet (241.5 mg/dl) were the lowest among all. Female wild rice group versus control showed a significant 42 % lower LDL cholesterol level. Furthermore, female mice also showed significantly lower values for LDL-cholesterol as much as 24 %, 48 %, and 51 % in the control, white rice, and wild rice groups, respectively, as compared to that in male mice fed the same treatment diets. However, HDL-cholesterol concentrations of female mice were comparable among all treatment groups. Female

mice fed with the control and white rice diets showed significantly lower HDL-cholesterol levels than that in the corresponding male counterparts.

Figure 7. Plasma lipoprotein profile (VLDL, LDL, and HDL-cholesterol concentrations)



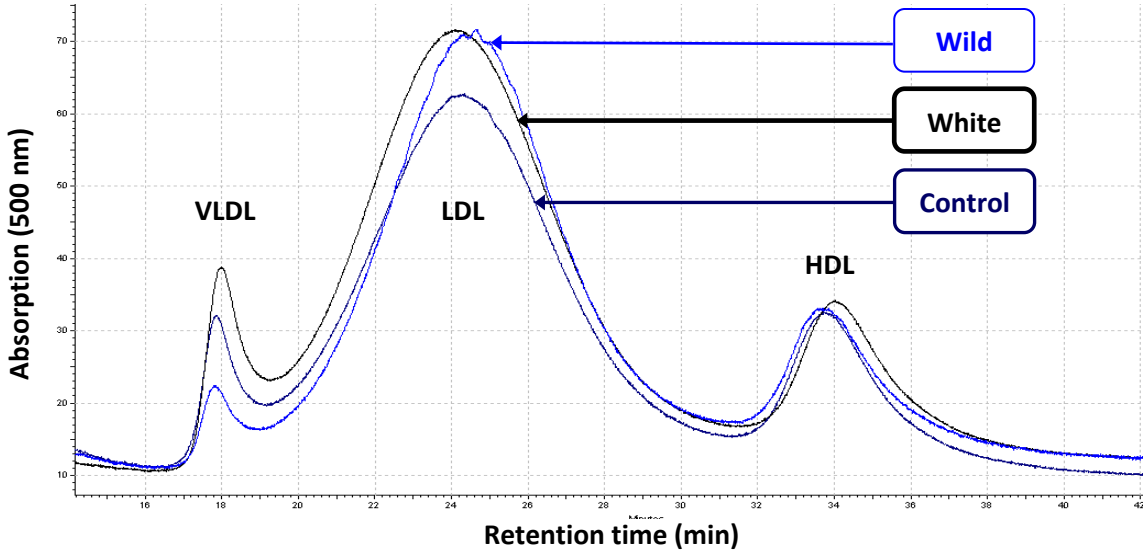
Data are presented as mean ± standard deviation (Male & female: control, n=6; white rice, n=6; wild rice, n=6)

[a] $p<0.05$ compared to control diet in the same gender group

[b] $p<0.05$ compared to white rice diet in the same gender group

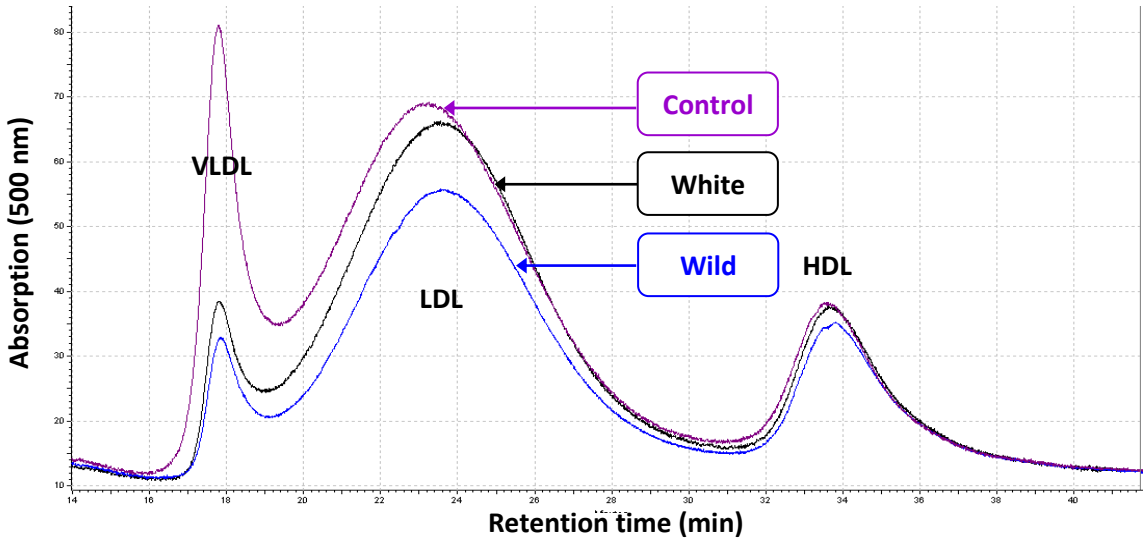
[c] $p<0.05$ compared to male fed with the same experimental diet

Figure 8. HPGC chromatograms of plasma lipoprotein profile of male mice



Representative plasma lipoprotein profile of male mice fed with control, white, or wild rice

Figure 9. HPGC chromatograms of plasma lipoprotein profile of female mice



Representative plasma lipoprotein profile of female mice fed with control, white, or wild rice

Table 8. Plasma lipoprotein profile (VLDL, LDL, and HDL-cholesterol %)

Gender	Groups	VLDL	LDL	HDL
MALE	Control	6.0 ± 2.2	77.0 ± 3.4	17.0 ± 4.2
	White	6.1 ± 1.3	79.7 ± 3.4	14.2 ± 3.3
	WILD	4.0 ± 1.6	80.2 ± 3.8	15.8 ± 5.2
FEMALE	Control	12.6 ± 2.7 [c]	71.5 ± 2.4 [c]	15.9 ± 1.4
	White	8.1 ± 1.6 [a]	74.8 ± 1.1 [c]	17.1 ± 2.4
	WILD	5.1 ± 2.0 [a]	69.4 ± 2.9 [b c]	5.6 ± 4.5 [a b c]

Data are presented as mean ± standard deviation

(Male & female: control, n=6; white rice, n=6; wild rice, n=6)

[a] $p<0.05$ compared to control diet in the same gender group

[b] $p<0.05$ compared to white rice diet in the same gender group

[c] $p<0.05$ compared to male fed with the same experimental diet

6.9. Liver weight

The proportion of liver weight to total body weight of the experimental mice is demonstrated in **Figure 10**. There was no significant difference among three treatment groups in either male or female mice. However, female mice in all groups had higher proportion liver weight as compared to male mice.

6.10. Total liver lipid concentrations

Liver lipid was extracted and expressed as percentage of total liver weight as presented in **Figure 11**. Total liver lipids were found to be comparable among control, white rice and wild rice diet groups in both genders. However, control female mice had about 28 % higher total lipid percentage than control male did.

6.11. Liver cholesterol concentrations

After liver lipid extraction, liver cholesterol levels were determined and presented in **Figure 12**. The differences between three treatment groups and among both genders were not statistically significant.

6.12. Liver triglyceride concentrations

Similar to the results of liver cholesterol levels, triglyceride concentrations of male and female mice did no differ significantly among the three different diet groups. Liver triglyceride concentrations of the mice are shown in **Figure 13**.

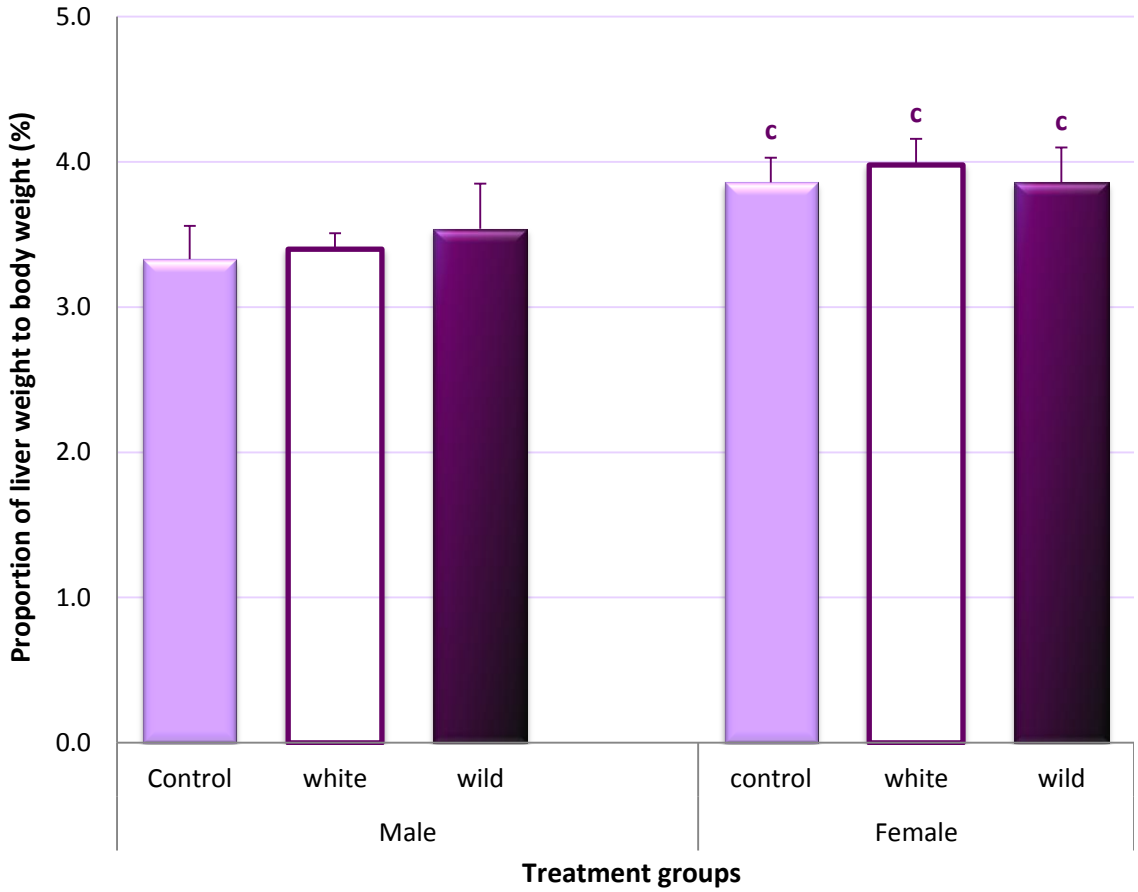
6.13. Liver malondialdehyde concentrations

Lipid peroxidation levels of liver tissues were assessed using TBARS assay. Results are illustrated in **Figure 14**. There was no significant difference among the three different treatment groups or between corresponding male and female mice.

6.14. Liver histology

Histological examinations of the sections obtained from the liver tissues were done, and the representative images are shown in **Figure 15.** Minimal fatty changes were noticed in all male mice (Panel A: control; Panel B: white; and panel C: wild) and female mice in control (Panel D), white rice (Panel E), and wild rice (Panel F) groups. However, the differences between three treatment groups and among both genders were not significant.

