Effects of Wheat Germ Supplementation on Hepatic and Cardiac Mitochondrial Function in a Model of Diet-induced Obesity and Insulin Resistance

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ABSTRACT

Obesity is strongly associated with insulin resistance, hyperglycemia, and hyperlipidemia, which are also linked to mitochondrial dysfunction and increased production of reactive oxygen species (ROS). Foods such as wheat germ (WG), which is rich in antioxidants, are thought to protect tissues from damage due to ROS and modulate some negative effects of obesity. This study examined the effects of WG supplementation on body composition, insulin resistance markers, and mitochondrial function in the liver and heart. Six-week-old male C57BL/6 mice were randomized into four dietary treatment groups (n=12 mice/group): control (C, 55% fat kcal), control + 10% WG (C+WG), high fat (HF, 55% fat kcal), or HF+WG. After 90 days of treatment, HF+WG mice had significantly less visceral fat and less insulin resistant compared to the HF group. Addition of WG to the control diet showed no considerable effect. HF feeding compared to control significantly elevated ($p<0.05$) cardiac oxygen consumption in complex 2, while WG supplementation to the HF diet stabilized this effect to the level of control. Consequently, *Sod2* and *Pgc1a* genes which mediate antioxidant defense and mitochondrial biogenesis respectively, were significantly reduced ($p<0.05$) in the heart of the HF group compared to control while WG supplementation tended to upregulate both genes. These effects of WG were not observed in the liver. Put together, these results showed that WG supplementation in HF diet reduced insulin resistance and improved the metabolic functions of the cardiac mitochondria.

1. Introduction

Obesity is a major public health problem worldwide. Data from the World Health Organization (WHO) showed that in 2014, nearly 2 billion people globally were overweight or obese (World Health Organization, 2016). Obesity is strongly associated with insulin resistance, hyperinsulinemia, hyperglycemia, and hyperlipidemia, which are all key components of type 2 diabetes (T2D) (LeRoith et al, 2008).

The mitochondria generate ATP by oxidation-reduction reaction using the electron carriers NADH or $FADH_2$. Electrons obtained from the oxidation of metabolic fuels are transported down the complexes of the electron transport chain to the final electron acceptor,

molecular oxygen, which is ultimately converted to water (Rolo et al, 2006). However, even in normal metabolism some oxygen (approximately 0.1%) leaks from the respiratory chain resulting in incompletely reduced oxygen molecules, or ROS, such as superoxide, which can damage the mitochondria and cell (Rolo et al, 2006). Mitochondrial function can be assessed in multiple ways, including changes in mtDNA encoded genes, altered expression or activity of enzymes involved in oxidative phosphorylation, and mitochondrial oxygen consumption rates (OCR) in response to various challenges (Montgomery, et al 2015). Mitochondrial function may be affected by a variety of factors including genetics, oxidative stress, diet, mitochondrial biogenesis, and aging (Kim et al, 2008).

Apart from stimulating insulin resistance and T2D, high fat (HF) diet-induced obesity has been shown to induce mitochondrial dysfunction and reactive oxygen species (ROS) generation, while modulating significant changes in fuel metabolism in the heart and the liver (Cole et al, 2011; Lim et al, 2014; Vial et al, 2010). At the genetic level, the mitochondrial master-regulator gene PGC-1a, as well as genes induced by PGC-1a, is downregulated in humans with T2D (Mootha et al, 2006). Moreover, Sreekumar et al (2002), reported lower gene expression levels of the antioxidant enzyme, superoxide dismutase (SOD-1 and SOD-2) in the skeletal muscle of rats fed a HF diet compared to rats fed a low fat diet, suggesting a loss of ability to cope with ROS generation. Therefore, it is vital to maintain the body's natural antioxidant enzymes levels in order to combat increased ROS production as a result of HF diet-induced mitochondrial dysfunction.

