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Grain sorghum whole kernel oil lowers plasma and liver cholesterol in male hamsters with minimal wax involvement

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Abstract

The lipid fraction of the grain sorghum whole kernel (GS-WK) (i.e., phytosterol rich oil or policosanol rich wax) responsible for lowering cholesterol in hamsters fed the crude lipid (wax + oil) was determined. As expected, hamsters fed an atherogenic diet for a four week period presented with higher plasma non-HDL plasma and liver esterified cholesterol than those on the low fat diet. However, the atherogenic diet containing 5% (w/w) oil significantly lowered non-HDL plasma and liver cholesterol. Although the 5% wax supplement did not affect either plasma or liver cholesterol, excreted neutral sterol and bile acid were slightly higher than produced by the atherogenic diet. Still, cholesterol excretion negatively correlated with liver cholesterol concentration ($r = -0.681$, $p < 0.001$) across diets with the oil fraction producing the greatest impact. These combined results indicate that oil plays the most significant role in modulating cholesterol, most likely by inhibiting absorption, but subtle interactions by the wax may be involved. However, the sorghum oil would be the most potent component to serve as a possible heart health ingredient in functional foods.

Keywords: grain sorghum, whole kernels, cholesterol, phytosterols, policosanols

Abbreviations: non-HDL, non high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; GS-WK, grain sorghum whole kernel; GS-DDGS, grain sorghum distillers dried grains with solubles; athero, atherogenic

1. Introduction

Lipids are important dietary components providing unique textures and flavors to foods. Yet, dietary fat has been linked to obesity (Newgard et al., 2009) and the onset of multiple types of diseases, including breast, pancreatic, colorectal cancers (Butler et al., 2009; Cho et al., 2003; Nöthlings et al., 2005), diabetes (Lindström et al., 2006), and cardiovascular diseases

(Brousseau & Schaefer, 2000). In particular, coronary heart disease (CHD) is strongly correlated to dietary fat intake (Jakobsen et al., 2009; Kwape et al., 2010). A primary risk for CHD is high low-density lipoprotein (LDL) cholesterol (Fernández & Webb, 2008). Cholesterol levels among the United States population is disproportionately high (28–32%) exceeding recommended 130 mg/dL levels (Heidenreich et al., 2011). It is expected that these statistics will be maintained or even increase

as dietary choices established during early ages are not easily altered (Frémeaux et al., 2011).

Increasing preference for natural interventions for lowering LDL, and the potential side effects of cholesterol lowering drugs, such as statins, have prompted the discovery several heart health food ingredients, including dietary fiber (Erkkilä & Lichtenstein, 2006), soybean protein (Pipe et al., 2009), tea catechins (Bursill & Roach, 2007), and grape polyphenols (Castilla et al., 2006). Among these dietary agents, phytosterols have been extensively investigated due to their potent LDL lowering properties (Batta, Xu, Honda, Miyazaki, & Salen, 2006; Racette et al., 2010). For example, meta-analyses of clinical trials have indicated a 10% reduction of LDL cholesterol when 2 g of phytosterols are consumed on a daily basis (Carr, Ash, & Brown, 2010). As a result, phytosterol-supplemented foods, such as margarine, yogurts, and cooking oils, are now commercially available in the US, Europe, and Australia (Chen, Jiao, & Ma, 2008). Other studies have shown a positive link with policosanols consumption and lower LDL cholesterol (Castaño et al., 2005; Gamez, Maz, Arruzazabala, Mendoza, & Castano, 2005).

Grain sorghum is critically underutilized in the US despite the presence of multiple types of lipids potentially able to prevent the onset of diseases currently afflicting western societies (Lee et al., 2010). In particular, Carr et al. (2005) showed potent cholesterol lowering properties the crude lipid (oil + wax) fraction extracted from grain sorghum whole kernels (GS-WK). Hamsters fed a high fat-cholesterol diet supplemented with 1% and 5% (w/w) GS-WK lipid presented with substantially lower plasma non high density lipoprotein (non-HDL = LDL + very low density lipoproteins (VLDL)) cholesterol levels and liver esterified cholesterol compared to the group on a high fat-cholesterol diet alone. Other benefits exerted by the 5% (w/w) GS-WK supplement included higher HDL levels and increased neutral sterol output. Considering the proliferation of evidence supporting the cholesterol lowering benefits of phytosterols (Batta et al., 2006; Racette et al., 2010), the positive results produced by the GS-WK lipids were attributed primarily to the phytosterol rich oil fraction, even though policosanols rich wax accounted for the most of the GS-WK lipid crude extract (3:1).

