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A Low-Fat Diet Containing Heated Soybean Oil Promotes Hyperglycemia in C57BL/6J Mice

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ABSTRACT

by Olivia Middleton

A Low-fat Diet Containing Heated Soybean Oil Promotes Hyperglycemia in C57BL/6J Mice

Introduction: The metabolic effects of consuming mildly oxidized lipids as compared to highly oxidized lipids are not well documented. Consumption of highly oxidized polyunsaturated fatty acids, even in a low-fat diet, may be a threat to one's health. Previous studies in our lab have shown that mice consuming soybean oil heated for 3 hours, compared to unheated, gain less body weight, but more fat pad mass, and 3T3-L1 adipocytes treated with soybean oil heated for ≥ 6 hours have abrogated triglyceride accumulation. Another study showed that rats fed highly oxidized oil (heated > 24 hrs), as compared to fresh soybean oil and fish oil, had lower fat pad mass and weight gain but developed glucose intolerance. This suggested that the extent of lipid oxidation determined the subsequent metabolic risk.

Objective: Our aim, in the current study, is to investigate if a low-fat diet with soybean oil heated for increasing time points (3h, 6h and 9h) alter fat mass and glucose tolerance.

Methods: Six week old, male, C57BL/6J mice were randomly divided into six groups (n=8/group). Three groups were fed a low fat diet with soybean oil heated for 3 (3hr-HO), 6 (6hr-HO), or 9 (9hr-HO) hours for 16 weeks. Another three groups were pair-fed to each of the 3hr-HO, 6hr-HO and 9hr-HO groups with a low-fat diet containing unheated oil (PF-UHO). Food consumption was recorded every 3-4 days, and body weights were recorded weekly. Soy oil in the diets was analyzed for products of oxidation. At 16 weeks, blood glucose levels were measured after a 6 hour fast; fat pad and liver weights were recorded, and blood was collected by cardiac puncture for serum insulin analysis.

Results: Final weight gain was not significantly different between all HO groups as a percent of their respective PF groups ($p > 0.05$). The feeding efficiency for 3hr, 6hr, and 9hr-HO groups as a percent of PF was 92.75, 113.02, and 111.28, respectively. Mean weights of all fat pads for HO groups decreased with heating time as a percent of PF, although these differences were not statistically significant. Blood glucose was lowest in the 3hr-HO group and significantly increased from 3hr-HO group to 6hr-HO group ($p=0.021$) as a percent of PF. Serum insulin levels decreased for the HO groups as heating time increased, although these differences were not statistically significant.

Conclusion: Consuming a diet with increasing amounts of oxidized lipids decreased fat pad mass and insulin levels, while increasing fasting glucose levels. This paradoxical relationship between increased glucose in the presence of decreased insulin in c57BL/6J mice could be due to either reduced insulin secretion or increased insulin resistance. Further research in our lab will aim to analyze triglyceride accumulation in the liver and muscle cells of these mice to determine if oxidized lipids promote ectopic fat deposition.

A LOW-FAT DIET CONTAINING HEATED SOYBEAN OIL PROMOTES
HYPERGLYCEMIA IN C57BL/6J MICE

by
Olivia Middleton

A Thesis

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TABLE OF CONTENTS

List of Tables	iv
List of Figures	v
Abbreviations	vi
Chapter	
I. INTRODUCTION	1
II. LITERATURE REVIEW	5
III. METHODS.....	12
IV. RESULTS.....	18
V. DISCUSSION AND CONCLUSIONS.....	24
REFERENCES	29

LIST OF TABLES

Table	Page
1. Mouse Diet Composition	13
2. Data for Mice Consuming ad-lib UHO chow	19

LIST OF FIGURES

Figure	Page
1. Feeding Design	15
2. Products of Oxidation in Oil Heated 3, 6, or 9 hrs	18
3. Mean Wright Gain of Micce Consuming the 3, 6, or 9 hr HO chow	20
4. Feeding Efficiency of mice consuming the 3, 6, or 9 hr HO chow	21
5. Mean organ weights of mice consuming the 3, 6, or 9 hr HO chow.....	22
6. Mean fasting glucose of mice consuming the 3, 6, or 9 hr HO chow	23
7. Mean serum insulin levels of mice consuming the 3, 6, or 9 hr HO chow	23

ABBREVIATIONS

ad-UHO: mice consuming unheated soybean oil ad libitum

CD: conjugated diene

CO: coconut oil

CT: conjugated triene

°C: degrees Celsius

DAR: Department of Animal Research

EWAT: epididymal white adipose tissue

g: gram

HF: high fat diet with fresh soybean oil

HFD: high fat diet

HIP: hexane isopropanol

HO: heated oil

HSO: heated sunflower oil

IBAT: intrascapular brown adipose tissue

kcal: kilocalories

kg: kilogram

L: liter

LF: low-fat diet with fresh soybean oil

LO: low-fat diet with heated soybean oil

LOOH: lipid hydroperoxides

mEq: milli equivalents

mins: minutes

n-6: omega 6

n-3: omega-3

nm: nanometer

OFO: oxidized frying oil

OGTT: oral glucose tolerance test

PAH: polycyclic aromatic hydrocarbons

PBS: phosphate buffered saline

pmol: picomole

PPAR- γ : peroxisome proliferator activated receptor gamma

PUFA: polyunsaturated fatty acid

PV: peroxide value

ROS: reactive oxygen species

RWAT: retroperitoneal white adipose tissue

SD: standard deviation

SPSS: Statistical Package for the Social Sciences

SREBP-1c: sterol regulatory enhancer binding protein-1c

TG: triglyceride

TZD: thiazolidinedione

UHO: unheated soybean oil

WHO: World Health Organization

13-HODE: 13-hydroxy linoleate

3hr-HO: mice consuming diet containing soybean oil heated for 3 hours

6hr-HO: mice consuming diet containing soybean oil heated for 6 hours

9hr-HO: mice consuming diet containing soybean oil heated for 9 hours

CHAPTER I: INTRODUCTION

Many Americans consume fried, fast and processed foods which are high in fat and calories and low in nutrients. As the rate of obesity in the United States continues to grow in both adults and children, many people and researchers are determined to discover the cause. According to the World Health Organization, obesity has more than doubled worldwide, since 1980. In 2008, more than 1.4 billion adults, 20 and older, were overweight, and of these, over 200 million men and 300 million women were obese¹. Studies both in humans² and in animals^{3,4} have shown that a high fat diet is generally associated with obesity and its related conditions such as metabolic syndrome⁵. In a recent study, C57BL/6J mice were fed a high fat diet for 18 months and developed glucose intolerance, hyperinsulinemia, and increased body weight; after the mice were switched to a low fat diet for 4 weeks, glucose tolerance was normalized, body weight was reduced, and insulin sensitivity was increased⁶. Diets high in saturated fatty acids may also put one at greater risk for developing the metabolic syndrome and its related conditions^{7,8,9,10}. Studies continue to show a beneficial cardiovascular effect with the use of mono- and polyunsaturated fatty acids, in place of saturated fatty acids^{11,12}. A study by Yang and colleagues fed five groups of Wistar rats high fat diets (HFD) with varying proportions of saturated/monounsaturated/polyunsaturated fatty acids (PUFA) and found that the HFD with most PUFAs prevented HFD-induced insulin resistance and significantly increased expression of adiponectin¹³. However, consuming a diet high in PUFA may not cure or prevent these conditions¹⁴. The type of fat and the condition in

which the fat was prepared may have an effect on physiologic and metabolic outcomes, even with a low-fat diet.