Figure 10. Proportion liver weight of total body weight

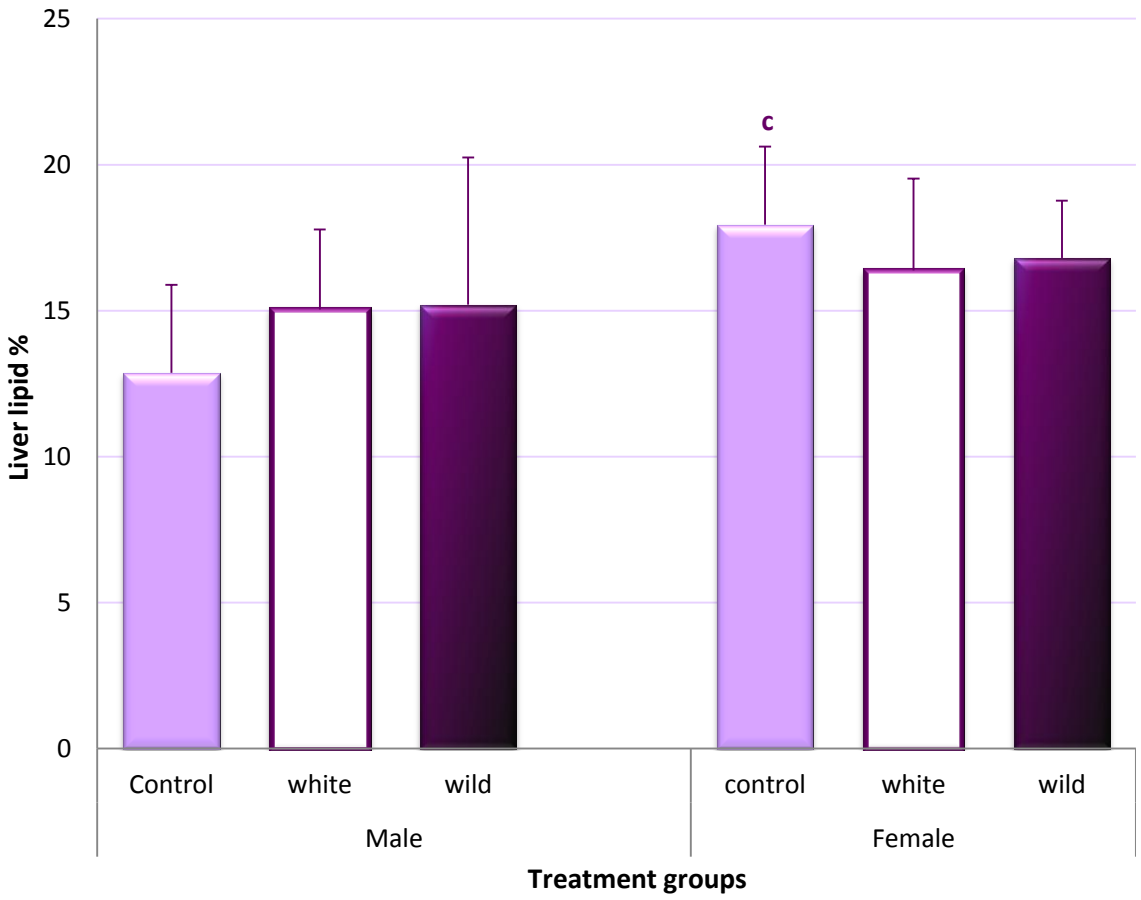


Data are presented as mean ± standard deviation

(Male & female: control, n=6; white rice, n=6; wild rice, n=6)

[c] $p<0.05$ compared to male fed with the same experimental diet

Figure 11. Total liver lipid expressed as percentage of total liver weight

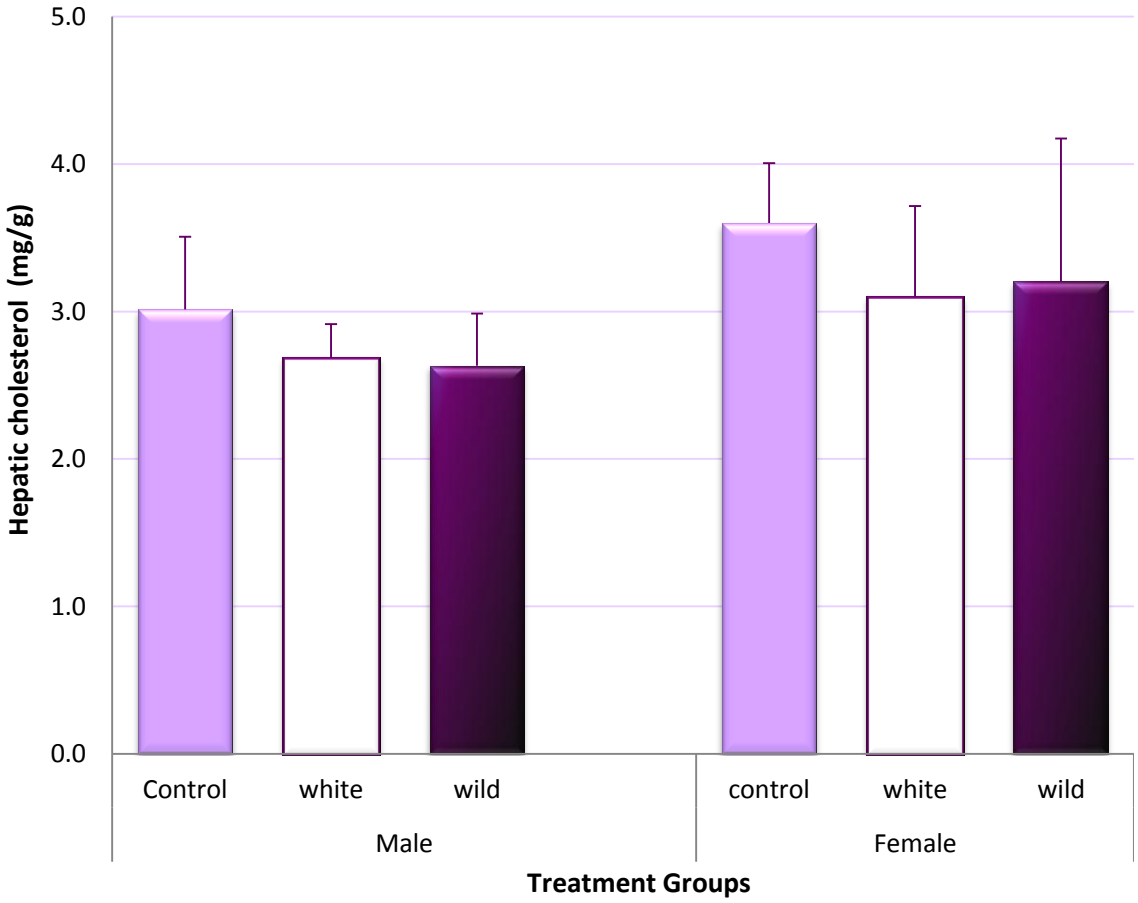


Data are presented as mean ± standard deviation

(Male & female: control, n=6; white rice, n=6; wild rice, n=6)

[c] $p<0.05$ compared to male fed with the same experimental diet

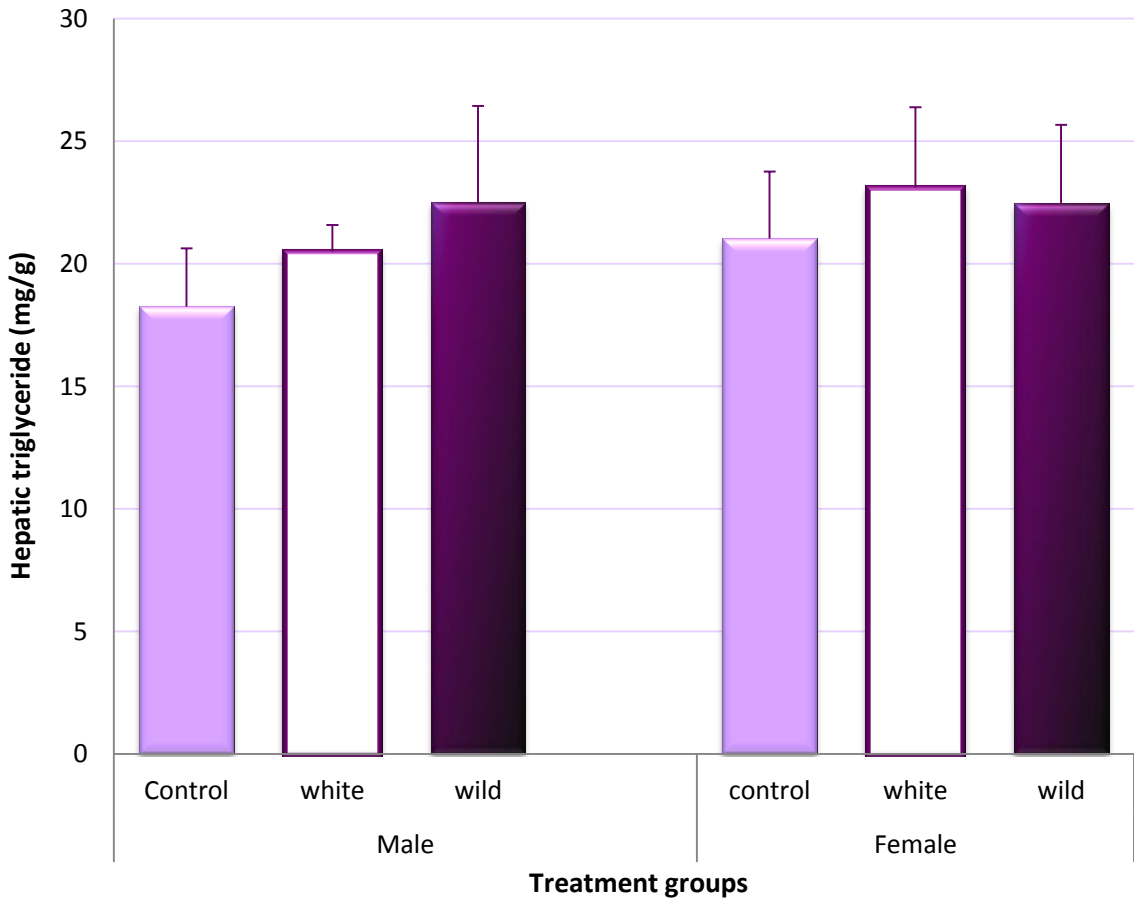
Figure 12. Liver cholesterol concentrations



Data are presented as mean ± standard deviation

(Male & female: control, n=6; white rice, n=6; wild rice, n=6)