Considering the increased prevalence of obesity and its related morbidities such as T2D, studies that investigate approaches and mechanisms to alleviate obesity-related T2D, oxidative stress, and mitochondrial dysfunction are needed. Wheat germ (WG) contains relatively large amounts of bioactive compounds known to have antioxidant properties such as tocopherols, carotenoids, flavonoids, and phytosterols (Cara et al, 1992). Traditionally, WG has been mostly ignored as a byproduct of wheat milling. When utilized, WG is most frequently used to produce WG oil or to add bulk to livestock feed (Brandolini et al, 2012). A previous study found a significant decrease in CD40 ligand, a protein with pro-inflammatory qualities, in patients with hypercholesterolemia who consumed a WG oil supplement for two months (Alessandri et al, 2006). Similarly, Cara, et al. (1992) reported that subjects with hypercholesterolemia and hypertriglyceridemia who supplemented their diets with WG had lower plasma cholesterol, VLDL cholesterol, LDL cholesterol, and plasma triglyceride levels after 4-14 weeks (Cara et al, 1992). WG's richness in antioxidant and bioactive compounds makes it a potentially useful natural food substance, which may play a role in preventing the oxidative damage to cells and mitochondrial dysfunction in obesity. However, there are no studies looking specifically at the effect of WG supplementation in a model of HF diet-induced obesity on oxidative stress and mitochondrial function. Therefore, the *purpose* of this study is to investigate the effects of WG supplementation on insulin resistance, mitochondrial oxidative capacity and oxidative stress in the liver and heart of C57BL/6 mice

fed a HF diet. We *hypothesized* that the bioactive compounds in WG will work in synergy to preserve mitochondrial function and reduce oxidative stress in HF diet-induced obese mice.

2. Experimental Details

Study design

Forty-eight, six-week-old male C57BL/6 (Charles River Laboratory) mice were housed in groups of four at the Oklahoma State University (OSU) Laboratory Animal Research facility in a temperature- and humidity-controlled environment and maintained on a 12 hr light:dark cycle with *ad libitum* access to deionized water. Mice were randomly assigned to four dietary treatment groups (n=12 mice/group: control (C, AIN-93M), C + 10% WG, high fat (HF), HF + 10% WG for 90 days (***Appendix 1***). WG were obtained from Shawnee Milling Co (Shawnee, OK), analyzed for its nutrient composition at NP Analytical Laboratories, (St Louis, MO) and added to the control or HF diets at a 10% wt/wt composition. The WG diets were adjusted to have the same macronutrient composition to the control and HF diets. Body weights were measured weekly and food intake was recorded 3 times/wk. After 90 days of dietary treatment, mice were fasted for 4 hrs and anesthetized with a mixture of ketamine/xylazine followed by exsanguination. The Institutional Animal Care and Use Committee at OSU approved all animal handling and procedures.

Glucose Tolerance Test (GTT)

After 90 days of treatment, mice were fasted for 6 h before undergoing a glucose tolerance test. Mice were given an intravenous injection of a 20% glucose solution (2 g glucose/kg body weight). Blood glucose levels from tail blood were recorded 0, 15, 30, 60, and 120 minutes after glucose injection using an AlphaTrak glucometer (Abbott Laboratories).

PCR Array and qPCR

Total RNA was isolated from approximately 100 mg whole liver or 50 mg whole heart sample using Trizol reagent (Thermo Fischer Scientific, Middletown, VA). RNA concentration and integrity were measured using a Nanodrop spectrophotometer (Thermo Fischer Scientific, Middletown, VA) and agarose gel electrophoresis. cDNA was synthesized using SuperScript II (Invitrogen, Grand Island, NY). The cDNA from the individual samples was used as a template for PCR according to the protocol for using SYBR green chemistry on an ABI 7900HT system (Applied Biosystems, Grand Island, NY). Primers were designed using Primer Express v. 2.0 (Applied Biosystems) as described in Davis, et al (Davis et al, 2012). Relative quantitation for each gene was determined using the $2^{-\Delta\Delta CT}$ method (Applied Biosystems User Bulletin #2) with Cyclophilin B (Cyclo) as the invariant control. Primer sequences used in these experiments are listed in ***Appendix 2***.

Plasma Measurements

Plasma lipids, inflammatory markers, and diabetes markers were assessed as we have previously described (Ojo et al, 2016). Briefly, plasma concentrations of triglycerides (TGs), non-esterified fatty acids (NEFA), total cholesterol (TC), and HDL cholesterol were

measured using a BioLis 24i automated analyzer (Carolina Chemistry). Using the Bio-Plex MAGPIX Multiplex Reader (Bio-Rad Laboratories Inc, CA), plasma diabetes markers (gastric inhibitory peptide [GIP], glucagon-like peptide 1 [GLP1], insulin, leptin, plasminogen activator inhibitor 1, and resistin) were assessed according to the manufacturer's instructions. Insulin resistance was estimated utilizing the homeostatic model assessment of insulin resistance (HOMA-IR) (Matthews et al, 1985) HOMA-IR = (fasting insulin (μU/mL) x fasting glucose (mmol/L))/22.5.