Frying foods in unsaturated oil, a typical way of food preparation in America, causes triglycerides in the oil to become oxidized, producing volatile and nonvolatile products of oxidation¹⁵. Primary products of lipid oxidation are lipid hydroperoxides (LOOH), which are highly labile and become reduced to hydroxyl fatty acids¹⁶. Prolonged heat exposure, oxygen exposure, and metal ion exposure further lead to the formation of secondary products of lipid oxidation, which include oxidized triglyceride monomers, dimers, and polymers and aldehydes, ketones, epoxides,^{16,17}. In fast food restaurants, the oil used for frying is typically heated up to 18 hours a day and is used for about 1 week before being changed to fresh oil¹⁸. Along with fried and fast foods, processed foods are known to contain oxidized lipids at the time of purchase¹⁹; primary and secondary lipid oxidation products can be absorbed and have a negative impact on one's health^{20,21}.

Although limited, there is research showing that the consumption of these thermally oxidized fats may be contributing to atherosclerosis^{22,23} and other harmful health outcomes²⁴, as a result of the increase in lipid peroxidation products in tissues²⁵. Outcomes related to these negative health conditions may vary depending on the species tested, the amount and the extent of oxidation of the oils, the type of oil used, and the length of exposure. Some studies have shown that dietary oxidized lipids may have a pro-atherogenic effect. Studies by Staprans and colleagues have shown in animal models that dietary oxidized lipids increase the formation of oxidized lipoproteins in circulation, which contributed to fatty streak formation in the aorta²⁶. An atherogenic effect, resulting

from increased aortic lesion development, was seen in LDL-receptor knock-out mice fed oxidized linoleic acid, but only in conjunction with a high fat, high cholesterol containing diet²⁷. Thermally heated oils have also been shown to have a carcinogenic effect in animals and cells. Human lung carcinoma A-549 cells treated with methanolic extracts from soybean, sunflower, or lard oil fumes lead to DNA damage and increased cytotoxicity; soybean oil fumes, compared to sunflower and lard, had the greatest cytotoxicity and genotoxicity related to its high lipid hydroperoxide formation²⁸. In a related study, Wistar rats fed repeatedly heated coconut oil (CO) had higher total polycyclic aromatic hydrocarbons (PAH), lipid peroxidation and reactive oxygen species (ROS), and increased formation of preneoplastic lesions in their liver, than rats fed once heated or unheated CO; this leads to the conclusion that repeatedly heated coconut oil may increase susceptibility toward cancer development²⁹. Consuming heated oils may also be associated with increased risk of hypertension. In a study by Leong and colleagues, male Sprague-Dawley rats fed diets containing repeatedly heated palm oil had significantly elevated blood pressure compared to rats fed fresh palm oil³⁰.

A previous study in our lab showed that C57BL/6J mice consuming soybean oil heated for 3 hours gained less body weight per gram of food consumed, although gaining more fat pad mass, compared to mice consuming unheated soybean oil³¹. On the other hand, a study by Chao *et al* showed that rats fed highly oxidized oil (heated for 6hrs/day for 4 days), as compared to fresh soybean oil and fish oil, had lower fat pad mass and weight gain but developed glucose intolerance³². This suggests that glucose homeostasis is negatively affected by PUFAs that have been extensively heated, although adipose mass decreases in the presence of extensively heated oils and increases in the presence of

mildly heated oils. Given these results, our lab studied the mechanism involved with triglyceride accumulation using 3T3-L1 cells. Our study showed that when treated with soybean oil heated ≥ 6 hours, 3T3-L1 adipocytes had a lack of triglyceride accumulation & reduction in genes, such as SREBP-1c and PPAR γ that promote adipocyte differentiation; this was not seen in cells treated with 3 hour heated oil, which were still able to differentiate and store triglycerides³³. With this preliminary evidence, we wanted to see if the accumulation of adipose mass and glucose tolerance is dependent on the extent to which soy oil is heated.

Objective: Our aim, in the current study, is to investigate if the extent of lipid oxidation determines fat pad mass and glucose homeostasis. This will be determined by comparing fat pad mass, fasting glucose, and plasma insulin in groups of C57BL/6J mice given a chow with either unheated soybean oil (UHO), soybean oil heated 3 hours (3hr-HO), 6 hours (6hr-HO), or 9 hours (9hr-HO) .

CHAPTER II: LITERATURE REVIEW

With obesity, diabetes, and atherosclerosis on the rise, there has been a focus on dietary lipids and their relation to insulin resistance and fat accumulation.

Polyunsaturated fatty acids (PUFA) are known as “good” fats and are recommended, by the World Health Organization (WHO), to be consumed in larger amounts than saturated fatty acids¹. There is convincing evidence that plasma LDL-cholesterol and the risk of coronary heart disease are lowered when saturated fat is replaced by PUFA in the diet³⁴.

Specifically, there is evidence that consuming n-6 PUFA lowers the risk of the components of metabolic syndrome and diabetes³⁴. However, it is not known if these beneficial effects of PUFA remain when they become oxidized during cooking and food processing. Polyunsaturated fatty acids come in two forms, n-6 and n-3. Linoleic acid, a commonly consumed n-6 fatty acid, is typically consumed in the form of vegetable oils, such as safflower, sunflower, sesame, corn and soybean oil. Oils are used in the frying process, a common way to prepare fast-food, which produces oxidized lipid products, such as hydroperoxy and hydroxy fatty acids, oxidized triglyceride monomers, dimers, and polymers and aldehydes, acids, keto and epoxy compounds. Research in our lab has shown that commonly consumed foods, such as walnuts, sunflower seeds, ground flax, fish oil capsules, and infant formula contain oxidized lipids at the time of purchase¹⁹.