Therefore, the objective of this study was to determine the fraction (wax or oil) in GS-WK responsible for lowering cholesterol. A hamster model was again fed an atherogenic diet mimicking current westernized diets, but supplemented with either oil or wax fractionated crude lipid extract at a 5% (w/w) dosage. Other groups consumed a diet low in fat or an atherogenic diet without GS-WK lipids. Plasma/liver cholesterol profiles and sterol output was monitored for the four groups. In addition, the oil and fractions were characterized for their phytosterols and policosanols content along with other lipid constituents.

2. Materials and methods

2.1. GS-WK lipid extraction and fractionation

Wax and oil fractions were prepared from WK white food-grade sorghum, *Sorghum bicolor* cultivar *Macia*, grown in Lincoln,

Nebraska. The kernels (1 kg) were refluxed in their whole form with 1 L of hexane for 30 min in a three-neck round bottomed flask at approximately 60 °C. After cooling to room temperature, the extract was filtered through a coffee filter followed by a Whatman No.2 filter. The filtrate was maintained at -15 °C for 12–16 h to precipitate the wax. The waxy precipitate was isolated by filtering the cooled extract through a Buchner funnel and Whatman No. 42 filter paper, which had been washed with hexane and dried prior to use. The retained wax material was completely dried, while the hexane was removed from the filtrate with a rotary evaporator to obtain the oil fraction. Both fractions were stored at -15 °C until sufficient amounts were obtained for the animal studies, which occurred approximately three months after the first fractions were prepared.

2.2. Characterization of WK-GS wax and oil

Wax and oil samples fractionated from the WK were analyzed for phytosterol and policosanols content by using a gas chromatograph (GC) equipped with flame ionization detector (FID) as described by Leguizamón, Weller, Schlegel, and Carr (2009). Briefly, 1 mL of 5 α -cholestane and 0.1 mL of C₂₇ were combined with 0.02 g of wax or oil suspended in chloroform. The chloroform was evaporated using a heat block (~60 °C) under a nitrogen stream, and the samples were allowed to cool for 3 min. The samples were saponified with 1 mL of 1 M methanolic KOH and the mixture was heated to 50 °C in a heat block for 1 h with occasional agitation. Non-saponifiable lipids were extracted by adding 1 mL of deionized water and 2 mL of hexane. The samples were shaken for approximately 2 min and centrifuged prior to transferring the hexane phase to another vial. Hexane was evaporated under gaseous nitrogen at a temperature of approximately 60 °C. The remaining residue was then derivatized with 50 μ L of Sylon BTZ (Supelco, Bellefonte, PA, USA) for at least 20 min at room temperature. The samples were injected onto a 0.32mm \times 30m DB-5 capillary column (J&W Scientific, Folsom, CA, USA) at a split ratio of 50:1 with helium serving as the carrier gas. The injector and detection temperatures were set at 270 and 300 °C, respectively, while the initial oven temperature of 100 °C was ramped to 300 °C at 5 °C/min. Stigmasterol, β -sitosterol, and campesterol standards were used to identify/quantitate the phytosterols, whereas hexacosanol (C₂₆), octacosanol (C₂₈) dotriacontanol (C₃₂) standards were used to characterize the policosanols. Internal standards, 5 α -cholestane and policosanols C₂₇, were also used for more precise determination. Total phytosterols and policosanols were determined by adding the contribution of each of the individual detected components.

Simple lipid profiles were monitored with thin layer chromatography (TLC). Samples of each fraction were suspended in chloroform at a concentration of 20 mg/mL and warmed to 40 °C to melt the solid or semi-solid material prior to analysis. Aliquots of each (10 μ L) were spotted onto a silica 60Å TLC plate (20 \times 20 cm, 250 μ m) and separated with 90:10:2 (v/v/v) hexane, diethyl ether-acetic acid. To visualize the lipid bands, the plate was submerged briefly in a 10% cupric sulfate in 8% phosphoric acid solution, air-dried in a chemical fume hood and charred at 165 °C for 10 min.

Lipid groups were identified against a cocktail of standards containing monoacylglycerols, 1,2-diacylglycerols, 1,3-diacylglycerols, triacylglycerols, fatty acid methyl esters, D- α -tocopherol acetate, three polyoxyethlenesorbitan monooleate, and octacosanol.

Gas chromatography with FID detection was also used to confirm the presence of aldehydes and hydrocarbons in each fraction as cited by Verleyen et al. (2001). Briefly, a test sample was dissolved in pyridine and combined with *N,O*-bis(trimethylsilyl) trifluoroacetamide containing trimethylchlorosilane. Lipid classes were resolved in a single run by using a CP-Sil 8 CB (Low bleed/MS 15m \times 0.25 mm, 0.1 μ m, Varian, Palo Alto, CA, USA) with helium serving as the carrier gas. The initial injection temperature was set to 60 °C and increased to 140 °C at 30°C/min. The oven temperature was ramped from 5 to 235 °C at 5 °C/min, followed by a 7 min heating hold, increased to 340 at 15 °C/min, followed by a 15 min hold. The detector temperature was maintained at 360 °C.