Seahorse Mitochondrial Flux Analyses

Freshly obtained liver and heart ventricle (60 mg) were homogenized on ice at low speed (Qiagen homogenizer) in 0.5 mL of mitochondrial isolation buffer (210 mM mannitol, 70 mM sucrose, 5 Mm HEPES,I mM EGTA and 0.5% (w/v) fatty acid-free BSA, pH 7.2), followed by a series of centrifugation steps as described earlier (Bharadwaj et al. 2015). Isolated mitochondria pellets were suspended in mitochondrial assay solution (1X MAS, 70 mM sucrose, 220 mM mannitol, 5 mM $MgCl_2$, 2 mM HEPES, 10 mM KH_2PO_4, 1 mM EGTA, 0.2% (w/v) fatty acid-free BSA) and protein concentration determined using Pierce BCA protein assay kit (ThermoScientific, Rockford, IL). Mitochondrial sample was re-suspended in 200 μg/mL for further analyses. Mitochondrial respiration was measured as oxygen consumption rates (OCR) using the Seahorse XF 96 flux analyzer (Seahorse Bioscience, Billerica, MA) following manufacturer's instructions. Briefly, isolated mitochondria from the liver and heart were seeded into a 96-well microplate at 4 ug/well and 1 ug/well, respectively. For the coupling assay, final concentrations of compounds after port injections were 4 mM ADP (port A), 1.5 μM oligomycin (port B), 4 μM FCCP (port C) and 4 μM antimycin A (port D). Final concentrations of compounds for the electron flow assay were 2 μM rotenone (port A), 10 mM succinate (port B), 4 μM antimycin A (port C) and 10 mM/100 μM ascorbate/TMP (port D). From these reactions, basal respiration, proton leak, maximal respiratory capacity, complex 1, 2, 4, and 5 OCR were calculated using the adjusted method from previous study (Lay et al. 2016) and as illustrated in Appendix 3. Results are reported as pmol O_2 min^{-1} μg^{-1} protein.

Statistical Analysis

All data are reported as means±SEM. Differences between treatment groups were analyzed using one-way ANOVA followed by *post hoc* Tukey test using SAS 9.4 software. P-value < 0.05 was considered statistically significant.

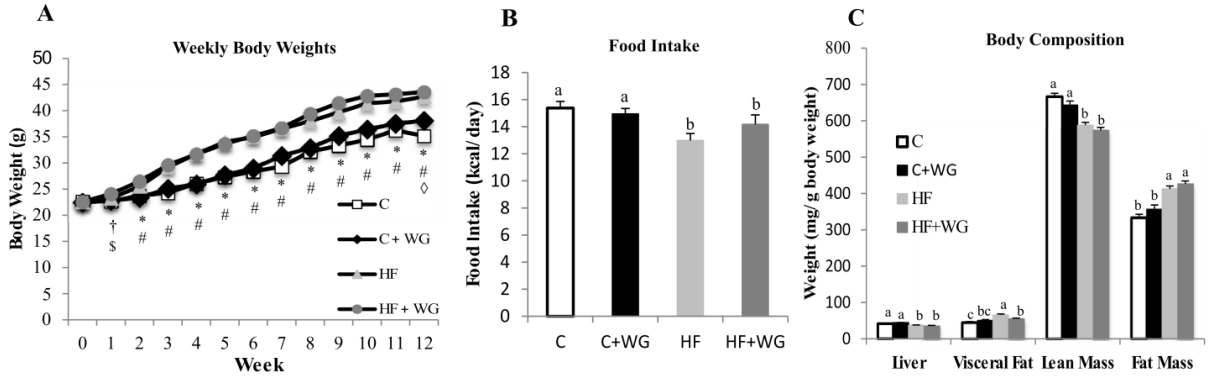
3. Results

WG supplementation decreased amount of visceral fat but did not affect total body weight

There were no differences in body weight between the 4 groups before treatment began. After 90 days of treatment, the mice in the HF group weighed significantly more than mice in the C group and C+WG mice weighed significantly more than C group mice (Fig. 1A). There was no difference in the final weights of the mice in the HF group compared to the HF+WG group (Fig. 1A). HF mice ate 40% fewer kcal per day than mice in the C group,

yet weighed significantly more at the end of the experiment (Fig. 1A and 1B). Notably, mice in the HF and HF+WG groups had lower caloric intake than mice in the C and C+WG groups throughout the 90-day experimental period (Fig. 1B). The livers of mice in the HF and HF+WG groups weighed significantly less than the livers of the C and C+WG mice (Fig. 1C). However, mice in the C group have significantly less visceral fat compared to mice in the HF group (Fig. 1C). Mice that consumed the HF+WG diet also had significantly less visceral fat than mice in the HF group (Fig. 1C). HF and HF+WG mice had significantly less lean mass and significantly more fat mass compared to C and C+WG groups (Fig. 1C).