Investigating the relationship between dietary oxidized lipids and glucose homeostasis is a new area of research. Specifically, our study is looking at the effects that dietary

oxidized lipids have on body weight gain, adipose tissue mass, and glucose tolerance in C57BL/6J mice.

a. Oxidized lipids and PPAR γ :

The peroxisome proliferator-activated receptors (PPARs) include nuclear hormone receptors that regulate several metabolic pathways by influencing gene expression³⁵. Of the PPAR isoforms, PPAR γ is of interest due to its role in adipocyte differentiation, triglyceride accumulation, glucose homeostasis, and inflammation³⁶. The role of PPAR- γ in adipocyte differentiation involves ligand binding; ligands are responsible for promoting or suppressing PPAR γ activity and its target genes³⁵. The primary biological ligand for PPAR- γ is still unknown; however, oxidized polyunsaturated fatty acids have demonstrated a greater ability to activate PPAR γ compared to un-oxidized fatty acids^{35,36}. Upon intestinal absorption, dietary oxidized lipids are incorporated into lipoproteins and tissues³⁵. Oxidized fatty acids found in oxidized low-density lipoprotein are also more potent activators of PPAR γ compared to their un-oxidized counterparts³⁵.

b. Oxidized lipids and body weight gain:

Dietary heated oils have been shown to have an effect on body weight in animals^{17,27,29,31,32,37,38,39}. While studies comparing heated oil intake and metabolic outcomes in humans are limited, evidence does show that higher amounts of fast food consumption are associated with increasing rates of severe obesity⁴⁰. Paradoxically, several animal studies have shown that the consumption of highly oxidized dietary

lipids leads to decreased body weight gain^{17,31,32,37}. In a study by Chao and colleagues, frying oil significantly reduced body weight gain in rats, compared to rats fed a high-fat diet with fresh soybean oil (HF). Interestingly, body weight gain in rats fed a low-fat diet with heated soybean oil (LO) did not differ significantly from rats fed a low-fat diet with fresh soybean oil (LF), even though the LO group had a significantly greater food intake compared to all other groups¹⁷. A more recent study by Chao also showed that rats fed a HF diet with oxidized oil (soybean oil heated at 205°C for 24 hrs) had a lower body weight gain in comparison to rats fed a HF diet with un-oxidized oil³². Similarly, in a study by Lopez *et al*, Wistar rats fed repeatedly heated sunflower oil (HO) had a decrease in body weight gain compared to controls with unused sunflower oil. Although the food intake of both groups was reported to be similar, the food efficiency ratio in the HO group was significantly decreased³⁷. We previously demonstrated that C57BL/6J mice fed oxidized soybean oil had a decrease in weight gain compared to mice fed fresh soybean oil, along with a decrease in food intake³¹. Other animal studies show no significant difference in body weight gain with animals fed oxidized lipids^{27,29,38}. In a study by Khan-merchant *et al*, body weight gain did not differ between mice fed a high-fat, high cholesterol diet with oxidized linoleic acid (13-HODE) for 11-13 weeks and mice fed un-oxidized oils²⁷. Similarly, Wistar rats fed repeatedly heated coconut oil (repeatedly heated >300°C, 3hrs), as well as rats fed fresh and single-heated coconut oil, had no change in body weight gain²⁹. A couple of animal studies showed an increase in weight gain when heated oil was consumed. Sprague-Dawley rats fed a diet consisting of 2% cholesterol with 5 times heated palm oil (heated at 180°C w/ fish chips, 10 mins, 5x; 50 mins total) resulted in the highest body weight gain compared to rats fed fresh

palm oil and once heated palm oil³⁹. A study by Leong *et al*, showed a step-wise increase in body weight with male Sprague-Dawley rats fed fresh palm oil, one time heated palm oil, five times heated palm oil, and ten times heated palm oil over 24 weeks, although differences in weight gain were not significant³⁰. A notable difference between studies showing no difference or an increase in weight gain and studies showing a decrease in weight gain is the duration for which the oil was heated. Studies using repeatedly or extensively heated oil (>9 hrs) showed a decrease in body weight gain, while a majority of the studies showing no difference or increase in weight gain used mildly heated oil (heated for 3hrs). Other differences in weight may be due to the type of oil used, the species tested, the length of time fed, the composition of the diet, and how much of the total fat came from oxidized lipids.

c. Oxidized lipids and fat pad mass:

Obesity is characterized by the increase in number and size of fat cells, which increases the risk of developing insulin resistance and cardiovascular disease⁴¹. Adipose tissue is an endocrine organ that secretes bioactive factors, or adipokines. Dysregulation of adipokines may be involved in mechanisms contributing to insulin resistance and metabolic disease⁴². Adipocyte differentiation is regulated by PPAR γ , and oxidized lipids have been shown to be potent PPAR γ ligands⁴³. Since a major function of adipose tissue is to store lipids and regulate metabolic homeostasis⁵, the interaction of dietary oxidized lipids and adipose tissue mass is an important area of study to determine how these interactions affect metabolic risks. Chao *et al* showed that Sprague Dawley rats fed a high fat diet with highly oxidized oil, had a decrease in body weight

gain and significantly lower weights of epididymal and retroperitoneal fat³². Similarly, Burenjargal showed that rodents fed oxidized lipids (PV, 3.6 mEq/kg) had a significant decrease in the weight of the retroperitoneal fat pad, as well as the amount of triglyceride accumulation in the fat pad, after 12 weeks⁴⁴. These results indicate that oxidized frying oil has a greater anti-adipogenic effect than un-oxidized oil on fat depots. On the other hand, our lab previously showed that mean fat pad weights were greatest in the mice fed low-fat diet with oxidized oil, heated only for 3 hrs³¹. As previously mentioned, the length of time used in heating the oils led to differences in the type and amount of oxidation products, which might explain the differences in fat mass accumulation. Given the studies mentioned, an increased heating time may be associated with decreased fat pad mass, while mildly heated oil (3 hrs) may have the opposite effect on fat pad mass.

d. Oxidize lipids and glucose tolerance:

Insulin resistance plays a major role in the development of type 2 diabetes and is inherently related to lipid metabolism. It is hypothesized that insulin resistance can result from the accumulation of triglycerides and acyl-coA in liver and muscle tissue⁴⁵. Studies show a strong correlation between intramuscular fat content and insulin resistance; one study showed that lean offspring of parents with type 2 diabetes had increased triglyceride accumulation in the muscle, which correlated with greater insulin resistance throughout the body^{46,47}. When lipoprotein lipase is over-expressed, triglycerides can accumulate in non-adipose tissue, causing insulin resistance⁴⁸. Glucose homeostasis and adipose tissue can be more thoroughly understood by looking at PPAR γ , a regulator of fat