Lastly, fatty acid profiles were determined with slight modifications to the method described by Schneider, Cowles, Stuefer-Powell, and Carr (2000). Boron trifluoride (14%) in methanol was added to a sample and heated to 100 °C for 30 min in sealed reaction vials. The samples were allowed to cool to room temperature before extracting with 1 mL of hexane and 2 mL water washes. The hexane phase was resolved with a GC DB-Wax column (30m \times 0.25 mm, J&W Scientific) and detected with FID. Injections were achieved with a split ratio of 10:1 with the starting temperature set at 185 °C for 12 min. The temperature was increased to 210 °C at 10 °C/min, held for 0–1 min, and increased to a final temperature of 230 °C at 10 °C/min, which was maintained for the remaining 15 min run time.

2.3. Animal study and diets

Four different AIN-93 M purified diets were formulated as shown in Table 1. The atherogenic (athero) diet contained coconut oil at the expense of cornstarch compared to the control diet, whereas 5% of the GS-WK oil or wax was added to the athero diet at the expense of soybean oil and corn starch, respectively.

Table 1. Composition of diets fed to hamsters.

Food component (g/kg)	Control	Athero	Athero + wax	Athero + oil
Cornstarch	455.7	354.5	304.5	354.5
Dextrinized cornstarch ^a	155.0	155.0	155.0	155.0
Casein	140.0	140.0	140.0	140.0
Sucrose	100.0	100.0	100.0	100.0
Coconut oil	—	100.0	100.0	100.0
Soybean oil	50.0	50.0	50.0	—
Insoluble fiber (cellulose) ^b	40.0	40.0	40.0	40.0
Soluble fiber (guar gum)	10.0	10.0	10.0	10.0
Oily fraction	—	—	—	50.0
Waxy fraction	—	—	50.0	—
Cholesterol	—	1.2	1.2	1.2
AIN-93 mineral mix	35.0	35.0	35.0	35.0
AIN-93 vitamin mix	10.0	10.0	10.0	10.0
L-cysteine	1.8	1.8	1.8	1.8
Choline bitartrate	2.5	2.5	2.5	2.5

a. Dyetose (Dyets, Bethlehem, PA).

b. Solka-Floc (International Fiber Corporation, North Tonawanda, NY).

Prior to combining the GS-WK lipids to the other dietary ingredients, the wax fraction was finely ground with a commercial blender, while the oil fraction was heated to approximately 60 °C.

Forty-four male Bio-F₁B Syrian hamsters (Bio Breeders, Watertown, MA, USA) were randomly assigned to four groups based on diet, each of which were housed individually in polycarbonate cages maintained at approximately 25 °C. The hamsters were allowed free access to fresh water and their assigned diet throughout the four-week feeding period. The sawdust serving as bedding was changed every week, and the fecal material was collected during the second and fourth weeks. Body weights and food intake were recorded on a weekly basis. At the end of the four-week feeding period, the hamsters were fasted for 14 h and then euthanized by CO₂ asphyxiation.

2.4. Characterization of plasma/liver samples for cholesterols

Fasting blood samples were collected directly from the heart by cardiac puncture using 10-mL syringes containing 10 mg of EDTA as an anticoagulant. Red blood cells were removed by centrifuging the blood at 1000g for 30 min at 4 °C; 2–4 mL plasma was recovered from each hamster. Aprotinin (1 mg/L) and phenylmethylsulphonyl fluoride (80 mg/L) were added to the plasma as preservatives. Plasma total cholesterol concentration was determined enzymatically using our microplate method (Carr, Andresen, & Rudel, 1993). Plasma HDL cholesterol concentration was measured after apolipoprotein B precipitation, and non-HDL cholesterol (VLDL + LDL) was calculated by difference. Previous hamster studies in our laboratory showed that the non-HDL plasma fraction contains >90% LDL cholesterol (Carr, Cai, Lee, & Schneider, 2000).

Livers were perfused with saline through the portal vein to eliminate residual blood before being excised. Gallbladder bile, which was collected with a preweighed 31-gauge syringe weight, was determined, and the volume was estimated assuming a density of 1 kg/L. The bile was transferred to a microfuge tube, and the syringe was rinsed several times with small volumes of saline.