Figure 1. Weekly body weights, food intake, and body composition of C57BL/6 mice fed either a control (C) or high fat (HF) diet supplemented with 10% WG for 90 days.

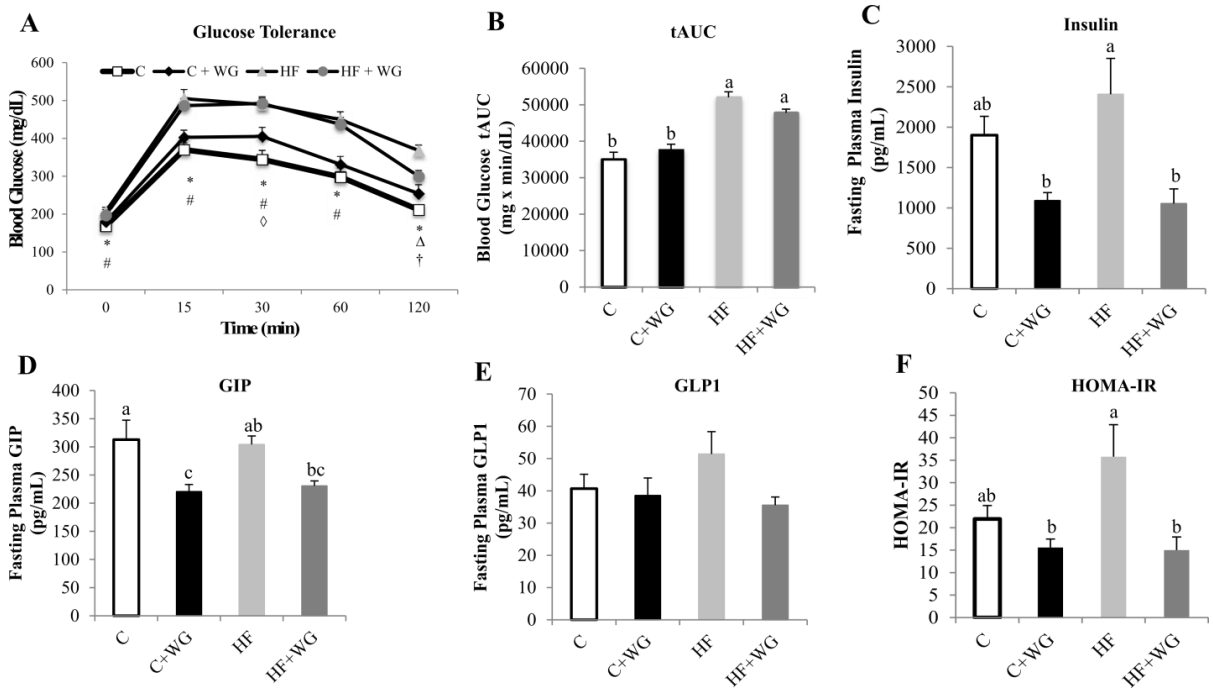


n=12/group; Different letters indicate significant (P<0.05) difference between groups; Data = mean ± SE
* C is different from HF and HF + WG; # C + WG is different from HF and HF + WG ; † C + WG is different from HF ; ◊ C is different from C + WG; $ C is different from HF + WG

WG supplementation improved GTT, fasting plasma insulin levels, and IR measurements

HF and HF+WG mice had significantly higher blood glucose levels at baseline than C and C+WG mice (Fig. 2A). Two hours post-glucose injection, HF+WG mice had significantly lower (18.5%) glucose levels than HF mice. However, the GTT total area under the curve (tAUC) was not different between HF and HF+WG groups (Fig. 2B). Fasting plasma insulin levels were 2.3 fold lower in HF+WG mice than HF mice (1054 pg/mL compared to 2407 pg/mL, respectively). Gastric inhibitory polypeptide (GIP) was lower in the C+WG group compared to the C group. There were no differences in GIP expression between HF and HF+WG mice (Fig. 2D). Glucagon-like peptide-1 (GLP1) levels were not different across any of the groups (Fig. 2E). Insulin resistance, assessed by HOMA-IR, was 58% lower (p=0.0080) in HF+WG group compared to the HF group (Fig. 2F).