cell formation. PPAR γ knock-out mice fail to develop adipose tissue, and people with dominant-negative mutations in a single allele *PPARG* have partial lipodystrophy and insulin resistance⁴⁵. TZDs, drugs used to treat diabetes, work by increasing insulin to promote glucose disposal in muscle and inhibit glucose output in the liver. TZDs are also agonists of PPAR γ , which promotes insulin sensitivity⁴⁵. The relationship between PPAR γ and insulin sensitivity can be explained by two possible mechanisms, one being that stimulating PPAR γ in adipose tissue improves its ability to store fat, reducing triglycerides in the liver, muscle, and circulation^{49,50}. PPAR γ agonists also stimulate adiponectin, which promotes endogenous fatty acid oxidation and insulin sensitivity^{51,52,53}. It is clear that PPAR γ in adipose tissue is a key regulator of adipose tissue homeostasis, as a study in our lab has shown that 3T3-L1 cells treated with highly oxidized soybean oil alter PPAR- γ expression in the early adipocyte differentiation process, resulting in a decrease of downstream adipogenic gene expression³³. These findings are supported by a study that shows a positive relationship between oxidized fat intake and insulin resistance in women; an inverse relationship between body fat percentage and oxidized fat intake was also reported⁵⁴. Along these lines, C57BL/6J mice and Sprague-Dawley rats fed oxidized frying oil developed glucose intolerance, even with a decrease in adipose mass³². Another study showed that C57BL/6J mice fed oxidized frying oil developed glucose intolerance due to impaired insulin secretion, observed in an oral glucose tolerance test (OGTT)⁵⁵. If there is less TG accumulation in adipose tissue due to alterations in PPAR γ expression, then the TGs are likely to accumulate in non-adipose tissue, thereby promoting insulin resistance.

The physiologic and metabolic effects that oxidized lipids have on the body is a new area of research that is important for determining the origin and possible prevention of the leading causes of death in the United States, including coronary artery disease, diabetes, and obesity. Specifically, our study is looking at the effects, given the extent of oxidation, that dietary oxidized PUFA have on body weight gain, adipose tissue mass, and glucose tolerance, using C57BL/6J mice. In contrast to previous studies that use unsaturated fatty acids as the only source of fat in the experimental diet, our study combines PUFA and saturated fatty acids (lard) to reflect a diet that contains a mixture of fatty acids. Methods and procedures included heating soybean oil, performing assays to analyze the primary and secondary oxidation products formed, incorporating the heated and unheated oils into mouse chow, performing the mouse study, and completing terminal procedures on all mice. Blood glucose values, adipose tissue samples, liver samples, and plasma samples were collected during the terminal procedures.

CHAPTER III: METHODS

a. Heating soybean oil

Soybean oil was heated on a hot plate, under a hood, at 190°C for a total of nine hours, and aliquots were removed at hours three, six, and nine separately and stored at -80°C. While the oil was heating, an air compressor supplied a continuous flow of oxygen throughout the oil. Before the oil was placed in the freezer, the aliquots were placed under nitrogen gas.

Unheated soybean oil was used in the control diets. Both the heated and unheated oil was incorporated into mouse chow by Research Diets, Inc. (New Brunswick, NJ). All diets were isocaloric. A total of four low-fat diets were produced: mouse food with (1) un-oxidized oil (UHO), (2) three hour heated oil (3hrs), (3) 6 hour heated oil (6hrs), and (4) nine hour heated oil (9hrs). The composition of the diets is shown in Table 1.

Table 1: Diet Composition

Ingredients	Unheated		Heated Oil	
	<i>gm</i>	<i>kcal (%)</i>	<i>gm</i>	<i>kcal(%)</i>
Protein	19.2	20	19.2	20
Carbohydrate	67.3	70	67.3	70
Fat	4.3	10	4.3	10
Ingredient	<i>gm/kg</i>	<i>kcal/kg</i>	<i>gm/kg</i>	<i>kcal/kg</i>
Casein	200	800	200	800
L-Cystine	3	12	3	12
Corn Starch	315	1260	315	1260
Maltodextrin 10	35	140	35	140
Sucrose	350	1400	350	1400
Cellulose, BW200	50	0	50	0
Heated Soybean Oil	0	0	15	135
Fresh Soybean Oil	15	135	0	0
Lard	30	270	30	270
Mineral Mix S10026	10	0	10	0
DiCalcium	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate, 1	16.5	0	16.5	0
Vitamin Mix	10	40	10	40
Choline Bitartrate	2	0	2	0

b. Quantifying oxidized lipids in mouse diets

i) Fat extraction: Lipids were extracted from the each of the four mouse diets using a solvent system of Hexane Isopropanol (HIP) in a ratio of 3:2⁵⁶. Samples were homogenized in the HIP solution and filtered through a Buchner funnel. The filtrate was mixed with sodium sulfate and dried under nitrogen gas to evaporate the HIP. Extracted fat was weighed and used to determine the amounts of the products of oxidation in the oil through measurement of the conjugated dienes (CD, primary product of oxidation),

conjugated trienes (CT, secondary product of oxidation) peroxide value (PV; primary product of oxidation) and aldehydes (secondary product of oxidation).

ii) Measuring Peroxide Value: Peroxide values were determined at an offsite laboratory, due to inconsistencies using the PeroxySafe kit.

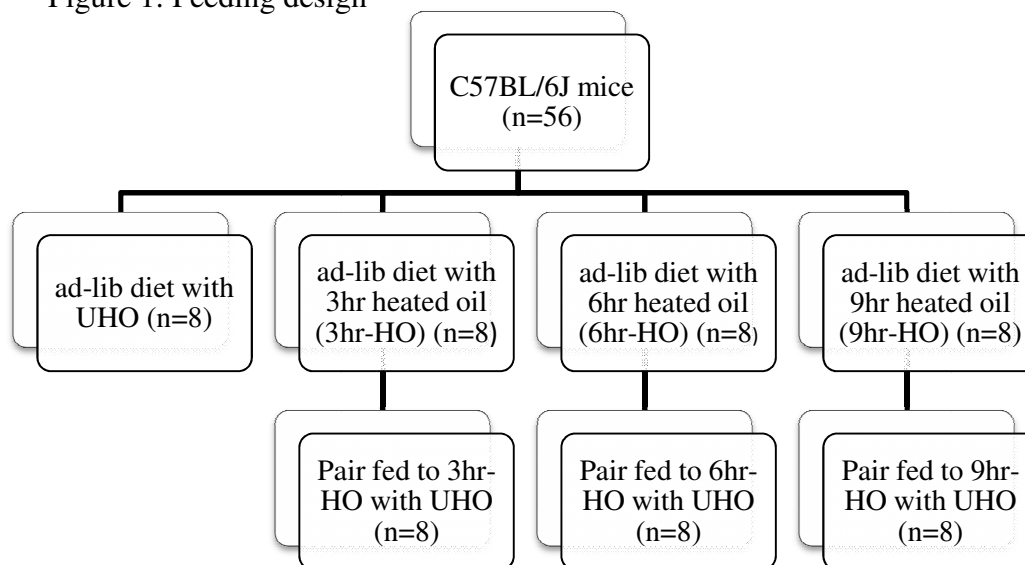
iii) Measuring conjugated dienes (CD) and trienes (CT): To prepare the samples, the sample oil was solubilized in hexane to create a 1% solution; this solution was diluted as necessary by a process of trial and error to achieve spectrophotometric readings in the target absorbance range of 0.2-0.8 at 234 nm for CD and 268 nm and 270 nm for CT. Hexane was used as the blank. Because hydroxy and peroxy fatty acids—primary products of oxidation—have CD structures that demonstrate maximum UV absorbance at 234 nm, the oil samples were read at that wavelength in an Epoch spectrophotometer (BioTek, Winooski, VT, USA). CD and CT levels are expressed as absorbance of a 1% solution at the respective wavelength; however, the calculation necessary for expressing them this way requires knowing the extinction coefficient of the sample, which itself requires knowledge of the specific fatty acid composition of each sample. Because this information was not available for the soybean oil, CD and CT levels were expressed in units of raw absorbance.