Diluted bile was adjusted to a final volume of 0.2 mL. Biliary cholesterol and phospholipid were determined enzymatically (Carr et al., 1993), and bile acids were quantified as described previously for fecal samples. The livers were weighed and immediately frozen at -80°C . Aliquots of frozen liver were minced, and the lipids were extracted into chloroform–methanol (2:1, v/v) (Folch, Lees, & Sloane-Stanley, 1957). Total cholesterol, free cholesterol, and triglyceride were quantified enzymatically (Carr et al., 1993), and liver esterified cholesterol was calculated as the difference between total and free.

2.5. Characterization of Fecal Biological Samples

Nonradioactive feces were collected during week 4 for quantification of cholesterol-derived neutral steroids and bile acids. The following neutral steroids were quantified and represent fecal cholesterol and its metabolites: cholesterol, coprostanol, dihydrocholesterol, epicoprostanol, epicholestanol, coprostanone, and cholestanone. Ground feces (100 mg) were acidified by adding 0.2 mL of 0.5 mol/L HCl. Lipids were then extracted with chloroform–methanol (2:1, v/v) (Folch et al., 1957) containing 10 mg/L 5-cholestane as an internal standard. The lower phase solvent was evaporated, and the samples were saponified in 2 mL of 1 mol/L methanolic KOH for 1 h at 50°C . After the addition of 2 mL deionized water, the fecal steroids were extracted into 5 mL of hexane. The hexane was evaporated under nitrogen, and the steroids were derivatized and analyzed by GC as described above for GS-WK sterols.

Fecal bile acid concentration was quantified enzymatically as reported previously (Schneider et al., 2000). Samples were measured at 340 nm before and after the addition of 3 α -hydroxysteroid dehydrogenase to account for background absorbance. The concentration of total bile acids was calculated by the difference of the 2 absorbance readings, compared against a cholic acid calibration curve. Excreted plant sterols were quantified by GC as described in the previous section for determining phytosterol content in the GS-WK wax and oil fractions.

2.6. Statistical analysis

Lipid characterization data are reported as mean \pm standard deviation (STD) of triplicate analyses, whereas the animal data are presented as the mean \pm standard error of the mean (SEM). After ensuring the animal data were normally distributed, significance of treatment differences ($p < 0.05$) were by one-way ANOVA using Tukey's-LSD multiple comparison procedure (GraphPad InStat, La Jolla, CA, USA). Pearson correlation was used to compare fecal cholesterol excretion and liver cholesterol concentrations.

3. Results and discussion

3.1. Animal study design and diets

A Bio-F₁B Syrian hamster model was selected for this study because their plasma lipoproteins, free/esterified cholesterol,

and phospholipid profiles in the lipoproteins are similar to those in humans (Bravo, Cantafora, Calcabrini, & Ortu, 1994; Kowala, Nunnari, Durham, & Nicolosi, 1991). In addition, these hamsters are more susceptible to diet-induced atherosclerosis and hyperlipidemia (Cheema & Cornish, 2007; Trautwein, Jingsheng, & Hayes, 1993). The hamsters were fed an AIN diet containing low fat (control), high fat-cholesterol (athero), or high fat-cholesterol supplemented with 5% (w/w) GS-WK wax (athero + wax) or 5% (w/w) GS-WK oil (athero + oil) (Table 1) over a four week feeding period. Analysis of the body weights (43–50 g) and total food intake (194–221 g) at the end of the feeding period were not significantly different between the four groups, indicating that both GS-WK supplements as high as 5% were palatable and did not adversely affect animal growth.

Both the wax and oil were supplemented at 5% (w/w) of the diet based in part to a previous study (Carr et al., 2005) that showed an athero diet containing 5% (w/w) of the crude GS-WK lipid (wax + oil) resulted in significantly lower non-HDL cholesterol (69% reduction) and higher plasma HDL cholesterol levels (19% increase) compared to the athero diet alone. The 0.5% and 1% GS-WK crude lipid supplemented diet had, respectively little or lower (36% reduction) impact on non-HDL cholesterol levels. Additionally, liver esterified cholesterol decreased (66%), whereas neutral sterol output (47%) and HDL (16%) increased in response to the 5% GS-WK crude lipid supplement relative to an athero diet. Considering that the GS-WK crude extract contained a 3:1 wax to oil ratio, the fractionated GS-WK oil and wax were supplemented at higher levels compared to the latter study, particularly the oil based diet. The 5% level was used for this study to provide a phytosterol dose that was within the effective cholesterol lowering dosage of 2 g/d or ~ 30 – 40 mg phytosterols/(kg·d) for a 70 kg adult (Carr et al., 2010). Based on the phytosterol present in the GS-WK oil fraction (8.4 ± 1.8 mg/g oil, Table 2), the amount consumed by the hamsters the last week of the feeding period was approximately 2.4–3.7 mg/d, or 48–90 mg of phytosterols/(kg·d).