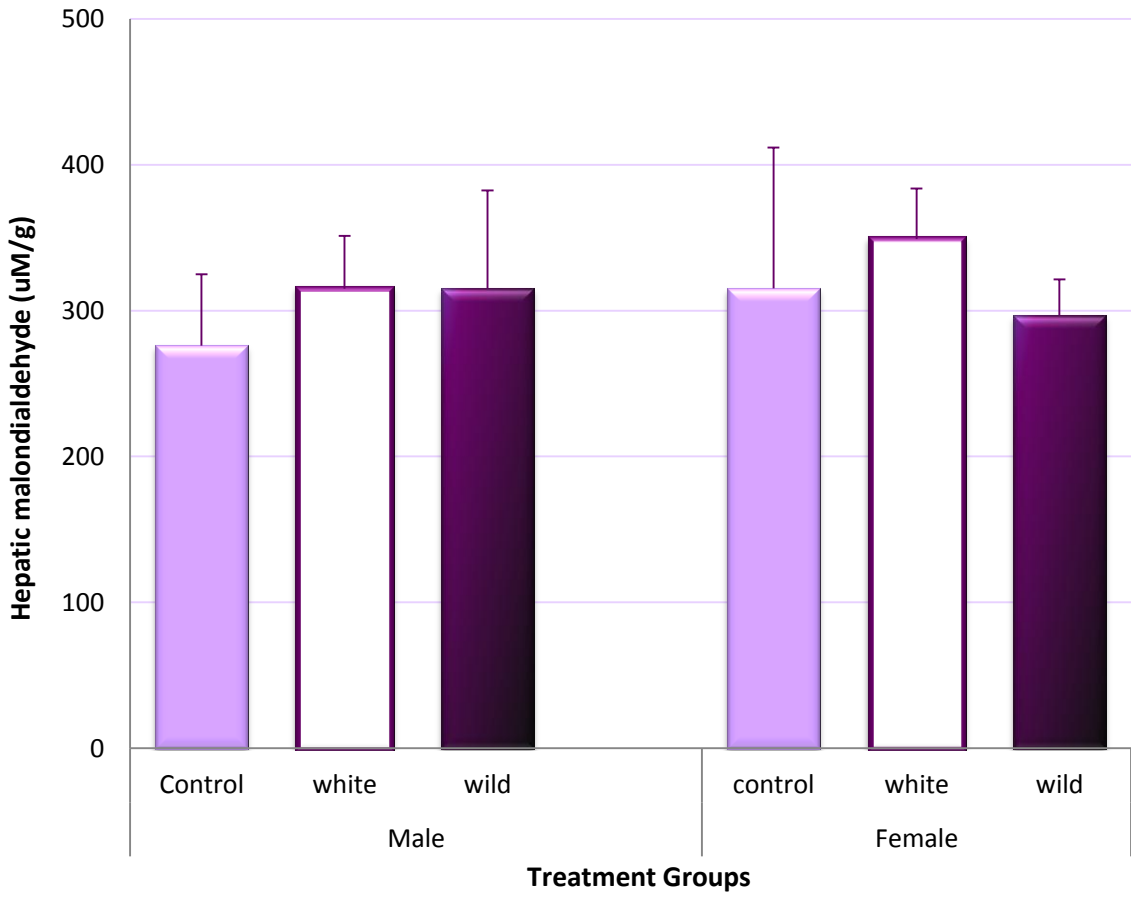
Figure 13. Liver triglyceride concentrations



Data are presented as mean ± standard deviation

(Male & female: control, n=6; white rice, n=6; wild rice, n=6)

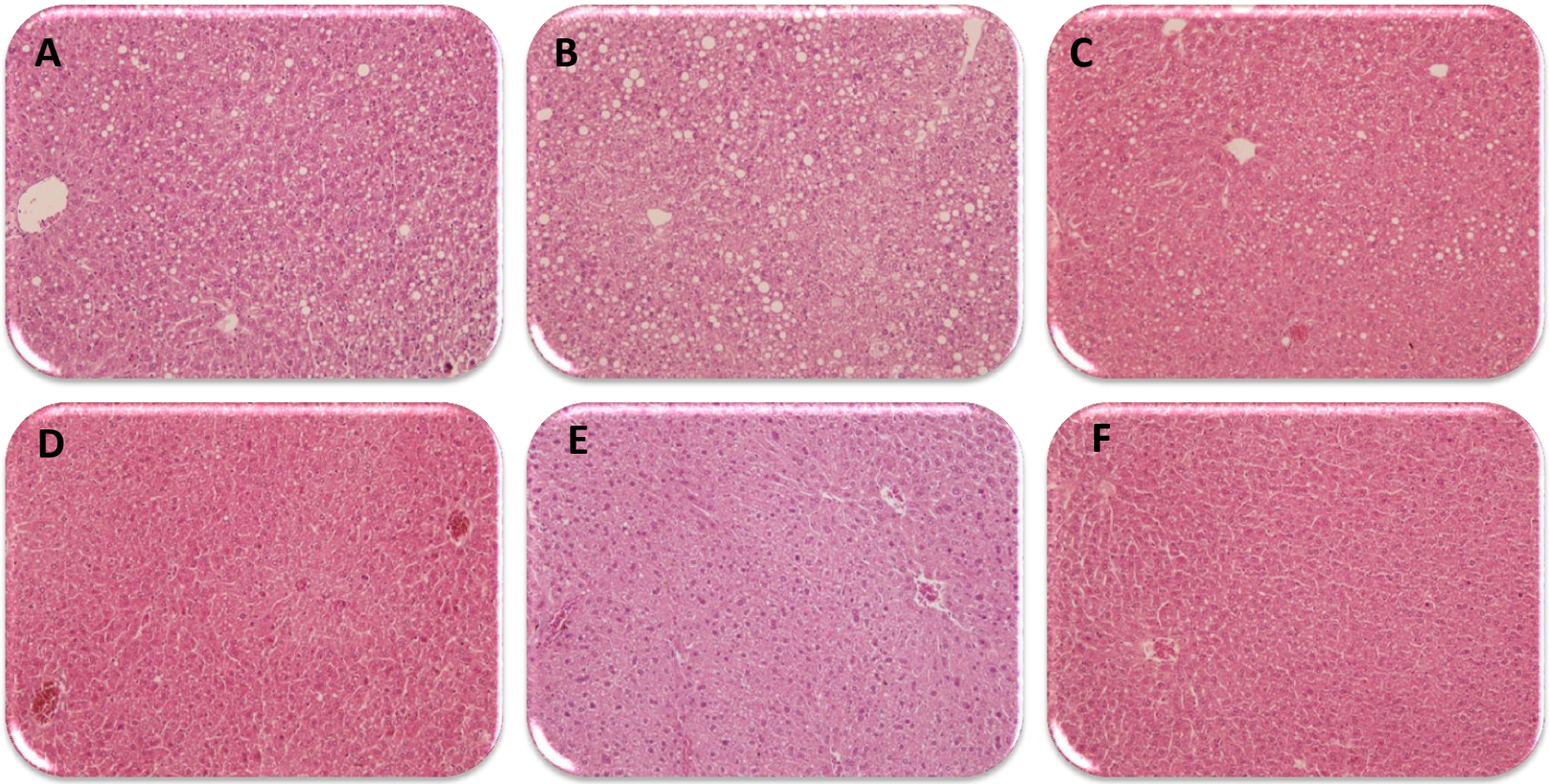
Figure 14. Liver malondialdehyde concentrations



Data are presented as mean ± standard deviation

(Male & female: control, n=6; white rice, n=6; wild rice, n=6)

Figure 15. Representative photomicrograph showing histological features of liver tissue of the experimental mice



Panel A: male control; **Panel B**: male white rice; **Panel C**: male wild rice,

Panel D: female control; **Panel E**: female white rice; **Panel F**: female wild rice

6.15. Total fecal lipid concentrations

Fecal samples were collected for lipid extraction, and fecal lipid percentages were calculated. As shown in **Figure 16**, male and female mice fed wild rice diet had significantly higher fecal lipid content than control and white rice diet groups. Male wild rice group had 21 % and 22 % higher fecal lipid percentages; while female wild rice group contained 34 % and 23 % higher lipid contents, respectively as compared to control and white rice diet groups.

6.16. Fecal cholesterol concentrations

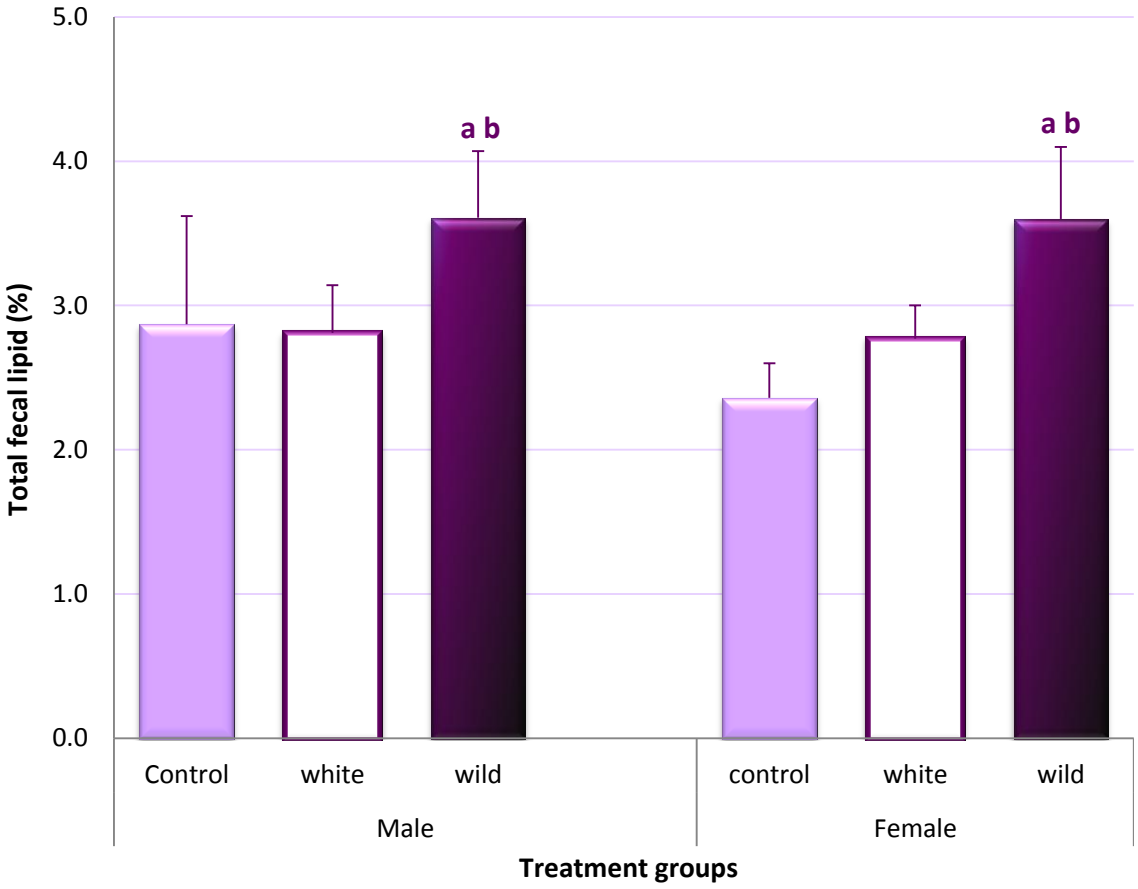
Extracted fecal lipids were subjected to cholesterol concentration determination, and results are presented in **Figure 17**. Male wild rice group had higher fecal total cholesterol levels, approximately 30 % higher than control group. In addition, female fed with wild rice diet contained significantly highest fecal cholesterol level among all groups. Fecal cholesterol concentration of female wild rice group was about 39 % and 32 % higher than that in female control and white diet groups, respectively.

6.17. Fecal triglyceride concentrations

Male and female mice fed wild rice diet contained significantly higher triglyceride levels compared to control and white rice diet groups. Addition of wild rice in the diet had significantly increased excretion of fecal triglyceride concentration of male mice at 54 % and 41 %; female mice at about 58 % and 48 %, as compared to mice fed control

and white rice diet, respectively. Moreover, fecal triglyceride levels of female with wild rice diet also significantly higher (24%) relative to male wild rice diet group. Triglyceride levels of fecal samples of the experimental animals at week 24 are illustrated in **Figure 18**.