Figure 2: Diabetes markers in C57BL/6 mice fed either a control (C) or high Fat (HF) diet supplemented with 10% WG for 90 days.



n=10-12/group; Different letters indicate significant (P<0.05) difference between groups; Data = mean ± SE
* C is different from HF and HF + WG; # C + WG is different from HF and HF + WG
† C + WG is different from HF; ◊ C is different from C + WG; Δ HF is different from HF + WG

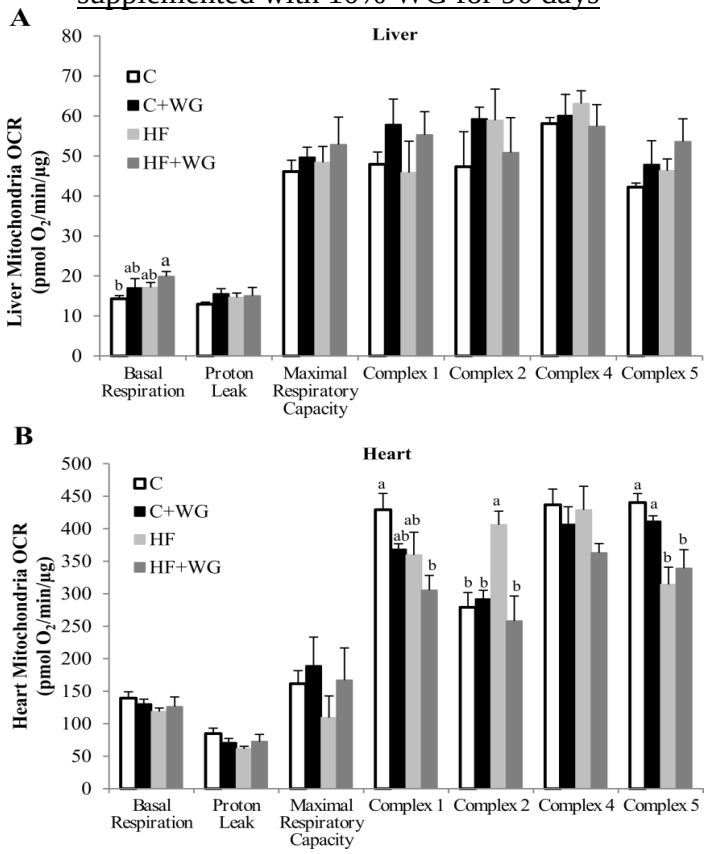
WG supplementation reduced OCR in complex 2 of the heart mitochondria

Mitochondrial basal respiration rate in the liver was lowest in the C group while HF+WG mice consumed the most oxygen (Fig. 3A). Apart from this, other measured components in the liver mitochondria were not different (Fig. 3A). In the heart, HF+WG group significantly lowered OCR in complex 1 compared to the C group (p=0.0285), but there were no differences between C and C+WG or between HF and HF+WG (Fig. 3B). In complex 2 of the heart mitochondria, OCR was 32% higher (p<0.05) in HF mice compared to all the other groups (Fig. 3B). HF feeding significantly reduced complex 5 OCR compared to C group mice, while the addition of WG to either C or HF diet had no significant effect (Fig. 3B).

Addition of WG to diets has modest effects on plasma lipids

Plasma triglyceride levels were significantly higher (p=0.0061) in HF diet mice compared to C diet mice (Fig. 4A). Addition of WG to the C has no effect on plasma triglycerides but tended (P=0.14) to be lower when added to the HF (Fig. 4A). Non-esterified fatty acid (NEFA) levels were higher in C+WG mice compared to C mice (Fig. 4B). NEFA levels were not different between HF and HF+WG mice (Fig. 4B). Total plasma cholesterol levels were 21% higher in HF+WG mice compared to HF mice (Fig. 4C).