As expected, phytosterols were not detected in the GS-WK wax, while policosanols were not present in the GS-WK oil (Table 2). As the GS-WK wax fraction contained 10.8 ± 2.2 mg of policosanols/g of wax, with hexacosanol and dotriacontanol comprising 84% of this lipid class (Table 2), an estimated 3.1–4.7 mg policosanols/d of diet were consumed by the hamsters fed the GS-WK wax supplemented diet, or 62–115 mg policosanols/(kg·d). The oil contained higher levels unsaturated fats, whereas the wax was composed of higher levels saturated lipids (Table 2). Long chained aldehydes were present in the wax fraction, but were not detected in the oil. Both fractions contained minimal levels of hydrocarbons as confirmed by TLC and GC-FID methodologies (data not shown).

3.2. Plasma cholesterol profiles

A strong link between plasma LDL cholesterol concentration and cardiovascular diseases has been well established (Fernández & Webb, 2008), whereas hypertriglyceridemia has been positively correlated with VLDL levels (Hegele & Pollex, 2009). Although non-HDL cholesterol is comprised mainly of LDL

Table 2. Profiles of lipid components of interest in grain sorghum (GS) oil and wax.

		GS-WK wax	GS-WK oil
Phytosterols ¹	Total amount (per mg of lipid)	—	8.4 ± 1.8
	Sitosterol	—	29.6%
	Stigmasterol	—	34.9%
	Campesterol	—	32.3%
	Stigmastanol	—	3.2%
Policosanols ²	Total amount (mg per g of lipid)	10.8 ± 2.2	—
	Octacosanol	2.9%	—
	Triacosanol	8.6%	—
	Hexacosanol	48.3%	—
	dotriacontanol	35.7%	—
	Lignoceryl alcohol	4.4%	—
Fatty acids ³	Myristic acid	—	0.3%
	Palmitic acid	24.7%	22.1%
	Palmitoleic acid	—	1.5%
	Unknown	—	0.3%
	Stearic acid	12.4%	3.2%
	Oleic acid	25.0%	31.8%
	Linoleic acid	27.3%	37.5%
	Linolenic acid	1.4%	3.4%
	Eicosanoic acid	9.3%	—

1. Phytosterols were analyzed by GC coupled with FID as described by Leguizamón et al. (2009).

2. Policosanols were analyzed by GC after derivatization and silylation as described by Verleyen et al. (2001).

3. Fatty acids were derivatized to volatile fatty acid methyl esters (FAMES) and the FAMES were analyzed by GC coupled with FID as described by American Oil Society (2000). Percentage results from the cited compositional analyses are based on relative amounts.

cholesterol, VLDL cholesterol is present at quantities of <10%. Therefore, all four hamster groups were evaluated for plasma non-HDL levels (Figure 1). Plasma non-HDL levels were not significantly different between the athero + GS-WK wax and the athero fed animals, but the athero + GS-WK oil fed animals presented with significantly lower non-HDL levels similar to the control group.

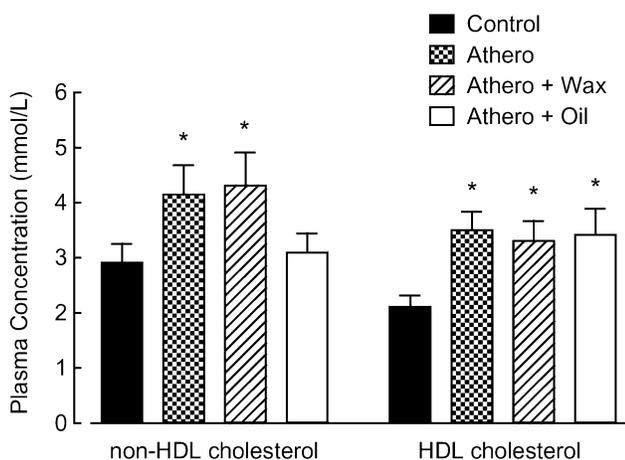


Figure 1. Plasma lipoprotein cholesterol concentration. 44 male Bio-F₁B Syrian hamsters separated into 4 groups of 11 were fed one of the following diets during 4 week feeding period: Control diet (Control), atherogenic diet (Athero), atherogenic diet containing 5% grain sorghum whole kernel wax (athero + wax), atherogenic diet containing 5% grain sorghum whole kernel oil (athero + oil). *Significantly different ($p < 0.05$) compared to control.