iv) Measuring aldehydes: Aldehyde levels were read at an offsite lab, due to limited availability of the SafTest AldeSafe kit.

c. Mouse Study

Fifty-six male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and were approximately 4-6 weeks old at the start of the study. The mice were randomly separated into groups as shown in Figure 1.

Figure 1: Feeding design



All of the mice were housed in the Georgia State University Animal Research Facility located in the Petite Science Center. The protocol for the animal study was approved by the IACUC committee at GSU and the animals were treated according to the guidelines in the protocol. The ad libitum fed mice were acclimated for one week and given regular chow by the Georgia State Department of Animal Research (DAR), before the start of the study. The pair fed mice were acclimated for two weeks, in order to calculate the amount of food needed based on food consumed by the mice in the ad libitum groups. On the first day of feeding for the ad libitum fed mice and pair fed mice

(although one week apart), normal chow was removed from all cages and replaced with the special diets described previously.

Four groups of eight mice each were fed ad libitum low-fat modified diets. The groups consumed an UHO diet, 3hr-HO diet, 6hr-HO diet, or 9hr-HO diet, respectively. The 24 remaining mice were pair fed with the UHO diet. The food amount, in grams, given to the pair fed mice was calculated to be parallel to the food amount, in grams, consumed by the ad libitum groups consuming the oxidized oil. Each week, we calculated the average amount of food consumed by each of the oxidized oil groups and approximately (± 1 gram) the same amount of food was given to the pair fed mice.

Mice were given fresh chow every 3-4 days and any remaining chow was weighed and discarded. Animals were weighed once a week, and a final body weight was taken, after 16 weeks, the morning of euthanasia.

On the day of euthanasia, each mouse was placed under isoflurane anesthesia and blood was collected by cardiac puncture. Blood samples were collected into a pre-prepared centrifuge tube and left at room temperature for 1 hour and then centrifuged for ten minutes to separate the serum. Serum was then removed from the centrifuge tube and stored in a clean tube at -80°C for future analysis of insulin levels. Epididymal and retroperitoneal white adipose tissue (EWAT, RWAT) and intrascapular brown adipose tissue (IBAT), along with liver, were harvested, washed in ice cold phosphate buffered saline (PBS), slightly blotted on paper, weighed and stored -80°C for each mouse.

d. Measuring Blood Glucose

After a 6 hour fast on the day of euthanasia (week 16), a drop of blood was drawn from the saphenous vein and the blood glucose levels were measured with a Contour glucose meter (Tarrytown, New York, USA).

e. Measuring serum insulin

An ELISA kit, purchased from B-Bridge International (Billerica, MA, USA), was used to measure serum insulin levels in all groups of mice, except the ad libitum UHO group. As explained later in the results, this group was not used for comparison purposes. Serum collected at 16 weeks was used according to manufacturer's instructions and absorbances were read on the Epoch spectrophotometer at 450 nm and 590 nm. Each absorbance was inserted into the linear equation generated from the calibration curve and expressed in pmol/L.

f. Statistical Analysis

Data were analyzed with SPSS version 18.0 (IBM Corporation). Data are shown as means \pm SD. Differences in the mean values, as a percent of pair-fed groups, between the 3hr-HO, 6hr-HO, and 9hr-HO groups were compared by one-way analysis of variance (ANOVA) for normally distributed data or by Kruskal- Wallis test for non-parametric data. Bonferroni was used as the post-hoc test. Statistically different means for non-normally distributed data were further analyzed by pairwise comparisons. A $p \leq 0.05$ was considered statistically significant.

CHAPTER IV: RESULTS

a. Products of oxidation

Figure 2 illustrates the amounts of primary (peroxides and conjugated dienes) and secondary (malondialdehyde and conjugated trienes) products of oxidation in the mouse chow at the beginning of the 16 week study. Conjugated dienes increased directly with heating time ($p=0.05$ for 3hrs vs 9hrs), whereas the peroxide value, conjugated trienes and the aldehyde value increased up to 6hrs and then decreased with the 9hrs time point. Levels of conjugated trienes 268 were significantly different between all HO time points ($p=0.05$). Significance of differences in aldehyde and peroxide values were not calculated, as the results were determined at an offsite laboratory.

Figure 2: Products of oxidation of soybean oil

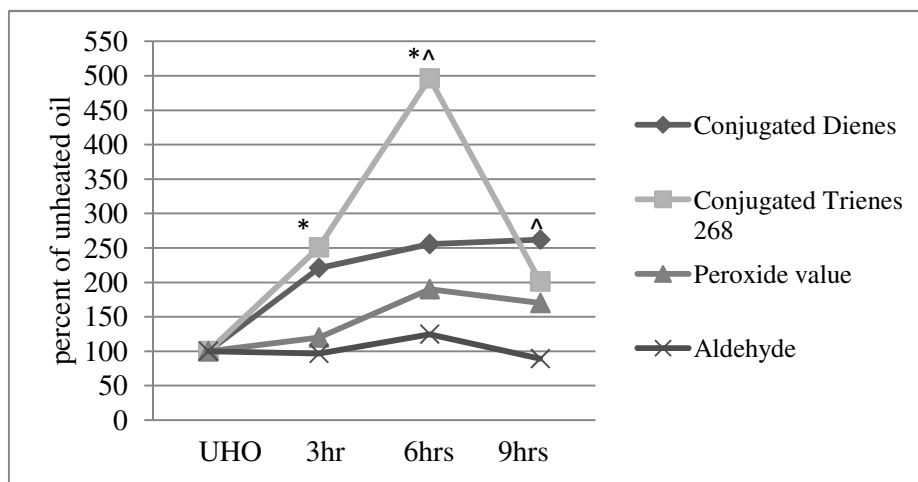


Figure 2: Values are expressed as mean%±SD (percent of UHO). Significant differences in mean levels of products of oxidation between the UHO and oil heated for 3, 6, or 9 hrs were determined by Kruskal Wallis test followed by Mann Whitney U. A p-value of ≤0.05 is considered significant.