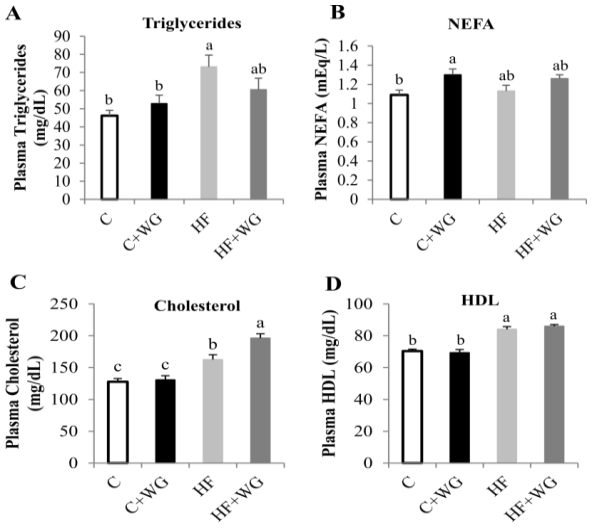
Figure 3: Mitochondrial oxygen consumption rates (OCR) in the liver and heart of C57BL/6 mice fed either a control (C) or high fat (HF) diet supplemented with 10% WG for 90 days



N=5/group; Different letters indicate significant (P<0.05) between groups; Data = mean ± SE

(Fig 5A).

Figure 4: Plasma lipid content of C57BL/6 mice fed either a control (C) or high fat (HF) diet supplemented with 10% WG for 90 days



N=12/group; Different letters indicate significance between groups | Data = mean ± SE

There were no differences in cholesterol levels between C and C+WG mice. Plasma HDL levels were not different between C and C+WG or HF and HF+WG mice (Fig. 4D).

HF diet fed mice showed reduced expression of the mitochondrial biogenesis marker, Pgc1a and the antioxidant enzyme, Sod2 in the heart

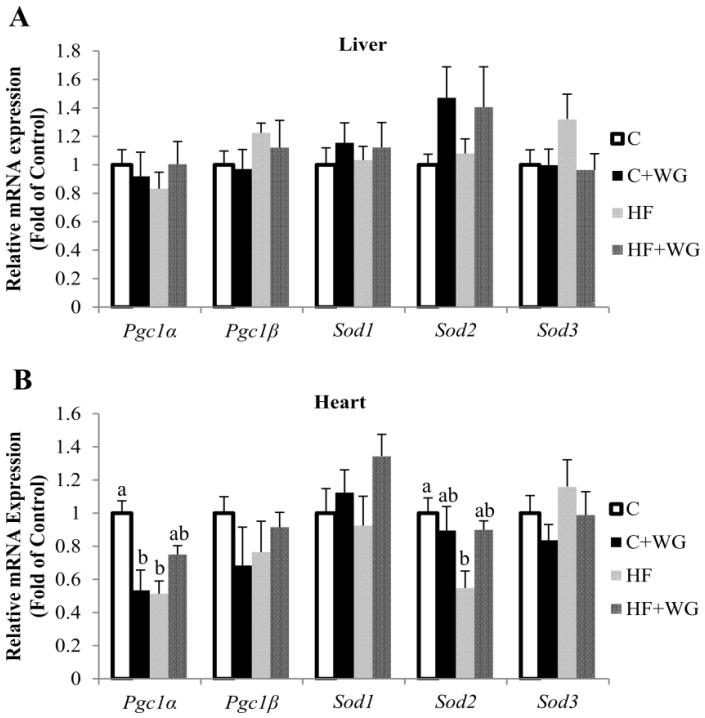
Heart *PGC1α* was expressed 40% less in C+WG mice compared to C mice. Interestingly, *PGC1α* tended (P=0.16) to be expressed more highly in HF+WG mice compared to the HF mice (Fig. 5B). A 45% decrease (p=0.028) in *Sod2* expression was observed in the heart of HF fed mice compared to C. *Sod2* in the heart also tended (P=0.11) to be more highly expressed in the HF+WG group compared to the HF mice (Fig. 5B). No liver genes tested showed significant differences in expression

4. Discussion and Conclusions

The aim of the current study was to examine the effects of WG supplementation on insulin resistance, oxidative stress, and mitochondrial function in C57BL/6 in mice fed a high-fat diet for 90 days. Mice fed a HF+WG diet demonstrated reduced visceral fat storage and insulin resistance compared to HF-diet fed mice. Results from a mitochondrial extracellular flux assay indicated increased oxygen consumption in complex II of HF-diet mice's hearts, but without any change in activity of complex V, which is responsible for ATP synthesis. HF-diet fed mice also demonstrated decreased expression of *Sod2* compared to C and HF+WG fed mice indicating a reduced ability to cope with ROS and oxidative stress generated by an inefficient mitochondria.