Comparison of plasma non-HDL levels in response to WKGS fractionated oil and to the GS-WK crude diet (Carr et al., 2005) suggest that the latter extract exerted a greater overall benefit even though lower amounts of oil were consumed, i.e., ~1.25% (w/w) for the 5% (w/w) crude lipid supplement based on 3:1 wax to oil ratio (Wang et al., 2005). As stated previously, the athero diet supplemented with 1% or 5% (w/w) GS-WK crude lipid resulted in a 36% and 69% reduction in plasma non-HDL levels, respectively, when normalized to the athero diet. Alternatively, a 25% reduction in non-HDL plasma cholesterol occurred in response to the GS-WK oil (Figure 1). This difference could be due to slight variations in athero diets used. In the Carr et al. (2005) study, the athero diet was prepared with 3% saturated fat and 0.05% cholesterol. Alternatively, the diets used for this study contained 5% saturated fat and 0.12% cholesterol, which may have resulted in higher cholesterol levels. If so, there is a possibility that the fractionated GS-WK oil could have exerted non-HDL responses comparable or even lower than produced by the GS-WK crude fraction (Carr et al., 2005). This hypothesis requires further testing as the latter study did not use a control diet as a point of reference (Table 1).

However, Hoi et al. (2009) showed that non-HDL levels decreased by 27% in hamsters fed an atherogenic diet similar to that used for the crude lipid experiment but supplemented with 5% (w/w) of grain sorghum dry distiller's grain with soluble (GS-DDGS). Lower GS-DDGS (0.5% and 1%) dosages did not affect non-HDL cholesterol. Moreover, analysis of the GS-DDGS showed that the wax fraction only accounted for 3.3% of the lipid extract (Hoi et al., 2009). These combined studies suggest that the oil and wax present in the GS-WK crude lipid may act as synergists or potentiates to elicit a greater benefit than the

Table 3. Liver lipid concentrations, $\mu\text{mol/g}$

	Control	Athero	Athero + wax	Athero + oil
Free cholesterol	4.3 ± 0.1^a	5.8 ± 0.2^b	5.7 ± 0.1^b	5.6 ± 0.1^b
Esterified cholesterol	0.6 ± 0.04^a	17.4 ± 1.9^c	13.1 ± 1.3^c	6.2 ± 0.9^b
Triacylglycerol	3.7 ± 0.3^a	4.5 ± 0.3^a	5.7 ± 0.2^b	5.7 ± 0.3^b

Values are means \pm SEM, $n = 11$. Means within a row having different superscripts are statistically different ($p < 0.05$).

individual fractions. Such dietary agent interactions have been reported for multiple food systems and biological targets (Wagner & Ulrich-Merzenich, 2009). Still, the absolute difference in plasma non-HDL cholesterol for the athero + WK-GS crude lipid fed group (Carr et al., 2005) and the athero + GS-WK oil fed animals was 0.6 and 1.05 mmol/L, respectively, relative to the group fed only the athero diet in each study.

HDL cholesterol levels were similar across the athero diets regardless of the supplement but higher compared to the control (Figure 1). These results support those reported by Hayes, Livingston, and Trautwein (1992) who showed that hamsters fed a high fat diet presented with higher HDL cholesterol compared to the control group. Additionally, Hoi et al. (2009) demonstrated that an athero + 5% GS-DGGS supplement, which contained only 3.3% wax, resulted in HDL levels similar to the athero diet. On the other hand, the 5% GS-WK crude lipid supplement produced a 16% increase in HDL (Carr et al., 2005). Combined, these results again suggest that the wax and oil may again be acting synergistically to elevate HDL levels.

For the study cited herein, the athero diet caused significantly higher plasma triacylglycerols (4.98 ± 0.46 mmol/L) relative to the control diet (3.05 ± 0.22 mmol/L) (data not shown in tabulated form). The WK-GS oil was not able to remediate the elevated triacylglycerols (4.75 ± 0.30 mmol/L), but the athero + WK-GS wax fed animals presented with even higher plasma triacylglycerol levels (6.49 ± 0.41 mmol/L). The effects of the GS-WK crude lipid on plasma triacylglycerols were not reported (Carr et al., 2005), but the GS-DDGS study showed that triacylglycerols were slightly elevated by the predominantly oil based extract at doses of 1 and 5% (w/w) (Hoi et al., 2009), which could be due to the small amount of policosanols present.

3.3. Lipid cholesterol profiles

Free liver cholesterol levels were comparable across the athero fed animals with each being slightly higher than the control (Table 3). Nonetheless, liver triacylglycerol levels were higher for only the animals fed the GS-WK oil and wax supplemented diets, which may have been due to lower VLDL secretion (Wang, Weller, & Hwang, 2005a). At this time we do not have another explanation for these results, but similar trends were reported for hamsters fed the athero diet supplemented with 5% GS-WK crude lipid and 1% and 5% GS-DGGS (Carr et al., 2005; Hoi et al., 2009). Liver esterified cholesterol was also significantly higher for the athero + GS-WK oil group compared to the control, but was considerably lower for the animals fed the athero and the athero + GS-WK wax diets (Table 3).