*Significantly higher than 9hrs. ^Significantly higher than 3hrs.

We noticed that the pair-feeding worked well as the pair-fed groups consumed a similar amount of food on average as their heated oil (HO) group and gained a similar amount of weight. For this reason, we decided to compare weight gain, fat pad mass, liver weights, fasting glucose, and insulin levels between the 3, 6, and 9hr HO groups as a percent of each of their pair fed counterparts. Table 2 shows the results from the ad libitum group fed un-oxidized oil (ad-UHO), n=8. This group was not used in comparisons to 3, 6, or 9hr HO groups, as we did not find these comparisons to be as valid as comparing the ad libitum HO groups to their respective pair fed groups. Serum insulin levels were not measured for the ad-UHO group.

Table 2: Data for mice consuming ad-lib UHO chow

Weight gain (g)	EWAT (g)	RWAT (g)	IBAT (g)	Liver (g)	Fasting BG (mg/dL)	Feeding Efficiency
9.57 ± 2.59	0.10	0.41	0.13	1.03	177.63	0.031

Table 2: Weight gain, fat pad and liver weights, serum glucose and feeding efficiency (mean±SD) in ad-libitum fed UHO mice. Feeding Efficiency = body weight gain (g)/ food intake (g).

b. Weight gain

Figure 3 illustrates the weight gained over 16 weeks for the 3, 6, and 9 hr HO groups, as a percent of their respective pair fed groups. Weight gained for all three HO

groups was close to 100% of the weight gained for each respective pair fed group. Weight gain, as a percent of pair fed, was $98.32 \% \pm 19.48\text{g}$ (3hr-HO), $104.04 \% \pm 15.01\text{g}$ (6hr-HO), and $103.88 \% \pm 17.04\text{g}$ (9hr-HO) with no significant difference between the groups ($p=0.925$).

Figure 3: Mean weight gain of mice consuming the 3,6 or 9hr-HO chow.

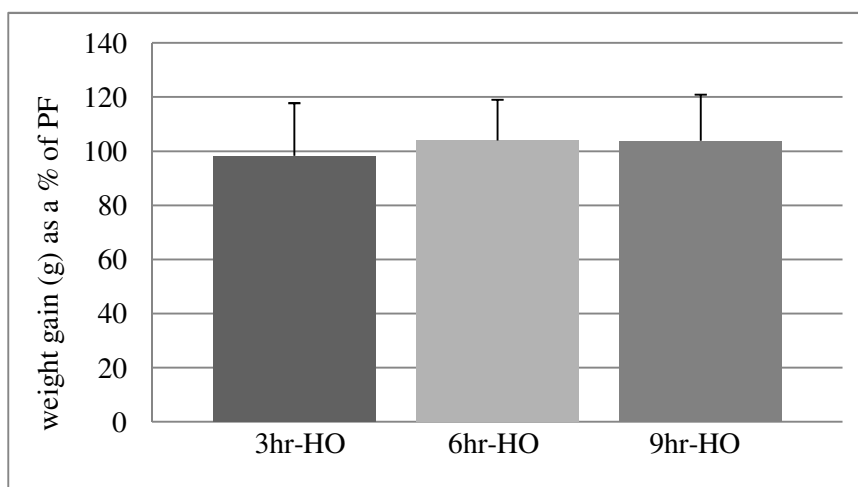


Figure 3: Values are expressed as mean% \pm SD (percent of PF). Significant differences in mean weight gain (g) between the 3hr-HO, 6hr-HO and 9hr-HO were determined by Kruskal Wallis test. A p -value of ≤ 0.05 is considered significant; $p > 0.05$ between all groups.

c. Feeding efficiency

Feeding efficiency was calculated as the ratio of total grams of weight gained per cage (4 mice/cage) by total grams of food consumed per cage. Values are given as mean percent of PF as seen in Figure 4. For all groups, since the $n=2$, standard deviation and significance were not calculated. On average, the 6hr-HO and 9hr-HO groups seem to gain more weight per gram of food consumed compared to the 3hr-HO group, suggesting

greater food efficiency. However, for the 3, 6, and 9hr HO groups, the feeding efficiencies were similar at 0.029, 0.030, and 0.028 respectively.

Figure 4: Feeding efficiency of mice consuming the 3,6 or 9hr-HO chow

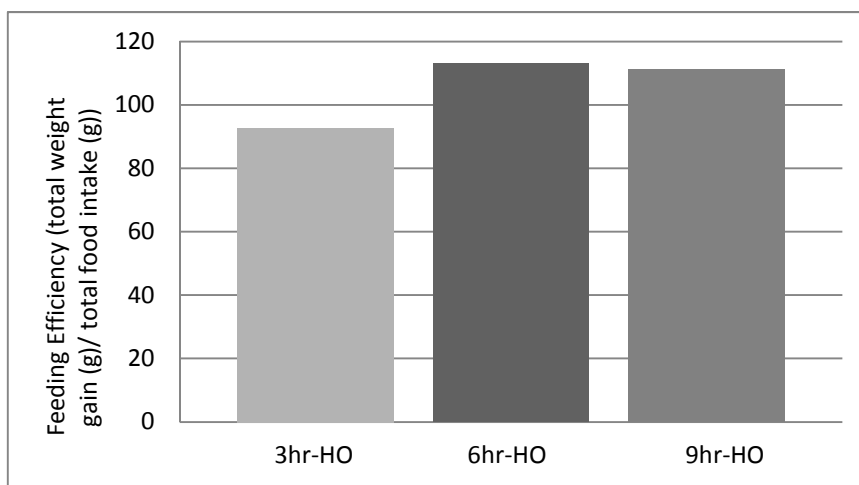


Figure 4: Values are expressed as mean%±SD (percent of PF). Significant differences and SD were not calculated (n=2).

d. Fat pad weights and liver weight

Figure 5 illustrates the weights of the epididymal white adipose tissue (EWAT), retroperitoneal white adipose tissue (RWAT), intrascapular brown adipose tissue (IBAT) and liver of the 3 hr-HO, 6 hr-HO, and 9 hr-HO groups as a percent of the organ weights of the respective pair fed groups after 16 weeks. Although the fat pad weights between the groups consuming the heated oils was not significantly different, EWAT and RWAT weights decreased by 10-12% as the heating time of the oil increased. In contrast, liver weights increased directly with heating time of the oil, although this increase was not statistically significant.

Figure 5: Mean organ weights of mice consuming the 3,6 or 9hr-HO chow.