HF and HF+WG mice consumed significantly fewer kcal than C and C+WG mice, but the HF diet-based groups weighed significantly more than the C and C+WG mice from week 2 of the experiment onward. This suggests the fat content of the HF diet contributes more to weight gain than simply the number of calories consumed. Our finding that visceral fat accumulation was reduced in HF+WG mice compared to HF mice agreed with the findings of Palmieri et al. (2006) who demonstrated that lower levels of antioxidants were strongly associated with abdominal fat storage due to systemic oxidative stress. The same study found an inverse relationship between vitamin E consumption and visceral fat accumulations. Since WG is high in vitamin E (Cara et al, 1992), this may have contributed to the reduction in abdominal fat storage in HF+WG mice.

Figure 5: Relative gene expression of mitochondrial biogenesis and oxidative stress markers in C57BL/6 mice fed either a control (C) or high fat (HF) diet supplemented with 10% WG for 90 days



N=6/group ; Different letters indicate significance between groups; Data = mean ± SE

HF+WG mice in this study demonstrated increased insulin sensitivity compared to HF mice, indicating that the addition of WG may modulate insulin resistance induced by a HF diet. GIP, which is thought to induce insulin secretion (Brubaker et al, 2002), was reduced by the addition of WG to both the C (P= 0.01) and HF (P= 0.07) diet, further suggesting WG supplementation increased insulin sensitivity. WG supplementation also improved the efficiency of mitochondrial complex II in the heart as the mitochondria isolated from mice on the HF+WG diet consumed less oxygen at complex II than mice from the HF diet. Although the OCR of complex II in HF-diet mice was significantly higher than other groups, this did not reflect in complex V OCR. Therefore, it is probable that in the HF group, electrons generated by oxygen consumption in complex II were not passed farther down the electron transport chain to complex V, which is the site of ATP synthesis. These lost electrons may have generated ROS within the heart mitochondria. Consequently, we noted significantly lower transcription of *Sod2* in HF diet mice compared to C diet mice indicating an inability of the heart in mice fed HF diet to cope with increased ROS production. Although not significant, *Sod2* expression tended to be higher in the hearts of HF+WG mice compared to HF mice suggesting that WG supplementation may have the potential of sustaining the ability of heart cells to cope with ROS generation.

There are limited animal and clinical studies that have investigated the health benefits of WG and its oil, but previous studies have provided evidence for wheat germ or wheat germ oil improving the response to oxidative stress. One study by Alessandri, et al. found that hypercholesterolemic patients who consumed a WG oil supplement showed reduced markers of oxidative stress, but did not have lower cholesterol levels. Our results, however, showed an increase in total plasma cholesterol levels in the HF+WG mice compared to HF diet mice suggesting that while wheat germ or wheat germ oil may be beneficial in reducing oxidative stress it may or may not actually lower cholesterol levels (Alessandri et al, 2006). The effects of WG on lipid metabolism need to be further investigated. Expression of *Pgc1α* was decreased in the hearts of C+WG fed mice compared to C mice. *Pgc1α* is known as the mitochondrial biogenesis master regulator and its decreased expression in C+WG mice suggests that WG supplementation may lower oxidative stress enough to reduce the need for mitochondrial biogenesis and production of new mitochondria to replace damaged ones.

A limitation of this study may be the possibility of achieving the 10% WG dose in humans. In a 70 kg adult, 10% wheat germ consumption per day would equate to about 50 g of wheat germ per day. At this time, it is not clear if a lower dose would create the same effects. Therefore, future studies may investigate the dose-dependent effects of WG supplementation in a model of diet-induced obesity. Nevertheless, the findings of this study indicate supplementing a HF diet with WG may increase the ability of C57BL/6 mice's hearts to cope with oxidative stress and other damage induced by HF diet, including additional visceral fat accumulation, increased insulin resistance, likely increased generation of ROS by inefficient mitochondria, and decreased innate antioxidant ability to cope with ROS.

4. Summary

WG is rich in antioxidants that may protect cells from the oxidative stress induced-damage due to a high fat diet. Since the mitochondria is a master regulator of ROS production, this study investigated the effects of WG supplementation on hepatic and cardiac mitochondrial function and innate antioxidant defense in C57BL/6 mice fed either a control or HF diet for 90 days. In this study, addition of WG to a diet high in fat tended to increase the ability of the heart to cope with oxidative stress by up regulating expression of *Sod2* compared to HF fed mice alone. WG supplementation also decreased visceral fat accumulation, decreased insulin resistance, and stabilized complex 2 oxygen consumption rate in the heart to the level of control. This study showed that supplementing a HF diet with WG has the potential to mitigate the negative effects of HF diet-induced obesity on insulin resistance and cardiac mitochondrial metabolism.