The unsaturated fatty acids present in the GS-WK oil may be partly responsible for the positive overall cholesterol responses (Valsta, Jauhiainen, Aro, Katan, & Mutanen, 1992), but the phytosterols are more likely providing the benefits (Figure 1, Table 2). Grain sorghum is a rich source of plant sterols compared to other commonly consumed cereal grains (Lee et al., 2010). The cholesterol lowering properties of phytosterols have been reported extensively for both humans and animal models (Batta et al., 2006; Racette et al., 2010). Several studies have linked phytosterols to the expression of enzymes involved in cholesterol synthesis, metabolism and transportation (Davis et al., 2004; Field, Born, & Mathur, 2004; Plösch et al., 2006). Vanstone, Raeini-Sarjaz, and Jones (2001) showed that subcutaneous injections of phytosterols suppressed higher levels of circulating cholesterol levels relative to orally consumed phytosterols. Other studies have shown that changes in gene expression were not directly caused by phytosterols but were rather a concomitant result followed by decreased cholesterol absorption efficiency (Calpe-Berdiel et al., 2005; Xu, Le, & Moghadasian, 2008). The generally accepted mechanism is that phytosterols displace dietary cholesterol and recirculating endogenous cholesterol when mixed micelles that transport lipids are formed in the small intestine resulting in reduced cholesterol absorption (Ostlund, 2007). Moreover, Kassis and Jones (2006) showed that the consumption of policosanols by human subjects did not positively affect plasma total, LDL, HDL-cholesterol or triglyceride level. These studies used authentic cane sugars as an isolated supplement to the individuals normal diets.

The 5% (w/w) GS-WK crude extract (Carr et al., 2005) as well as the 5% GS-DGGS (Hoi et al., 2009) were able to reduce liver esterified cholesterol. As stated previously, the animals used for both studies were fed the same types of diets in terms of fat content with only the GS supplement differing. The 5% GS-DDGS diet was not able to decrease liver cholesterol (48%) as effectively as the 5% (w/w) crude lipid (66%), whereas the fractionated WK-GS oil resulted in a 64% reduction. We have no explanation for these results except that the minimal amount of wax present in the GS-DGGS extract (3.3%) may be adversely affecting hepatic liver lipids. Many components acting as synergists or potentiates for a given health benefit can be antagonists for other outcomes (Junio et al., 2011).

3.4. Lipid excretion profiles and correlation with liver cholesterol

Fecal samples were measured for cholesterol output markers to determine if the fractionated GS-WK oil and/or wax affected cholesterol absorption. Total sterol (neutral + bile salts)

Table 4. Fecal sterol excretion

	Control	Athero	Athero + wax	Athero + oil
	$\mu\text{mol day}^{-1} 100 \text{ g}^{-1} \text{ body weight}$			
Bile acids	1.05 ± 0.13^a	1.42 ± 0.13^b	2.01 ± 0.12^c	1.67 ± 0.08^b
Neutral sterols	1.76 ± 0.11^a	4.55 ± 0.25^b	6.85 ± 0.47^c	10.24 ± 0.53^d
Phytosterols	0.64 ± 0.08^a	0.42 ± 0.07^a	0.61 ± 0.19^a	3.72 ± 0.21^b

Values are means \pm SEM, $n = 11$. Means within a row having different superscripts are statistically different ($p < 0.05$).

output was significantly higher for all the athero fed animals compared (Table 4). The excreted sterols were primarily neutral sterols, i.e., cholesterol and its metabolites, such as dihydrocholesterol and coprostanol. Cai and Carr (1999) also reported that neutral sterols represented the major cholesterol output in a hamster model fed a high cholesterol diet.

Although the GS-WK wax supplement did not lower plasma or liver cholesterol, neutral sterol output was 51% higher compared to the athero fed group (Table 4). These results are similar to those reported for the GS-WK crude lipid fed animals (47%) (Carr et al., 2005) but substantially lower than produced by the athero + GS oil diet (125%). As expected, high levels of phytosterols were detected in the fecal material collected from the athero + GS-WK oil fed animals (Table 4) as these agents were only present in the oil (Table 2). The high phytosterol output indicates that not all of these components were absorbed.

Interestingly, fecal bile acid output was higher for the athero + GS-WK wax fed group (Table 4). Of the components present in the wax fraction, the policosanols class is the most plausible agent eliciting this effect (Table 2). Ng, Leung, Huang, and Chen (2005) reported a significant increase in excreted bile acids in a hamster model fed a policosanols supplemented diet at dosages of 0.75 or 1.5 g/kg of diet, but the 0.35 g supplement had no effect. As stated previously, the policosanols content in the athero + GS-WK wax diet was 4.3–0.65 g/kg of diet. These levels are higher than present in the 5% GS-WK crude lipid (0.24 g/kg) (Carr et al., 2005), which had no effect on bile acid excretion.