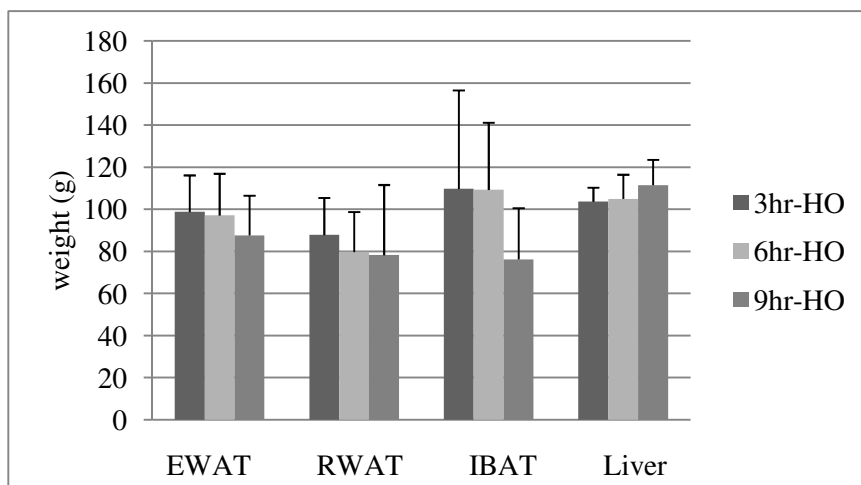


Figure 5: Values are expressed as mean%±SD (percent of PF). Significant differences in mean organ weights(g) between the 3hr-HO, 6hr-HO and 9hr-HO were determined by ANOVA (EWAT and RWAT) and Kruskal-Wallis (IBAT and Liver). A p -value of <0.05 is considered significant; $p>0.05$ between all groups for each organ.

e. Blood glucose and serum insulin

As seen in Figure 6, fasting blood glucose levels of the 3, 6, or 9 hr HO groups, as a percent of pair fed groups, increased significantly from 3hr-HO (89.01%) to 6hr-HO (122.35%), $p= 0.021$. Fasting glucose also increased from 3hr-HO to 9hr-HO, although this difference was not significant. Insulin levels, seen in Figure 7, were not calculated as a percent of pair-fed. Therefore, serum insulin levels are reported as mean values for 3, 6, and 9hr HO groups. There are no significant differences in insulin levels between groups.

Figure 6: Mean fasting glucose of mice consuming the 3,6 or 9hr-HO chow.

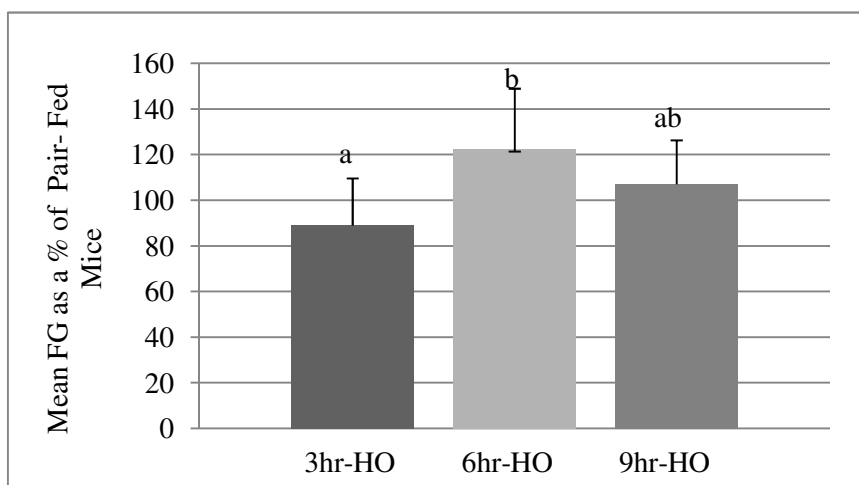


Figure 6: Values are expressed as mean%±SD (percent of PF). Significant differences in fasting blood glucose (mg/dL) between the 3hr-HO, 6hr-HO and 9hr-HO were determined by ANOVA followed by Bonferroni. A p -value of ≤ 0.05 is considered significant. Groups with different letters above error bars are significantly different than one another.

Figure 7: Mean serum insulin of mice consuming the 3,6 or 9hr-HO chow.

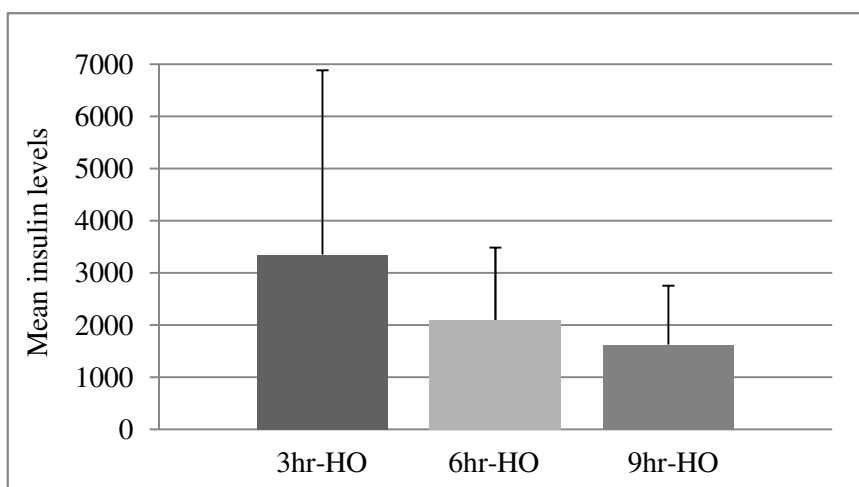


Figure 7: Values are expressed as mean%±SD. Significant differences in serum insulin (pmol/L) between the 3hr-HO, 6hr-HO and 9hr-HO were determined by Kruskal-Wallis. A p -value of ≤ 0.05 is considered significant; $p \geq 0.05$ between all groups.

CHAPTER V: DISCUSSION and CONCLUSION

Our study investigated the effects of the extent of lipid oxidation on adiposity and glucose homeostasis in C57BL/6J mice by feeding diets containing increasing amounts of oxidized lipids. Our results suggest that consuming increasing amounts of oxidized oil does not change weight gain but increases fasting glucose levels in the mice consuming 6hr-HO diet. The small 10-12% decrease in fat pad mass and serum insulin levels and the small increase in liver weight seen as the heating time increased were not statistically significant. Based on previous work by us and others, we had predicted that the increase in serum glucose levels could be due to the ectopic deposition of the triglycerides in the liver and muscle, suggesting insulin resistance. Triglyceride accumulation in the liver and muscle will be determined at a later time. However, the reduction in fasting insulin between the 3, 6, and 9hr HO consuming animals suggests more of a problem with insulin secretion rather than insulin resistance.