Figure 16. Total fecal lipid concentrations



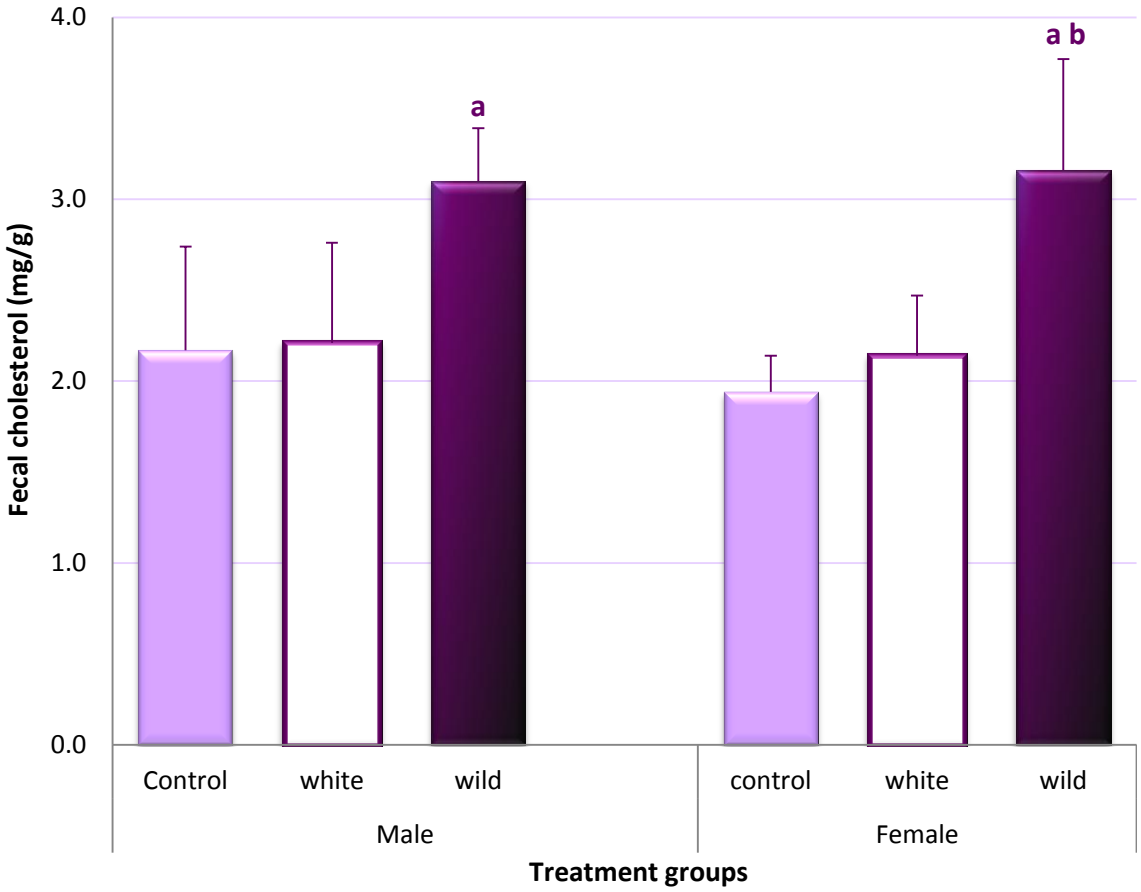
Data are presented as mean ± standard deviation

(Male & female: control, n=6; white rice, n=6; wild rice, n=6)

[a] $p<0.05$ compared to control diet in the same gender group

[b] $p<0.05$ compared to white rice diet in the same gender group

Figure 17. Fecal cholesterol concentrations



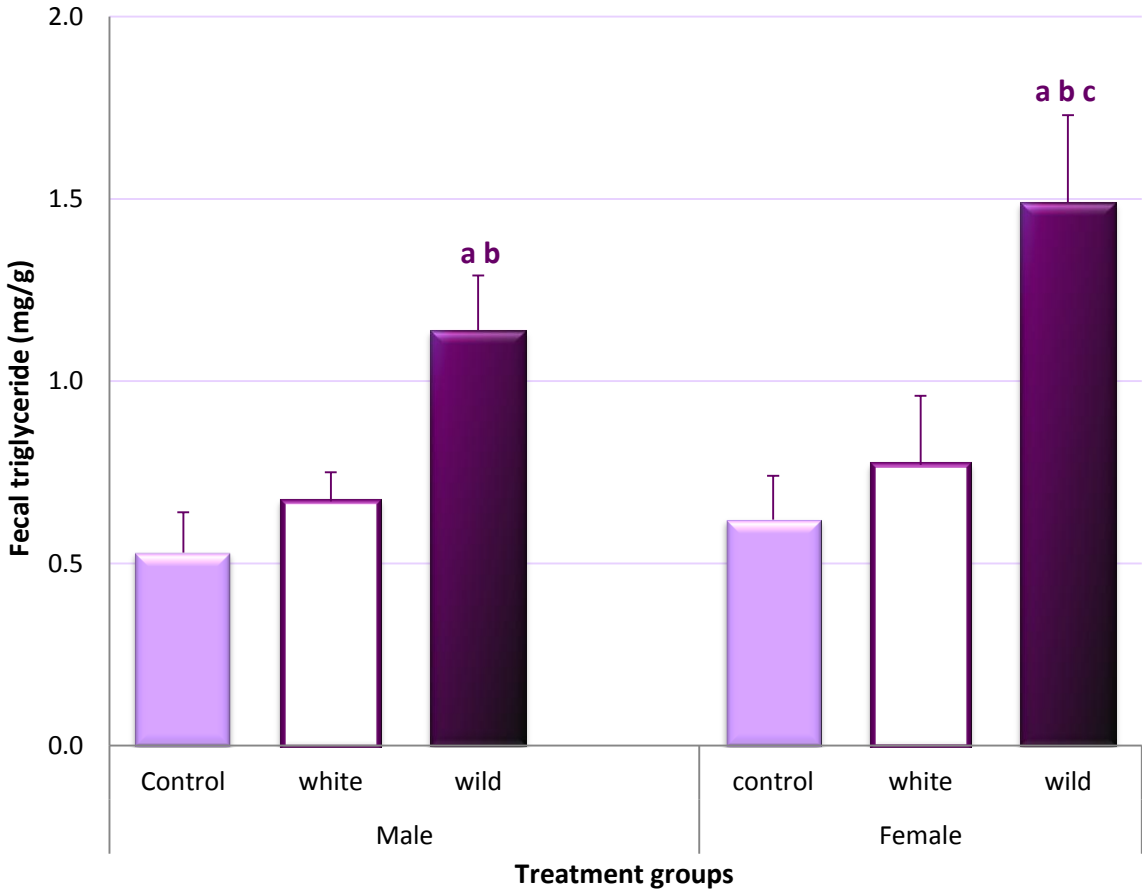
Data are presented as mean ± standard deviation

(Male & female: control, n=6; white rice, n=6; wild rice, n=6)

[a] $p<0.05$ compared to control diet in the same gender group

[b] $p<0.05$ compared to white rice diet in the same gender group

Figure 18. Fecal triglyceride concentrations



Data are presented as mean ± standard deviation

(Male & female: control, n=6; white rice, n=6; wild rice, n=6)

[a] $p<0.05$ compared to control diet in the same gender group

[b] $p<0.05$ compared to white rice diet in the same gender group

[c] $p<0.05$ compared to male fed with the same experimental diet

6.18. Plasma total phenolic contents

As shown on **Figure 19**, plasma total phenolic contents were similar among three different diet groups in both male and female mice. However, significantly higher total phenolic contents were detected in plasma samples of female mice. Female mice fed with control, white rice, and wild rice diet each had an increase of 29 %, 27 %, and 35 % of plasma total phenolic contents, respectively as compared to male mice fed with the same treatment diet.

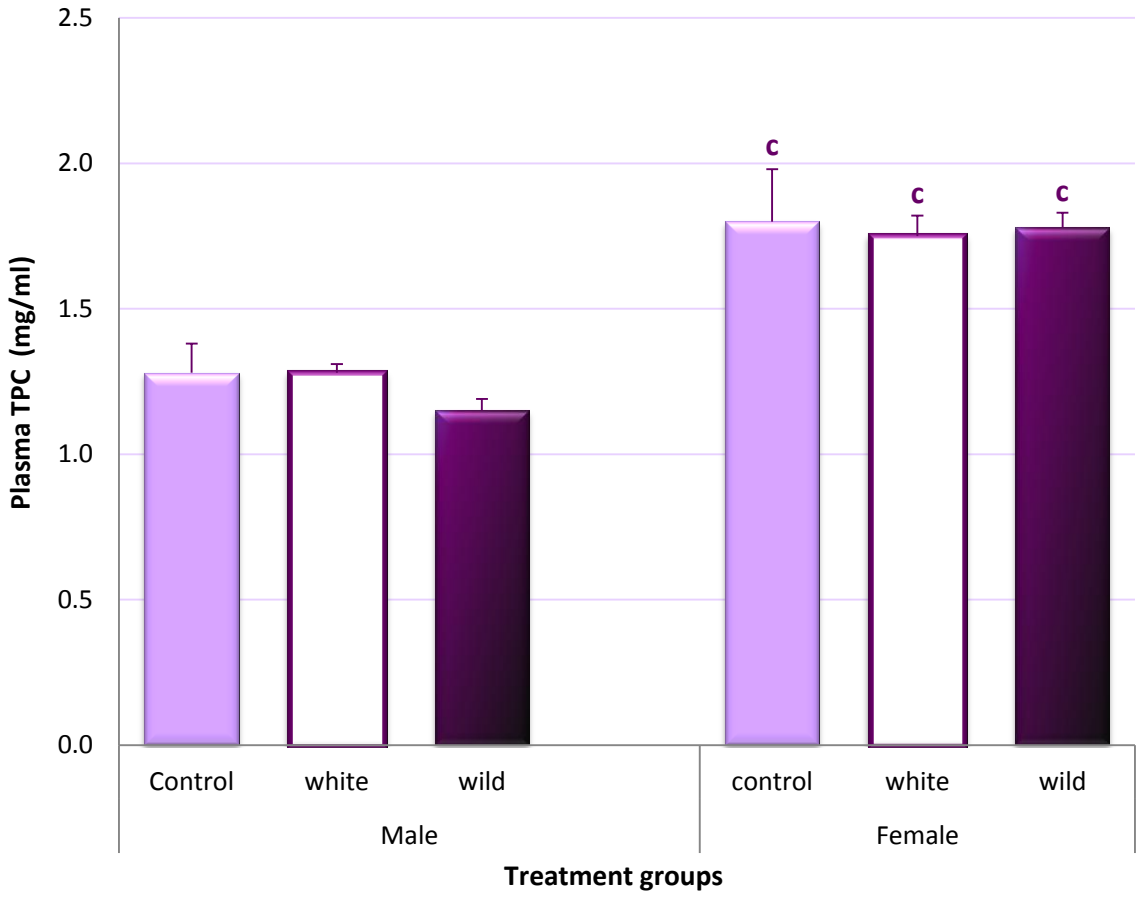
6.19. Plasma malondialdehyde levels

Plasma malondialdehyde (MDA) levels of the mice were measured at the end point, and are presented on **Figure 20**. There were no significant differences in plasma MDA concentrations among three different diet groups in both genders. Yet, female mice in all groups were found to have significantly lower plasma MDA levels than that of male mice fed with similar diet. Compared to male mice, female mice had 40 %, 62 %, and 59 % lower plasma MDA levels in control, white rice, and wild rice diet group, respectively.