Other studies have demonstrated that policosanols may play a role in reducing the activity of HMG-CoA, i.e., the rate-limiting enzyme in cholesterol synthesis and in increasing LDL receptor activity (Crespo et al., 1999; Menéndez et al., 2001). The results reported herein do not support these studies, as the athero + GS-WK wax diet did not positively impact other metabolic profiles (Figure 1 and Table 3), but were consistent with the results presented by Wang and Jones (2003), Kassis and Jones (2006); Kassis, Marinangeli, Jain, Ebine, and Jones (2007). It must be noted that this fraction contained higher amounts of saturated fatty acids (Table 2), which have been linked to elevated LDL levels (Siri-Tarino, Sun, Hu, & Krauss, 2010). Long chain aldehydes were also present in the GS-WK wax, but studies have not been completed on their participation in cholesterol absorption to our knowledge.

Sterols (in the form of bile acids, cholesterol, or its metabolites) have been correlated with and liver and plasma cholesterol levels (Charach, Rabinovich, Argov, Weintraub, & Rabinovich, 2012; Meissner et al., 2011). For example, Carr et al. (2005) showed a positive correlation between plasma non-HDL

cholesterol levels and cholesterol absorption in response to higher GS-WK crude lipid consumption ($r = 0.07$, $p = 0.034$), suggesting that dietary GS-WK crude lipid lowers non-HDL cholesterol, at least in part, by inhibiting dietary cholesterol absorption. On the other hand, GS-DGGS diet produced a strong negative correlation between fecal cholesterol excretion and liver cholesterol concentration in a dose dependent manner ($r = -0.97$, $p = 0.026$), indicating a dependency of intestinal cholesterol uptake on liver cholesterol levels.

For these studies, a negative correlation ($r = -0.687$, $p < 0.001$) (Figure 2) existed between cholesterol output (in the form of neutral sterols) with total liver cholesterol concentration that trended with diet. The GS-WK oil and athero based diets produced the greatest and lowest impact, respectively, with the wax diet falling in between. The results indicate that GS-WK oil plays the major role in reducing liver cholesterol levels by inhibiting dietary cholesterol absorption, but the policosanols containing wax may also provide such benefits, albeit not as potent. A review by Jones, Dassis, and Marinangeli (2009) has reported on the controversy of the cholesterol lowering ability of policosanols, concluding that there is no strong data to suggest that policosanols are effective cholesterol lowering compounds. Indeed, we agree with this assertion as isolated compounds; however,

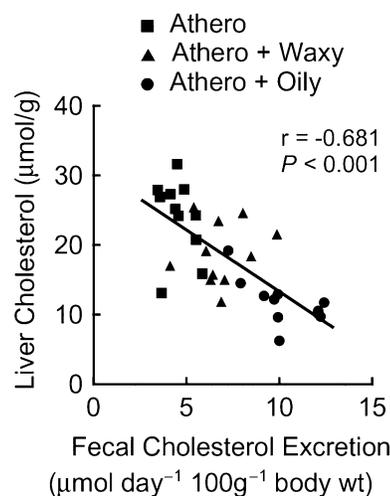


Figure 2. Correlation between cholesterol output and total liver cholesterol concentration. 44 male Bio-F,B Syrian hamsters separated into 4 groups of 11 were fed one of the following diets during 4 week feeding period: Control diet (Control), atherogenic diet (Athero), atherogenic diet containing 5% grain sorghum whole kernel wax (athero + wax), atherogenic diet containing 5% grain sorghum whole kernel oil (athero + oil).

we do also recommend that policosanols could act as a synergist or potentiates in complex systems in light of the health benefits data generated from WK and GS-DDGS story.

4. Conclusion

Comparison of two fractionated GS-WK lipid (wax and oil) shows that the oil is primarily responsible for lowering both plasma and liver cholesterol levels. A possible mechanism for this benefit is dietary cholesterol absorption inhibition. Nonetheless, consumption of GS wax resulted in elevated sterol excretion in the form of bile acids and neutral sterols. Although this fraction did not directly impact plasma and liver cholesterol, lipid profiles were not adversely impacted. These results in combination with other reported studies (Carr et al., 2005; Hoi et al., 2009) suggest that the wax may act as a synergist or potentiate by eliciting a greater effect than the sum of the individual parts. More studies are needed to test this hypothesis. This study does show that the oil is the most post cholesterol lowering fraction and thus has a great potential as a heart health functional food ingredient. Such an application would provide other opportunities for grain sorghum development for western markets.

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