We were successful in creating a mouse chow that had increasing amounts of CD as the heating time increased. With the exception of CD, all other products of oxidation seem to decrease after the 6hrs time point. We used four different methods to determine products of oxidation in the mouse chow, because of the inherent issues with measuring products of oxidation. Peroxide values are the least reliable measurement as they are very labile and reduce to hydroxides; and thus, a peroxide value does not necessarily increase with increasing heating time. This is especially true with prolonged heating times and high temperatures. For example, Chao and colleagues prepared oxidized chow by frying

wheat dough sheets in soybean oil that was heated at 205°C for 6 hrs a day for 4 days¹⁷; a much longer heating time compared to our study at 9hrs total. The peroxide value (PV) of the oxidized frying oil (OFO) in Chao's study was 6.34 mEq/kg compared to 2.85 mEq/kg in the fresh soybean oil (a 2.2 fold increase), and the CD value in the OFO was 4525 (OD) compared to 407 (OD) in the fresh oil (a 11.12 fold increase)¹⁷. In addition, peroxide values reported in different studies do not necessarily correlate well with the hours of heating. For example, the PV value in Schneider's study where soybean oil was heated at 190°C for 3 hours was 5.81³¹, while the PV in Chao's aforementioned study was 6.34 for a total heating time of 24 hours¹⁷. For this reason it is better to compare the relative fold change in the PV value of the oxidized oil as compared to the unheated oil when evaluating various studies. In the studies that were reviewed^{17,25,31,32,44,57} the fold increase in PV value ranged from 2.2¹⁷ to 204²⁵ where the PV value in this study only increased as much as 1.9 fold. In a similar fashion comparing the increases in the CD values the present study had only up to a 2.62 fold increase as compared to a fold change of 2.08³¹ to 11.0¹⁷. Thus we can conclude that in this study the products of oxidation in the mouse chow were at a lower level and only increased by 1.9 (PV) to 2.55 (CD) fold between the unheated and 6hr-HO. This might partially explain why we failed to see significant differences in fat pad mass and insulin levels in mice consuming the heated versus unheated oil containing chow.

A notable difference in our study, compared to other studies^{17,30,32,37,55} is that our mouse chow consisted of both polyunsaturated and saturated fatty acids for all groups, which is more representative of a normal, mixed, diet. The diets in our study had a fat content of 4.3g/100g, with only one-third of the total fat coming from soybean oil (the

other 2/3 coming from lard). The previous studies cited had a fat content ranging from 5g/100g to 20g/100g, and 100% of the fat in the diet came from oxidized or un-oxidized PUFA^{17,30,32,37,55}. We had chosen to incorporate oxidized oil only as a third of the total fat because we hypothesized that a typical person would not consume all of the fat in the diet in the oxidized form. The fact that we and others^{58,59} have previously demonstrated that oxidized lipids are absorbed to a lesser extent than un-oxidized lipids suggests that the actual concentration of the oxidized oil at the cellular level might have been too low. Still, the fact that the present study was able to show decreasing trends with fat pad mass and insulin and increases in fasting glucose shows that increased heating time of soybean oil, specifically 6hr-HO, impacts glucose homeostasis in mice. Although results for fat pad mass were not statistically significant, a decrease of 10-12% fat mass can be biologically significant⁶⁰. Although it is difficult to pinpoint which products of oxidation are associated with outcomes related to fat pad mass, liver weight, and glucose and insulin levels, it appears that the peak accumulation of products of oxidation at the 6hrs time point also correlate with the highest fasting blood glucose levels.

As mentioned earlier, changes in weight gain and fat mass in animals fed oxidized lipids seem to vary a great deal. Again, these differences could be due to the type of oil used, the extent of oxidation, the amounts of primary and secondary products of oxidation formed, the animal model used, and/or the diet composition as a whole. Although differing in animal model, oil type, and oxidation conditions, our study results are in accordance with previous studies by Srivastava S *et al*, Gabriel HG *et al*, and Chao *et al*, which show no difference in weight gain between rodents fed oxidized vs. un-oxidized oil^{17,29,38}. Opposingly, a study in our lab previously reported an increase in fat

pad mass ($p < 0.05$) in C57BL/6J mice fed soybean oil heated for 3 hours compared to unheated soybean oil³¹. A notable difference in our current study compared to Schneider's study is the difference in peroxide value for the mice fed 3hr heated oil (5.81 mEq/kg³¹ vs 2.32 mEq/kg in the current study). However, our results of decreased adiposity in the presence of dietary oxidized oil are supported by others^{32,45,54,55}.

Current research shows that the type 2 diabetic profile of hyperglycemia and insulin resistance is mostly associated with overweight and obesity^{61,62,63}. However, recent studies, investigating effects of dietary oxidized lipids, including our own, show that glucose intolerance can develop even in the presence of decreased adiposity^{32,55,64}. In our study, although fasting glucose for the 6hr-HO and 9hr-HO groups was higher than that of 3hr-HO, serum insulin levels decreased with heating time. As previously mentioned, elevated fasting serum insulin levels are typically seen with elevated fasting glucose levels, displaying an insulin resistant profile seen in type 2 diabetes⁶³. While unexpected, our results agree with studies by Chiang *et al*, Liao *et al*, and Farrah *et al*^{55,65,57}. Liao and colleagues found that feeding oxidized frying oil to C57BL/6J mice led to glucose intolerance by an OGTT while displaying hypoinsulinemia. Liao determined that insulin secretion was suppressed in the pancreatic beta-cells during the early phase of an OGTT⁵⁵. Chiang *et al* also showed that C57BL/6J mice fed a high-fat diet with oxidized frying oil (OFO) had significantly increased fasting glucose and significantly decreased serum insulin as compared to mice fed high-fat and low-fat diets with fresh soybean oil⁶⁵. Chiang investigated the mechanism associated with this phenomenon and discovered that mice fed OFO had oxidative damage and impaired insulin secretion from pancreatic islets, a profile reflective of type 1 diabetes.

There are some differences when comparing our results for products of oxidation to a previous study done in our lab¹⁹. Our previous study determined in soybean oil that was unheated, or heated for 3, 6, or 9 hours, the levels of CD, CT, and aldehydes increased with each time point. In the current study only CD levels increased at each time point. Our previous study, in accordance to our current study, did find that PV peaked at 6hrs. However, the current study also found that levels of CT and aldehyde were also highest at 6hrs. These differences between studies could be related to the fact that the previous study analyzed pure soy oil (no inclusion into food), whereas our current study analyzed oil extracted from mouse chow. This proves a need to determine if there are differences in levels of products of oxidation in plain oil vs. oil extracted from food. There is also a need to develop a method which correlates oxidation values with heating time. At the present time, we cannot say that a soybean oil with a PV of 5.6 has been heated for a longer amount of time compared to a soybean oil with a PV of 2.4. Conjugated diene values are given as optical density, which is a reading from the spectrophotometer. These values can vary depending on the handling of the spectrophotometer and the spectrophotometer itself. Determining a method to correlate oxidation values of oils with heating time will allow accurate comparisons of results regarding adiposity and glucose homeostasis among various studies.

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