6.20. Erythrocyte catalase and superoxide dismutase activity

Both erythrocyte catalase and superoxide dismutase activities were comparable between all treatment groups and among both male and female mice. Erythrocyte catalase activities of the experimental animals are presented on **Figure 21**; and superoxide dismutase activities shown on **Figure 22.**

Figure 19. Plasma total phenolic contents



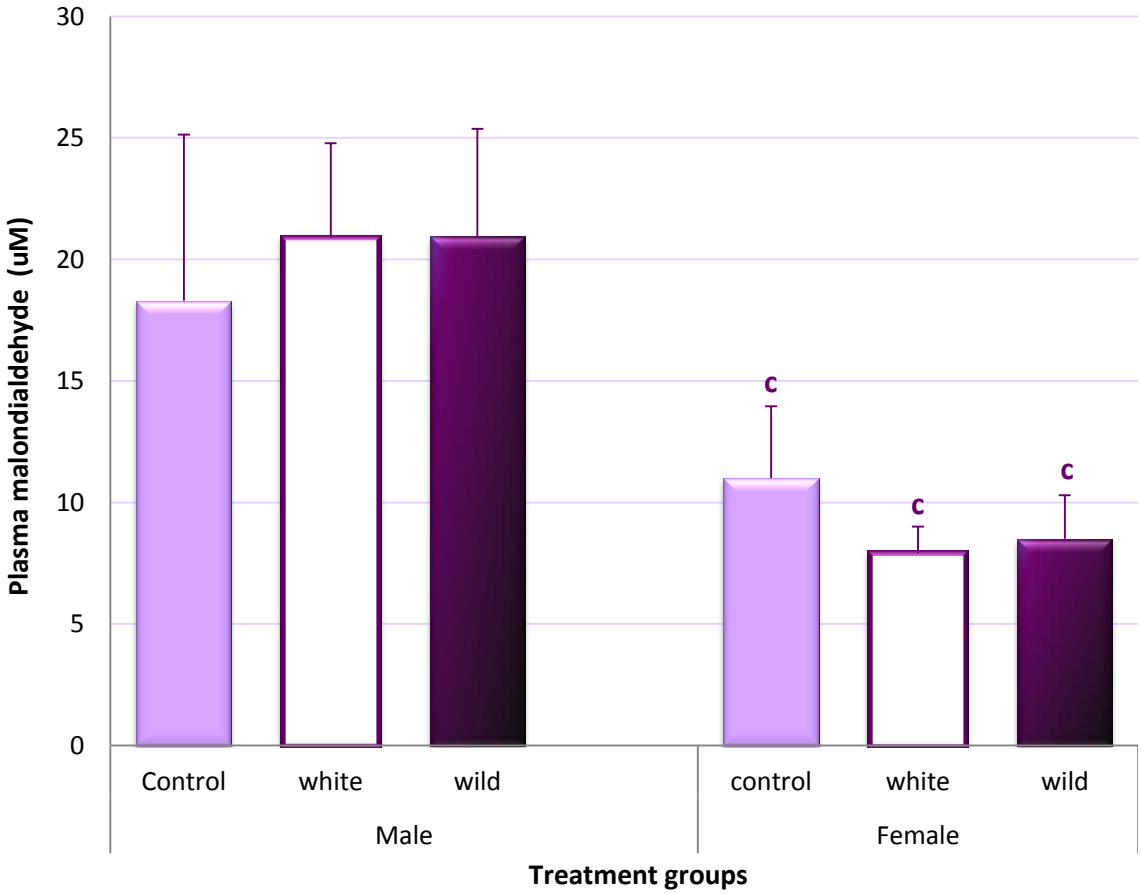
Data are presented as mean ± standard deviation

Values are presented as mg of gallic acid equivalent (GAE) per ml of sample

(Male & female: control, n=6; white rice, n=6; wild rice, n=6)

[c] $p<0.05$ compared to male fed with the same experimental diet

Figure 20. Plasma malondialdehyde levels

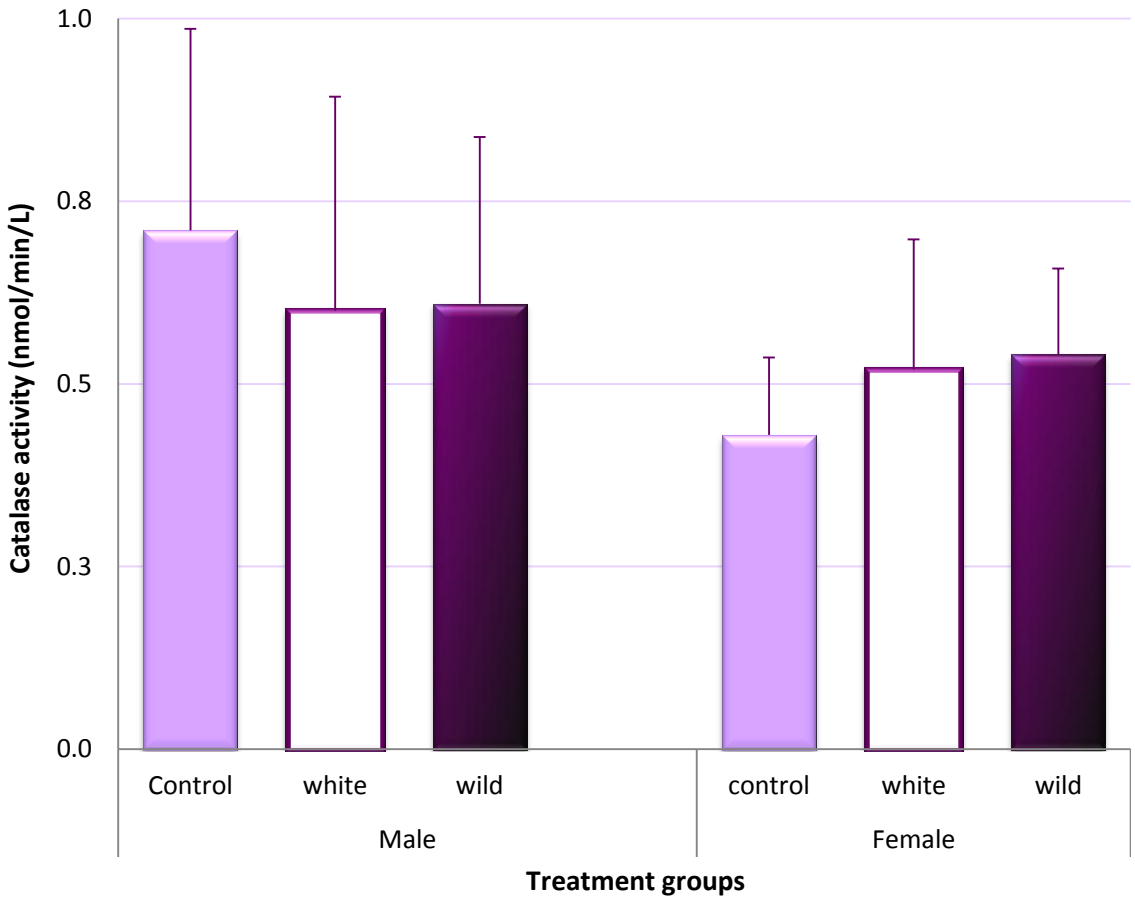


Data are presented as mean ± standard deviation

(Male & female: control, n=6; white rice, n=6; wild rice, n=6)

[c] $p<0.05$ compared to male fed with the same experimental diet

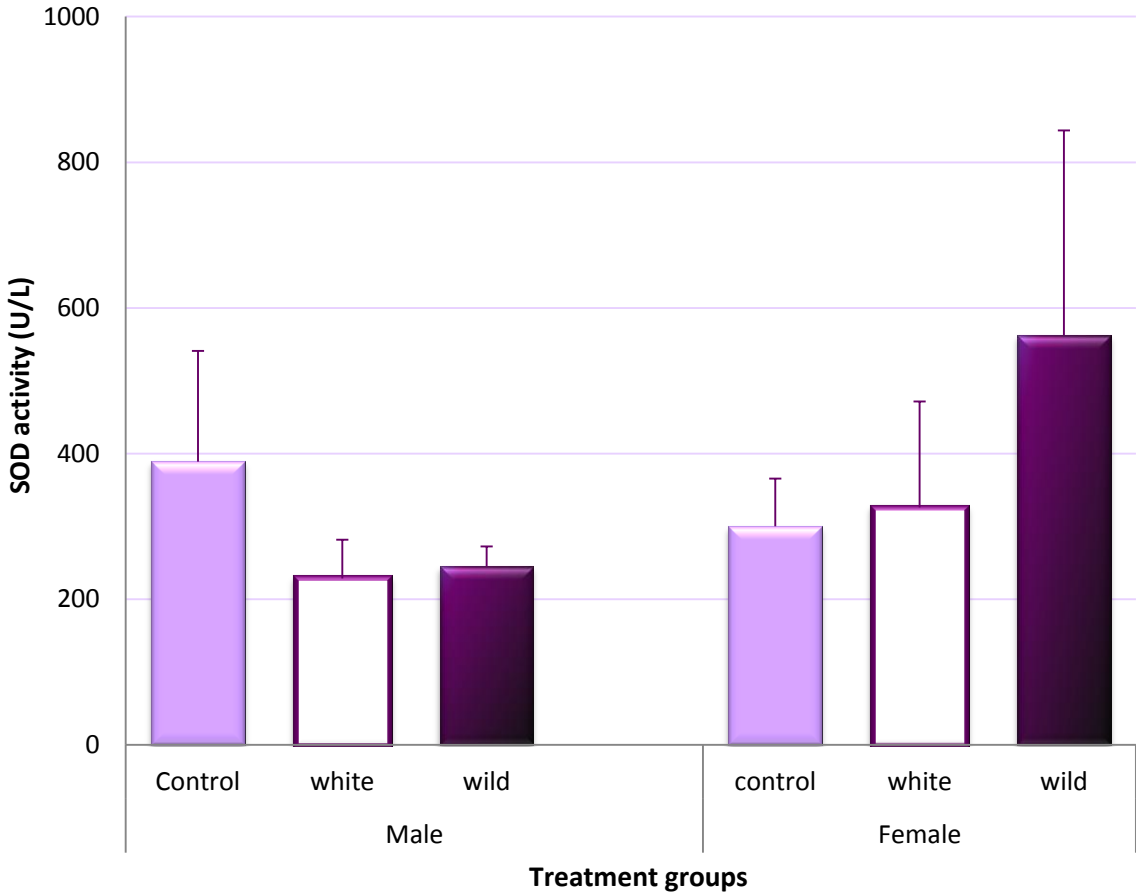
Figure 21. Erythrocyte catalase activity



Data are presented as mean ± standard deviation

(Male & female: control, n=6; white rice, n=6; wild rice, n=6)

Figure 22. Erythrocyte superoxide dismutase activity



Data are presented as mean ± standard deviation

(Male & female: control, n=6; white rice, n=6; wild rice, n=6)

Table 9. Summary of the results from 2-way ANOVA and Tukey's test

Attributer	Gender (G)	Mean values for treatment groups (Trt)			ρ-value		
		Control	White	Wild	Trt	G	Trt x G
Food intake (g)	M	3.3 (0.1)	3.3 (0.1)	3.4 (0.1)	NS	< 0.001	NS
	F	2.9 (0.0)	2.8 (0.1)	3.0 (0.1)			
Body weight (g)	M	33.3 (1.5)	36.1 (1.7)	36.2 (1.0)	NS	< 0.001	NS
	F	23.2 (0.6)	22.8 (0.4)	22.4 (0.7)			
Abdominal fat (g)	M	0.99 (0.18)	1.35 (0.27)	1.49 (0.12)	NS	< 0.001	NS
	F	0.01 (0.01)	0.00 (0.00)	0.00 (0.00)			
Liver (g)	M	1.11 (0.06)	1.23 (0.07)	1.29 (0.08)	NS	< 0.001	NS
	F	0.89 (0.03)	0.91 (0.03)	0.86 (0.01)			
Spleen (g)	M	0.07 (0.00)	0.08 (0.00)	0.07 (0.00)	NS	NS	NS
	F	0.07 (0.01)	0.07 (0.00)	0.07 (0.00)			
Kidney (g)	M	0.35 (0.01)	0.38 (0.00)	0.39 (0.02)	NS	< 0.001	NS
	F	0.26 (0.01)	0.26 (0.01)	0.26 (0.01)			
Heart (g)	M	0.14 (0.01)	0.15 (0.00)	0.15 (0.00)	NS	< 0.001	NS
	F	0.11 (0.00)	0.11 (0.00)	0.11 (0.00)			
Plasma triglyceride	M	141.7 (11.6)	166.7 (7.8)	153.8 (12.3)	NS	< 0.001	NS
(mg/dl)	F	124.6 (16.0)	95.0 (3.7)	101.3 (12.7)			
Plasma total cholesterol	M	746.4 (54.7) [a]	790.1 (34.2) [a]	600.5 (32.7) [b]	< 0.001	< 0.001	NS
(mg/dl)	F	586.7 (43.3) [a]	445.7 (22.0) [b]	354.0 (24.2) [b]			
Plasma VLDL (mg/dl)	M	37.5 (6.1) [a]	47.8 (7.0) [a,b]	23.4 (5.8) [a,c]	< 0.001	NS	< 0.01
	F	67.6 (8.0) [a]	32.2 (1.9) [b]	18.5 (4.3) [b]			

