DIACLYGLYCEROL: MECHANISM AND EFFICACY AS A FUNCTIONAL OIL

BY

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TABLE OF CONTENTS

ABSTRACT I
ACKNOWLEDGEMENTS II
DEDICATION III
ABBREVIATIONS IV

PART 1. Literature Review
1. Introduction 1
2. Safety of DAG oil consumption 2
3. Metabolism of DAG oil 4
4. DAG oil consumption on energy expenditure and fat oxidation 6
5. Action of DAG oil consumption on lipid profiles 8
a) The impacts of DAG oil consumption on lipid profiles by animals with insulin resistance/glucose intolerance 8
b) The impacts of DAG oil consumption on lipid profiles by healthy animals 9
c) The impacts of DAG oil consumption on lipid profiles by human subjects with lipoprotein lipase deletion, insulin resistance and glucose intolerance 9
d) The impact of DAG oil consumption on lipid profiles by healthy human 12
e) Antithrombotic effect of DAG oil consumption in animals 12
6. DAG oil consumption and body composition 13
a) DAG oil consumption and body composition in animals 13
b) DAG oil consumption and body composition in humans 14
7. Conclusion 15

PART 2. Study Rationale 16

PART 3. Hypotheses 17

PART 4. Sample Size Calculation 18

PART 5. Paper 1: Diacylglycerol oil fails to increase total energy expenditure or fat oxidation but reduces body fat in overweight and mild hypertriglyceridemic women 19

PART6. Bridge 41

PART 7. Paper 2: Diacylglycerol oil fails to alter the blood lipid profiles or hepatic lipogenesis in overweight and mild hypertriglyceridemic women 42

PART 8. Summary and Conclusions 65

PART 9. Directions for Future Research 67

PART 10. References 68

PART 11. Appendix 77
1. Calibration Methods 77
2. Graphs 81

List of Tables

1. Acute and long-term energy expenditure and fat oxidation per kilogram fat free mass of overweight women after consumption of DAG and control oil diets 32
2. Change in adipose tissue of overweight women after 4-week of consumption of DAG and control oil diets 36
3. Change in lean tissue of overweight women after 4-week consumption of DAG or control oil diets 37
4. Changes in blood lipids in overweight and mild hypertriglyceridemic women after 4 weeks of consumption of DAG or Control oil 57
5. Percentage changes of lipid profile parameters after 4-week test oil consumption 58

List of Figures

1. The enrichment of body water in two treatment phases 56
2. Total energy expenditure introduced by two test oils 83
3. Beginning and endpoint oxidation introduced by two test oils 84
4. Endpoint body weight in two study phases 85
5. Endpoint body weight in two study phases 86
6. Baseline body fat of two study phases 87
7. Endpoint body fat of two study phases 88
8. Baseline lean mass of two study phases 89
9. Endpoint lean mass of two study phases 90
10. Endpoint lipid profiles of two study phases 91
11. Fractional synthesis rate of triacylglycerol over 24 hours 92
12. Correlation between hepatic lipogenesis and fast serum TG 93

Abstract

BACKGROUND: Diaclyglycerol (DAG) oil has the potential as an effective weight control agent as well as an agent to modify overweight related complications. OBJECTIVE: We aim to examine the efficacy of DAG oil (Enova oil™) on regulating energy expenditure (EE), fat oxidation, body composition, lipid profiles and hepatic lipogenesis in comparison with conventional oils. DESIGN: Twenty-six overweight hypertriglyceridemic women consumed DAG or control oil for 28 days separated by a 4-week washout period using a randomized crossover design. Forty grams of either DAG or control oil were consumed daily by each study subject. RESULTS: DAG oil consumption for a period of 4-week did not alter total EE, fat oxidation, lean mass, fasting lipid profile or fatty acids synthesis rate, but effectively reduced ($p<0.05$) body weight and adiposity. CONCLUSION: DAG oil maybe useful as an agent in the battle against obesity. However, its body weight/composition control effects may not come from increasing of lean mass, or postprandial EE and fat oxidation. The consumption of DAG oil for a period of 4-week did not necessarily modify fasting lipid profiles or hepatic lipogenesis to reduce risk of coronary heart diseases in overweight hypertriglyceridemic women.

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Dedication

This master's thesis is dedicated to my mother Suping Xiao and my father Jiarui Yuan who are the biggest support of my life, and great role models in life's lessons all the time. You always love me and have showed me how to love, to others, to my work and to my life.