5. Appendices

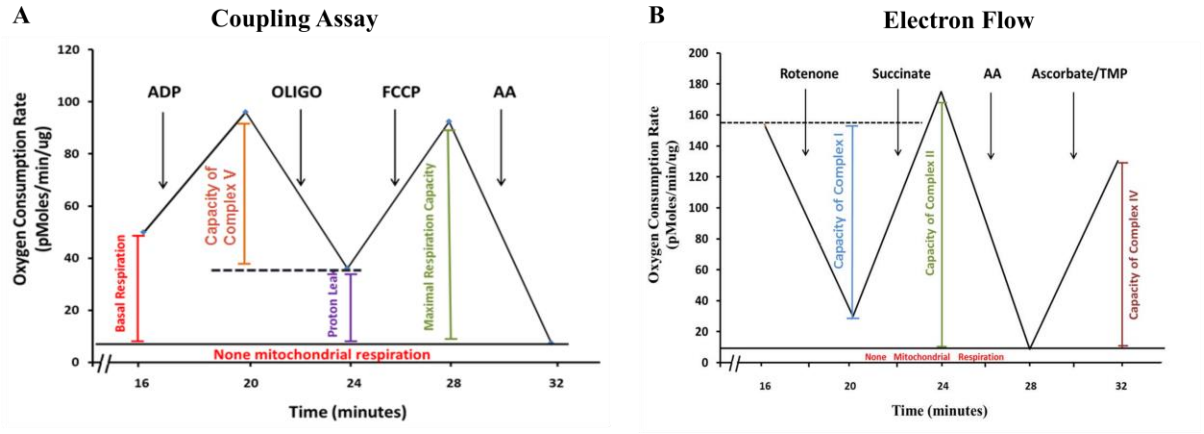
Appendix 1: Diet Composition (g/kg diet) for control (C), high fat (HF), control + wheat germ (C+WG), and high fat + wheat germ (HF+WG) diets

	C	HF	C+WG	HF+WG
Wheat Germ	--	--	100	100
Carbohydrates				
Total	722.1	372.26	724.77	374.93
Cornstarch	466	0	412.9	0
Sucrose	100	270	100	270
Dextrinized Cornstarch	155	100	155	46.9
WG	--	--	53.1	53.1
Protein				
Total	140	180	140	180
Casein	140	180	116.6	156.6
WG	--	--	23.4	23.4
Fat				
Total	40	350	40	350
Soybean Oil	40	40	33.35	33.35
Lard	0	310	0	310
WG	--	--	6.65	6.65
Fiber				
Total	50	50	50	50
Cellulose	50	50	45.84	45.84
WG	--	--	4.16	4.16
Vitamin Mix	10	10	10	10
Mineral Mix				
Total	35	35	35	35
Calcium	25.9	25.9	25.7	25.7
Calcium from WG	--	--	0.06	0.06
Sodium Phosphate	5.6	4.8	3.8	3.02
Potassium Phosphate	2.4	2.06	1.65	1.3
Phosphorous from WG	--	--	0.8	0.8
Sucrose	1.1	2.3	3.76	4.92

Appendix 2: PCR Primers

Target gene	Primer Sequence
Pgc1α	F 5' AAC-CAC-ACC-CAC-AGG-ATC-AGA 3' R 5' TCT-TCG-CTT-TAT-TGC-TCC-ATG-A 3'
Pgc1β	F 5' GAG-GGC-TCC-GGC-ACT-TC 3' R 5' CGT-ACT-TGC-TTT-TCC-CAG-ATG-A 3'
Sod1	F 5' GCC-CGG-CGG-ATG-AAG-A 3' R 5' CGT-CCT-TTC-CAG-CAG-TCA-CA 3'
Sod2	F 5' CTC-TGG-CCA-AGG-GGA-GAT-GTT 3' R 5' GTC-CCC-CAC-CAT-TGA-ACT-TC 3'
Sod3	F 5' CAG-ACA-AAG-GAG-CGC-AAG-AAG 3' R 5' TGA-GGC-TTA-AGT-GGT-CTT-GCA 3'

Appendix 3: Seahorse Mitochondria Analysis Graphs



Papers Published

Poster: Effects of Wheat Germ Supplementation on Mitochondrial Function in Mice Fed a High-Fat Diet presented at Oklahoma State University Research Week

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