Attributer	Gender (G)	Mean values for treatment groups (Trt)			ρ-value		
		Control	White	Wild	Trt	G	Trt x G
Plasma HDL (mg/dl)	M	122.7 (8.7)	120.3 (7.4)	105.3 (8.0)	NS	< 0.001	NS
	F	92.6 (9.5)	77.8 (3.1)	86.8 (4.4)			
Proportion of liver weight of body weight (%)	M	3.3 (0.1)	3.4 (0.0)	3.5 (0.1)	NS	< 0.001	NS
	F	3.9 (0.1)	4.0 (0.1)	3.9 (0.1)			
Liver lipid %	M	12.9 (1.2)	15.0 (1.1)	15.2 (2.1)	NS	< 0.05	NS
	F	17.9 (1.1)	16.4 (1.3)	16.8 (0.8)			
Liver cholesterol (mg/g)	M	3.0 (0.2)	2.7 (0.1)	2.6 (0.1)	NS	< 0.01	NS
	F	3.6 (0.2)	3.1 (0.3)	3.2 (0.4)			
Liver triglyceride (mg/g)	M	18.3 (1.0)	20.5 (0.4)	22.5 (1.6)	NS	NS	NS
	F	21.0 (1.1)	23.1 (1.3)	22.5 (1.3)			
Liver malondialdehyde (mM/g)	M	275.9 (20.0)	315.1 (14.8)	315.2 (27.4)	NS	NS	NS
	F	315.3 (39.4)	349.1 (14.1)	296.8 (10.0)			
Fecal lipid (%)	M	2.9 (0.3) [a]	2.8 (0.1) [a]	3.6 (0.2) [b]	< 0.001	NS	NS
	F	2.4 (0.1) [a]	2.8 (0.1) [a]	3.6 (0.2) [b]			
Fecal cholesterol (mg/g)	M	2.2 (0.2) [a]	2.2 (0.2) [a,b]	3.1 (0.1) [b,c]	< 0.001	NS	NS
	F	1.9 (0.1) [a]	2.1 (0.1) [a]	3.2 (0.2) [b]			
Fecal triglyceride (mg/g)	M	0.5 (0.0) [a]	0.7 (0.0) [a]	1.1 (0.1) [b]	< 0.001	< 0.01	NS
	F	0.6 (0.0) [a]	0.8 (0.1) [a]	1.5 (0.1) [b]			
Plasma TPC (mg/ml)	M	1.28 (0.04)	1.28 (0.01)	1.15 (0.02)	NS	< 0.001	NS
	F	1.80 (0.07)	1.75 (0.03)	1.78 (0.02)			

Attributer	Gender (G)	Mean values for treatment groups (Trt)			ρ-value		
		Control	White	Wild	Trt	G	Trt x G
Erythrocyte catalase (nmol/min/L)	M	0.7 (0.1)	0.6 (0.1)	0.6 (0.1)	NS	< 0.05	NS
	F	0.4 (0.0)	0.5 (0.1)	0.5 (0.0)			
Erythrocyte SOD (U/L)	M	388.82 (62.07)	228.74 (21.56)	244.65 (11.39)	NS	< 0.05	< 0.01
	F	300.40 (26.63)	326.05 (59.33)	562.47 (114.93)			

Values are means (followed in brackets by the standard error of the mean) for each treatment groups (Control, n=6; white rice, n=6; wild rice, n=6) in both male (M) and female (F) mice.

[a, b, c] Values with different superscripts within the same row indicate significant differences ($p < 0.05$); NS= not significant, $P \geq 0.05$.

7. Discussion

7.1. Summary of current knowledge and hypothesis

Health benefits of whole grain are recognized by Health Canada and the new Canada's Food Guide suggests replacing at least half of the grain products intake to whole grain. Natural dietary antioxidants have been shown to have cumulative evidence on oxidant defense and cardio-protective role in cardiovascular diseases. Current research also shows that whole Chinese wild rice positively modified lipid profile and antioxidant activities in male Sprague Dawley rats (Zhang, Cao, Agellon, & Zhai, 2009). Manitoban wild rice (Zizania palustris) was recently reported to exhibit high antioxidant capacity (Qiu et al., 2010). However, its beneficial effects on cardiovascular risk factors are unclear. Herein, we hypothesized that replacing refined carbohydrate source with Manitoban wild rice, will mitigate cardiovascular risk in LDLr-KO mice.

7.2. Effect of incorporation of wild rice into daily diet on cardiovascular risk factors

Treatment diets with wild rice as the main carbohydrate source displayed ameliorating effects on cardiovascular risk factors in LDLr-KO mice. Overall, beneficial effects particularly with regard to the improvement in blood lipids profile were observed in wild rice group compared to white rice and control groups. Total cholesterol, VLDL, and LDL cholesterol concentrations were reduced with increased sterol excretion through fecal samples. However, no significant improvement was seen in hepatic lipid profile as evaluated by histological analysis. Lipid peroxidation level and endogenous

antioxidant enzymes activities were also shown to be minimally affected by consumption of wild rice.

7.2.1. Food intake, body weight and abdominal fat

Obesity is often associated with increased risk of chronic health issues, such as hypertension, type II diabetes, cardiovascular diseases, and several types of cancers. Evidence from numerous epidemiological studies suggests an inverse relation between the consumption of whole grain and prevalence of obesity. Outcomes obtained from Nurses' Health Study (NHS), concluded that lower body weight gain was consistently observed in women who consumed more whole grain food products over twelve years, whereas higher intakes of refined grain contributed to greater weight gain (Liu et al., 2003). Similar findings were recorded from the analyses of The Health Professionals Follow-up Study (HPFS), which involved 27,082 men, aged 40-75 years old (Koh-Banerjee et al., 2004; Liu et al., 2003). Koh- Banerjee et al. (2004) reported that for every increment of 40 g/day of whole grain intake from all foods, 1.1 kg reduction of long term weight gain (8 years) was achieved. Recently, McKeown et al. (2010) reported that adults who consumed ≥ 3 servings of whole grains per day such as whole grain breakfast cereal, oatmeal, dark bread, and brown rice, had significantly lower body mass index, waist circumference, subcutaneous and visceral adipose tissues (McKeown et al., 2010). In 2009, Zhang et al. replaced white rice and processed wheat starch with wild rice as a chief carbohydrate source in a high fat cholesterol diet. Rats fed with wild rice diet had shown to have lower body weight and abdominal fat mass, despite similar

dietary energy intake among experimental groups (Zhang et al., 2009). Nevertheless, body weight and abdominal fat mass of the mice in this study did not significantly differ between diet groups, given that similar amounts of diets were consumed. Female mice were observed to have a lower body weight, as well as smaller abdominal fat mass, possibly due to lesser amounts of food ingestion compared to male mice.

7.2.2. Plasma, hepatic and fecal lipid profile

The associations of serum cholesterol concentrations and the risk of cardiovascular disease are widely recognized. In the present study, the effects of wild rice on circulating levels of plasma lipids and lipoproteins were determined. Wild rice diet was observed to exhibit plasma cholesterol lowering effects on the experimental animals. Wild rice group significantly reduced plasma total cholesterol (male, -19.5 %; female, -39.7 %), VLDL (female, -72.7 %), and LDL cholesterol (female, -42.3 %) concentrations compared to control. On the other hand, male mice fed with wild rice diet were also shown to have lower plasma total cholesterol (-24.0 %), VLDL (-51.3 %), and LDL cholesterol (-21.8 %) concentrations compared to white rice diet. 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). The cholesterol lowering properties of wild rice might be accounted for its other functional components.

Wild rice is accepted as whole grain given that the principal components, 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. In terms of whole grain and lipid profile, conflicting results had been observed in randomized clinical trials. Brownlee et al. (2010) reported that increased whole grain consumption (60 g and 120 g) for a 16 week period did not showed significant impact on plasma cholesterol levels of overweight individuals (Brownlee et al., 2010). Similarly, no changes in lipid profile were noticed in a randomized, crossover dietary intervention study with a 6-week consumption of whole grain rich diet (A. Andersson et al., 2007). In contrast, Giacco et al. (2010) noted significant reduction of fasting plasma cholesterol, as well as LDL cholesterol levels among healthy individuals after 3 weeks of wholemeal wheat foods. Likewise, total cholesterol concentration was lowered in obese individuals after consuming a starch-reduced whole grain diet, with replacing at least two daily meals, including snacks, preferentially with a daily consumption of 200 g of whole grain products for 4 weeks (Rave, Roggen, Dellweg, & Heise, 2007). In addition, evidence from cross-sectional Baltimore Longitudinal Study of Aging (BLSA), Health Professionals Follow-Up Study (HPFS) and Nurses' Health Study II (NHS II), as well as Framingham Offspring Study also showed inverse association between whole grain intakes with total cholesterol and LDL cholesterol levels (Jensen et al., 2006; McKeown, Meigs, Liu, Wilson, & Jacques, 2002; Newby et al., 2007).

Possible cholesterol lowering properties of whole grains remain to be elucidated. Whole grains (wheat, barley, rye, oats, millets, brown and wild rice) are known to be a

rich source of fibre, minerals, vitamins, phenolic compounds, and several other phytochemicals (Jones & Engleson, 2010; Okarter & Liu, 2010a; Slavin, 2003; Slavin & Slavin, 2004). The synergy of all protective components in grains had been suggested to contribute to their health benefits in cardiovascular diseases. Among all, dietary fibre has received the most attention and remains as the leading candidate which has been shown to improve serum lipid concentration (Anderson, Hanna, Peng, & Kryscio, 2000; Giacco et al., 2010; Jensen et al., 2006; Jones & Engleson, 2010; Newby et al., 2007; Okarter & Liu, 2010b; Slavin, 2003). Zizania wild rice has a higher content of dietary fibre, approximately 5.2 % of total dietary fibre, 3.3 % insoluble and 0.8 % of soluble fibre (Bunzel, Allerdings, Sinwell, Ralph, & Steinhart, 2002; Dreher, 2001), which is much greater than white rice with only 1.3 to 2.8 % of total dietary fibre, 0.3 % insoluble and negligible amount of soluble fibre content (Dreher, 2001; Li et al., 2002). Furthermore, total dietary fibre content of the wild rice diet used in this study, was higher than that in white rice diet(8.3 g/ 100 g vs. 5.3 g/ 100 g), or control diet (8.3 vs. 6.5 g/ 100 g). The cholesterol reduction effects of wild rice diet may be partially due to its higher content of dietary fibre compared to other treatment diets. Mechanisms that have been suggested to explain the hypocholesterolemic effects of dietary fibre include reduced absorption of cholesterol and reabsorption of bile acids in the intestinal lumen, as well as binding and increased fecal excretion of bile acids. Excess excretion of bile acids promotes uptake of circulating cholesterol for bile acids synthesis in order to replenish the bile acids pool, thus lowering concentration of plasma cholesterol (Andersson, Ellegård, & Andersson, 2002; Ellegård & Andersson, 2007; Erkkilä & Lichtenstein, 2006;

Lundin et al., 2004; Sayar et al., 2006; Yang, Kim, Lee, Lee, & Moon, 2003). Besides, low glycemic index of dietary fibre is associated with reduced insulin secretion, which potentially leads to the reduction of hepatic cholesterol synthesis by attenuating the activation of 3-hydroxy-3-methylglutaryl-co-enzyme A (HMG-CoA) reductase (Erkkilä & Lichtenstein, 2006; Juntunen et al., 2003; Mann, 2007). Lastly, short chain fatty acids (SCFA) such as acetate, butyrate, and propionate resulting from colonic fermentation of dietary fibre have also been demonstrated to lower plasma cholesterol level (Bridges, Anderson, Deakins, Dillon, & Wood, 1992; Marcil, Delvin, Garofalo, & Levy, 2003; Wolever et al., 1995; Wong et al., 2006).

Phytosterol composition of seven wild rice samples obtained from different growers across United States and Canada were analyzed and determined by Przybylski et al. Wild rice appeared to carry a rich source of phytosterol, mainly consisting of campesterol, β-sitosterol, and cycloartenol. Total sterol content of wild rice lipid ranged from 70 g to 145 g of sterol/ kg of wild rice lipid, which is at least 2.6 times higher than brown rice, 27 g/ kg (Przybylski et al., 2009); and averaging 3.3 times higher than rice bran (20 g/ kg), wheat bran (18 g/ kg), and germ (21 g/ kg) (Jiang & Wang, 2005). Recently, a randomised, double-blind, crossover study reported that incorporation of 20 g phytosterol enriched spread into regular daily dietary intake for 4 weeks, successfully lowered total cholesterol (-4.4 %), and LDL cholesterol (-5.6 %) in eighty mildly hypercholesterolemic volunteers. No changes were observed in HDL cholesterol and triglycerides levels (Eady, Wallace, Willis, Scott, & Frampton, 2011). Similar findings

were noticed in sixty non-hypercholesterolemic healthy subjects given 3 cups of low fat yogurt with 1 g of plant stanol esters (Mensink, Ebbing, Lindhout, Plat, & van Heugten, 2002). Similar findings were also reported by several other clinical trials (Miettinen, Vuoristo, Nissinen, Järvinen, & Gylling, 2000; Mussner et al., 2002; Plat, Onselen, Heugten, & Mensink, 2000). This may support our findings from the present study that plasma total and LDL cholesterol were statistically lowered regardless of any significant changes in HDL cholesterol and triglyceride levels between wild rice and control or white rice diet groups. Cholesterol lowering properties of plant sterol are highly recognised, mainly due to its similarity in structure with cholesterol, which allow it to act as a competitor in micellar binding, resulting in reduced absorption of intestinal cholesterol and consequently increased the excretion of unabsorbed cholesterol markedly. Moreover, several other mechanisms were speculated to be involved, such as promoting the expression of intestinal ATP binding cassette A1 (ABCA1) that may facilitate free cholesterol efflux back to the intestinal lumen. Plant sterol also may interfere with Acyl CoA: cholesterol acyltransferase (ACAT) activity which reduced the esterification of free cholesterol for chylomicron packaging and lead to reduced cholesterol absorption (Trautwein et al., 2003).

In addition, clinical studies have shown that moderate (459 mg) and high (2059 mg) plant sterol consumption (Racette et al., 2010) or incorporation of margarine with 2 g of plant stanol (Miettinen et al., 2000), has a significant impact on lowering serum cholesterol concentration through reducing intestinal cholesterol absorption and

increased excretion of fecal cholesterol. This corresponds with our study that mice fed wild rice diet excreted statistically higher amounts of total fecal lipid percentage (male, 20.5 %, female, 34.4 %) compared to control, (male, 22.2 %, female, 23.1 %) compared to white rice, and total cholesterol (male, 30.0 %, female, 38.6 %) compared to control, (female, 32.3 %) compared to white rice. The unabsorbed cholesterol and bile acids are excreted from the body as fecal neutral and acidic sterols, the major route of sterol elimination from the body (Wang, 2007). Racette et al (2010) proposed that phytosterol may play a critical role in aiding the efflux of cholesterol from peripheral tissues or arterial wall to liver for excretion. This reverse cholesterol transportation in arterial wall may beneficially improve the condition of atherosclerotic development (Ye et al., 2010). Besides, Ye et al. (2010) also had suggested that ABCA5 may play a part in macrophage cholesterol homeostasis, hence assist in regulation of the atherosclerotic lesion development. Moreover, increased excretion of bile acid was found to be inversely correlated to the elevated of cholesterol levels and the prevalence of coronary artery disease (Charach et al., 2011; Charach, Rabinovich, Argov, Weintraub, & Rabinovich, 2012).

Wild rice is also rich in γ-oryzanol (Przybylski et al., 2009). Hypocholesterolemic properties of γ-oryzanol were observed in C57BL/6 mice fed with high fat diet for 7 weeks (Son, Rico, Nam, & Kang, 2010), as well as in Golden Syrian hamsters given high cholesterol diet with 0.5 % oryzanol for 10 weeks (Wilson et al., 2007). These may possibly be due to γ-oryzanol's effect on promoting excretion of fecal cholesterol.

Besides, cholesterol lowering effects of γ-oryzanol were also reported in 32 men with slightly high cholesterol level after receiving 0.05 g or 0.8 g of γ-oryzanol per day for duration of 4 weeks; this treatment resulted in 6.3 % reduction in total cholesterol, 10.5 % reduction in LDL-C, and 18.9 % reduction in LDL-C/ HDL-C ratio (Berger et al., 2005). Other than that, the favourable fatty acid composition (Przybylski et al., 2009), such as high percentages of polyunsaturated fatty acids, linolenic and linoleic acids, low ratios of n-6 to n-3 fatty acids of wild rice may also positively impact the lipid profile of the experimental animals.

Several potent phenolic acids had been identified in wild rice, with ferulic acid being noted to be the highest among all (Qiu et al., 2010). Potential health benefits of ferulic acid, including its hypocholesterolemic properties has attracted a considerable amount of interest. Dietary supplementation of 0.5 % ferulic acid was reported to lower total cholesterol concentration in Golden Syrian hamster fed high cholesterol diet (Wilson et al., 2007). Despite total cholesterol lowering, increased excretion of total, neutral and acidic sterol, hepatic HMG-CoA reductase and ACAT activities were effectively reduced in rats fed high cholesterol with 0.2 % ferulic acid (Yeh et al., 2009). Identical results were obtained with incorporation of minimal amount of ferulic acid (0.013 %) in rats given high cholesterol diet, with no changes in hepatic total cholesterol and triglyceride levels (Kim et al., 2003). Inhibition of HMG-CoA reductase, rate limiting enzyme in cholesterol biosynthesis, leads to decreased intracellular cholesterol concentration (Trautwein et al., 2003). The reduction of ACAT activities potentially limit

esterification of cholesterol, thereby limiting assembly of VLDL and leading to reduction of VLDL secretion from the liver (Wang, 2007).

All female mice in the present study had lower plasma total cholesterol and LDL cholesterol concentration compared to male mice fed the same treatment diets. These may partly be due to the gender differences in sex steroid hormones on cardiovascular risk factors. Numerous findings suggested that endogenous estrogen is potentially cardio-protective. In addition, epidemiological studies have identified the protective role of postmenopausal hormone replacement therapy (HRT) on reducing the morbidity and mortality rate of cardiovascular disease (Vitale, Mendelsohn, & Rosano, 2009). However, controversial results have been observed in clinical trials primarily due to variations in HRT start time. It has been hypothesized that the initiation of HRT is effective only if it was done before advanced atherosclerotic lesions development (Mendelsohn & Karas, 2005). In animal models, reduction of total cholesterol, non-HDL cholesterol, aortic lesion areas were observed in diet-induced atherosclerosis female hamster compared to male (Wilson, Nicolosi, Lawton, & Babiak, 1999). On the contrary, plasma cholesterol levels of endogenous estradiol- female LDLr-KO mice were noticed to be similar with estradiol deficiency female mice, and both of which contained higher level of cholesterol than male mice (Marsh, Walker, Curtiss, & Banka, 1999). Nonetheless, it was concluded that endogenous estrogen or exogenous estrogen treatments are strongly associated with delayed onset and development of atherosclerosis lesion, despite unchanged lipid profile (Hodgin & Maeda, 2002; Knopp et al., 2005; Mendelsohn &

Karas, 2005). The mechanisms underlying the lipid regulation of male and female LDLr-KO mice need to be elucidated to better understand the gender related differences in this particular animal model.

Hepatic lipid profile in this study did not differ significantly among three different diet groups in both genders. Histological structure of all hepatic tissues was shown to be similar with unremarkable fatty change. Hepatic biochemical profile was in agreement with histological features, suggesting minimal effects and a high degree of safety profile for long term wild rice consumption on the liver in this particular mice model.

7.2.3. Total phenolic content of treatment diets and plasma

Phenolic compounds are consistently shown to have potent antioxidant properties that act as an essential role in preventing oxidative stress- induced diseases, like cardiovascular disease (Soobrattee, Neergheen, Luximon-Ramma, Aruoma, & Bahorun, 2005). A wide variety of plant derived foods that are high in phenolic compounds, such as fruits, vegetables and cereal grains have been investigated in several in vitro, in vivo and clinical trials to further determine their potential health benefits (Morton et al., 2000). Qiu et al. (2010) reported that wild rice consists of high total phenolic content with 10 folds of antioxidant activity compared to white rice. In the present study, incorporation of wild rice in animal diet also distinctly boosted the total phenolic content about 4 times more than white rice and control diet. However, high antioxidant capacity of wild rice diet did not reflect on plasma total phenolic content in mice supplemented with wild rice in both genders.

7.2.4. Plasma and hepatic lipid peroxidation and erythrocyte enzyme activities

Oxidative stress induced LDL cholesterol oxidation is widely recognized as the key step in the initiation of atherosclerosis development. Therefore, thiobarbituric acid-reactive substances (TBARS) are often used as an indicator of lipid peroxidation and oxidative stress levels in clinical and animal studies. Besides, measurements of SOD and catalase activities, with TBARS value enable prediction or indication of oxidative level. Existence of sufficient amount of various endogenous antioxidant compounds and enzyme activities are crucial to counteract the increasing of oxidative stress in the body system. Superoxide dismutase (SOD) and catalase (CAT) have high affinity to catalyze the excess amount of their substrates, superoxide radical and hydrogen peroxide, respectively (Stocker & Keaney Jr, 2004). Determinations of SOD and catalase activities in erythrocyte are steady as compared to measurement of these activities in the liver or other tissues in which the cell contains nuclei and continuous synthesis of new protein (Nelson, Bose, Grunwald, Myhill, & McCord, 2006). In contrast, once mature erythrocyte enter circulation for a life span of 120 days, they do not induce new enzyme production due to the absent of nuclei. Erythrocyte SOD is crucial to protect red cells from accumulation of superoxide due to haemoglobin auto-oxidation (Wallace, Houtchens, Maxwell, & Caughey, 1982). Furthermore, erythrocyte SOD has been recognized as a reflection in the build-up of reactive oxygen species in red cells, as well as in other tissues, possibly attributed to its anion channel that allow transition of superoxide and

other radicals into red cells (Lutoslawska et al., 2003; Richards, Roberts, Dunstan, McGregor, & Butt, 1998).

In Zhang's et al. study (2009) , antioxidant properties of wild rice managed to inhibit the increased of lipid peroxidation by lowering serum and hepatic malondialdehyde (MDA) concentrations with increased SOD activity. Similar to most cereal grains, ferulic acid appears to be the most abundant phenolic acid in wild rice, and may be responsible for the antioxidant capacity of wild rice. Yeh et al. (2009) reported that ferulic acid may act as a good scavenger by suppressing the rate of plasma and hepatic lipid peroxidation, and increasing hepatic SOD activities in rats fed high cholesterol diet supplemented with 0.2 % dietary ferulic acid. In addition, high fat diet with 0.5 % ferulic acid was also shown to reduce plasma TBARS level in C57BL/6 mice (Son et al., 2010). In contradiction to previous findings, wild rice diet did not effectively suppress the lipid peroxidation level nor increase the erythrocyte SOD and catalase activities in LDLr-KO mice in the present study. Nevertheless, all female mice had significantly higher plasma total phenolic content and lower plasma MDA level than male mice fed with similar diet. These may partially explain the existence of endogenous estrogens in female mice, a protective source of antioxidants that is lesser or absent in male. A study showed that baseline lipid oxidation in healthy male was higher than female volunteers through determination of plasma TBARS and MDA values (Actis-Goretta, Carrasquedo, & Fraga, 2004). Ide et al. (2002) also reported a lower incidence of oxidative stress in pre-menopausal women than men through TBARS evaluation,

suggesting that reduced lipid oxidation may potentially be attributed to the antioxidant properties of estrogens. Despite the absence of correlation between plasma 17β-estradiol and TBARS level, no increase in plasma antioxidant enzymes (SOD and catalase) were observed (Ide et al., 2002).

7.3. Summary of main finding and conclusion

Incorporation of Manitoban wild rice as a carbohydrate source (50 %) in animal diet significantly increases its total phenolic contents. In addition, wild rice diet beneficially modified plasma lipid profile, mainly through reducing circulating plasma total cholesterol, VLDL, and LDL cholesterol levels. The hypocholesterolemic properties of wild rice mainly attributed to the increased rate of fecal cholesterol excretion. However, hepatic lipid profiles, as well as lipid peroxidation levels in liver were not positively improved with the consumption of wild rice. Hepatic biochemical profile was in an agreement with histological features, indicating minimal effects of long-term wild rice consumption on the liver. No difference was found in plasma and erythrocyte antioxidant enzymatic activities between wild rice and white rice or control diets. In addition, female mice had better lipid profile and plasma lipid peroxidation value compared to male, which was probably related to its endogenous estrogen content.

Potential health benefits of Manitoban wild rice are probably due to the synergy of its functional components and phytochemical compounds. The outer bran layer and inner germ contain various dietary fibres, vitamins, minerals, polyphenols, and other

phytonutrients. Therefore, wild rice is shown to demonstrate higher potential health benefits compared to white rice and other refined carbohydrate sources.

In conclusion, long term consumption of Manitoban wild rice has desirable protective roles on improving plasma lipid profile in LDLr-KO mice. Substituting white rice or other refined grain products with Manitoban wild rice (*Zizania palustris*) may potentially lower cardiovascular risk through its hypocholesterolemic effect.

7.4. Strengths and limitations

A major strength of this study is the length of the experimental course, 24 weeks, which was sufficient to observe effects of both time and treatment on any risk factors measured. The extended period also enabled determination of long term effect and safety profile of treatment diets. Secondly, LDLr-KO mice were chosen as the animal model used in the present study. This animal model is one of the most extensively used animal models of dyslipidemia and atherosclerosis (Getz & Reardon, 2006). The plasma lipoprotein profile of this particular mice model mimics that of humans, as well as genetic disorder disease, familial hypercholesterolemia of humans (Zadelaar et al., 2007). Next, the inclusion of male and female mice in the present study also allowed observations of wild rice effect on both genders. Besides, several limitations were found in the present study. There was absence of wild type C57BL/6 mice to act as a control in cardiovascular risk factors measured. The size of the animal model limits the total amount of blood collection. Thus, insufficient of blood samples were obtained for analysis at every desired time point to oversee the progression across the study duration.

7.5. Future research

Future studies on assessing the effects of Manitoban wild rice on cardiovascular risk factors and the underlying mechanisms of actions are warranted. One potential area includes the investigation of the degree of absorption, metabolism and distribution of dietary phenolic compounds from wild rice. Besides polyphenol compounds, other functional components of wild rice, such as phytosterols, soluble fibres, resistant starch, and others should be examined to identify their potential health benefits. Reduction in plasma total cholesterol and non-HDL cholesterol were noticed in the present study. The mechanism to elucidate the effect of wild rice on cholesterol metabolism should be studied. Determination of HMG-CoA reductase, ACAT, and ABCG5, ABCG8 activities may be an initial step. Next, plasma pro-inflammatory cytokines can be analyzed to provide inflammatory profile of the experimental animal. Furthermore, a complete assessment of progression of atherosclerosis is also an interesting area of future investigation. The evaluation of the area of atherosclerotic lesion in aortic root, estimation of the lesion size and the lesion to lumen ratio will present a comprehensive report of atherosclerosis development. Lastly, investigation of the reason behind different responses obtained from male and female mice relative to any risk factors is crucial to better understand the gender associating mechanisms, especially on cholesterol lowering effects of wild rice.

7.6. Implications in the field of nutrition

Results from this study contribute to the body of research of Manitoban wild rice (*Zizania palustris*) on cardiovascular risk. Findings obtained also provide additional evidence for cholesterol lowering properties of whole grain consumption. Moreover, our data may promote intake of wild rice as a functional food, and the development of a safe and effective nutraceutical product from phytochemical extract of wild rice samples. Finally, the data obtained may encourage the growth of Manitoban wild rice industry, thus positively impact agriculture system in Manitoba, Canada.

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9. Appendix

Appendix A. Summary of the results from 2-way ANOVA and Tukey's test (without treatment group x gender interaction)

Attributer	Gender (G)	Mean values for treatment groups (Trt)			ρ-value	
		Control	White	Wild	Trt	G
Food intake (g)	M	3.3 (0.1)	3.3 (0.1)	3.4 (0.1)	NS	< 0.001
	F	2.9 (0.0)	2.8 (0.1)	3.0 (0.1)		
Body weight (g)	M	33.3 (1.5)	36.1 (1.7)	36.2 (1.0)	NS	< 0.001
	F	23.2 (0.6)	22.8 (0.4)	22.4 (0.7)		
Abdominal fat (g)	M	0.99 (0.18)	1.35 (0.27)	1.49 (0.12)	NS	< 0.001
	F	0.01 (0.01)	0.00 (0.00)	0.00 (0.00)		
Liver (g)	M	1.11 (0.06)	1.23 (0.07)	1.29 (0.08)	NS	< 0.001
	F	0.89 (0.03)	0.91 (0.03)	0.86 (0.01)		
Spleen (g)	M	0.07 (0.00)	0.08 (0.00)	0.07 (0.00)	NS	NS
	F	0.07 (0.01)	0.07 (0.00)	0.07 (0.00)		
Kidney (g)	M	0.35 (0.01)	0.38 (0.00)	0.39 (0.02)	NS	< 0.001
	F	0.26 (0.01)	0.26 (0.01)	0.26 (0.01)		
Heart (g)	M	0.14 (0.01)	0.15 (0.00)	0.15 (0.00)	NS	< 0.001
	F	0.11 (0.00)	0.11 (0.00)	0.11 (0.00)		
Plasma triglyceride (mg/dl)	M	141.7 (11.6)	166.7 (7.8)	153.8 (12.3)	NS	< 0.001
	F	124.6 (16.0)	95.0 (3.7)	101.3 (12.7)		
Plasma total cholesterol (mg/dl)	M	746.4 (54.7) [a]	790.1 (34.2) [a]	600.5 (32.7) [b]	< 0.001	< 0.001
	F	586.7 (43.3) [a]	445.7 (22.0) [b]	354.0 (24.2) [b]		

Attributer	Gender (G)	Mean values for treatment groups (Trt)			ρ-value	
		Control	White	Wild	Trt	G
Plasma HDL (mg/dl)	M	122.7 (8.7)	120.3 (7.4)	105.3 (8.0)	NS	< 0.001
	F	92.6 (9.5)	77.8 (3.1)	86.8 (4.4)		
Proportion of liver weight of body weight (%)	M	3.3 (0.1)	3.4 (0.0)	3.5 (0.1)	NS	< 0.001
	F	3.9 (0.1)	4.0 (0.1)	3.9 (0.1)		
Liver lipid %	M	12.9 (1.2)	15.0 (1.1)	15.2 (2.1)	NS	< 0.05
	F	17.9 (1.1)	16.4 (1.3)	16.8 (0.8)		
Liver cholesterol (mg/g)	M	3.0 (0.2)	2.7 (0.1)	2.6 (0.1)	NS	< 0.01
	F	3.6 (0.2)	3.1 (0.3)	3.2 (0.4)		
Liver triglyceride (mg/g)	M	18.3 (1.0)	20.5 (0.4)	22.5 (1.6)	NS	NS
	F	21.0 (1.1)	23.1 (1.3)	22.5 (1.3)		
Liver malondialdehyde (mM/g)	M	275.9 (20.0)	315.1 (14.8)	315.2 (27.4)	NS	NS
	F	315.3 (39.4)	349.1 (14.1)	296.8 (10.0)		
Fecal lipid (%)	M	2.9 (0.3) [a]	2.8 (0.1) [a]	3.6 (0.2) [b]	< 0.001	NS
	F	2.4 (0.1) [a]	2.8 (0.1) [a]	3.6 (0.2) [b]		
Fecal cholesterol (mg/g)	M	2.2 (0.2) [a]	2.2 (0.2) [a,b]	3.1 (0.1) [b,c]	< 0.001	NS
	F	1.9 (0.1) [a]	2.1 (0.1) [a]	3.2 (0.2) [b]		
Fecal triglyceride (mg/g)	M	0.5 (0.0) [a]	0.7 (0.0) [a]	1.1 (0.1) [b]	< 0.001	< 0.01
	F	0.6 (0.0) [a]	0.8 (0.1) [a]	1.5 (0.1) [b]		
Plasma TPC (mg/ml)	M	1.28 (0.04)	1.28 (0.01)	1.15 (0.02)	NS	< 0.001
	F	1.80 (0.07)	1.75 (0.03)	1.78 (0.02)		

Attributer	Gender (G)	Mean values for treatment groups (Trt)			ρ-value	
		Control	White	Wild	Trt	G
Erythrocyte catalase	M	0.7 (0.1)	0.6 (0.1)	0.6 (0.1)	NS	< 0.05
(nmol/min/L)	F	0.4 (0.0)	0.5 (0.1)	0.5 (0.0)		

Values are means (followed in brackets by the standard error of the mean) for each treatment groups (Control, n=6; white rice, n=6; wild rice, n=6) in both male (M) and female (F) mice.

[a, b, c] Values with different superscripts within the same row indicate significant differences ($p < 0.05$); NS= not significant, $P \geq 0.05$.

Treatment group and gender interactions (Trt x G) were not significant in most of the listed attributes in Table 9, except plasma VLDL and erythrocyte superoxide dismutase values. Therefore, non-significant interactions with the error were pooled, according to the methods described in O'Mahony (1986). New table: Appendix A. Summary of the results from 2-way ANOVA and Tukey's test (without treatment group x gender interaction) was generated to obtain the new p-value for treatment groups and genders, the p-value of the attributes might change due to exclusion of interaction between treatment group and gender in the statistical analysis. The increased of error sums of squares and the degree of freedom (df), result in the higher mean square (MS) value, thus decrease the F-value, which means higher p-value might be obtained and affect the significant of the results.

Nevertheless, the significant values of the results in this study were not affected with the exclusion of treatment group and gender interaction, mainly due to the highly significant value ($p < 0.01$ or < 0.001) in both treatment groups and genders.