Abbreviations

CHD Coronary heart disease

CVD Cardiovascular diseases

T2DM Type 2 diabetes mellitus

DAG Diacylglycerol

TAG/TG Triacylglycerol

EE   Energy expenditure

TC   Total cholesterol

HDL   High density lipoprotein

LDL   Low density lipoprotein

RLP Remnant lipoprotein

WTD Weston type diet

FFA Free fatty acid

MAG Monoacylglycerol

PKC Protein kinase C

IP3 Inostol triphosphate

MTP Microsomal triacylglycerol transfer protein

GRAS Generally recognized as safe

FDA Food and drug administration

FOSHU   Foods for specific health use

FSR Fractional synthesis rate

TG-FA Triacylglycerol fatty acid

NEFA   Nonesterified fatty acids

TEF   Thermic effect of food

Part 1. Literature review

Introduction

Obesity has been identified as one of the most important modifiable risk factors associated with coronary heart disease (CHD) and type 2 diabetes mellitus (T2DM). The lifestyle modification, pharmacological, and surgical approaches are strategies to manage body weight. Although short term weight reduction can be achieved by restricting energy intake (food consumption) and increasing energy expenditure (lifestyle modification), long term modification of body weight is usually unsuccessful. Safety concerns regarding pharmacological and surgical approach for modification of body weight and body compositions have recently emerged. Typical North American diets contain 38% of energy from fat [Akoh 1995]. Body weight and adiposity are positively correlated to dietary lipid levels [Astrup et al., 2008]. Thus, in view of these safety and efficacy issues, the need for non-pharmacological and nonsurgical body weight/composition therapies, which possess no risk of adverse events, has become apparent.

One such natural oil based compound that has been suggested for its function for body weight/composition modification is diacylglycerol (DAG) oil. DAG oil appears in numerous cooking oils range from 0.8% in rapeseed oil to 9.5% in cottonseed oil [Flickinger et al., 2003]. DAG oil is also a digestive intermediate for the lipid digestion processes that pancreatic lipase removes fatty acids on 1(3) position to form 1(3), 2-DAG. Among the two types of DAG namely 1, 2-DAG and 1, 3-DAG, the

latter may exert more beneficial impacts on human health, since fatty acids attached to sn-2 position is reserved to be packed into chylomicrons, and therefore to increase the viscosity of blood or to be stored as fat [Hunter, 2006]. In previous studies, DAG oil exerts capacities including modifying lipid profiles [Yamamoto et al., 2005; Tada et al., 2005; Takase et al., 2005; Ijiri et al., 2006; Kimura et al., 2006; Kristensen et al., 2006; Yamamoto et al., 2006; Ai et al., 2007, Yanai et al., 2008], body weight [Osaki et al., 2008] and composition [Nagao et al., 2000; Maki et al., 2002; Yanagisawa et al., 2003; Teramoto et al., 2004] via increasing total energy expenditure (EE) [Saito et al., 2006], fat oxidation [Kamphuis et al., 2003; Kimura et al., 2006; Saito et al., 2006; Osaki et al., 2008]. DAG oil is made by natural vegetable oils in the presence of an immobilized lipase according to the method reported by Watanabe et al [Watanabe et al, 2003] and is designated as "Generally Recognized as Safe" (GRAS) by worldwide scientists. FDA (food and drug administration) and FOSHU (foods for specific health use) have reviewed and accepted their conclusions. Therefore, DAG oil is available in Japan as a healthy cooking since 1999, and has become the #1 cooking oil. In 2005, DAG oil launched nationwide in United States.

However, the beneficial impacts of DAG oil on human health remain controversial. The objective of this review is to discuss the impacts of DAG oil on human health including safety, total EE, fat oxidation, modification effects on lipid profile and body composition. The potential mechanism will also be discussed.

Safety of DAG oil consumption

In order to assess the safety of DAG oil consumption, studies have done to assess

the relationships between DAG oil consumption and protein kinase C (PKC) activation, cancer development, genotoxic effects and interactions with other nutrients.

Increased PKC activity is closely associated with carcinogenic actions [Berg et al, 2006]. In the cytosol, DAG oil works with inositol triphosphate (IP3) to increase the level of PKC [Berg et al., 2006]. Wistar rats fed with 5 to 23% of either DAG or TAG oil with similar side chains exerted no differences in cytosolic or membrane PKC activities in lingual, esophageal, gastric, small intestinal, cecal, proximal and distal colonic mucosal tissue [Meguro et al, 2007].

However, one recent study investigated the effect of DAG oil on the activation of PKC and carcinogenic effects on transgenic rats [Tsuda et al, 2007] showed that after 12 and 20 weeks of dietary intervention, DAG oil consumption increased the incidence of squamous cell carcinomas of tongue, in comparison with TAG oil with similar side chain fatty acids including 46.6% linoleic acids and 38.9% oleic acids.

Other studies including four weeks and 24 months multi-organ carcinogenesis bioassay in rats fed with up to 5.5% dietary DAG or TAG oil incorporated showed similar clinical condition and pathological parameters between the two treatments [Chengelis et al., 2006; Ichihara et al,. 2008]. A 12 months beagle dog [Chengelis et al., 2006] model study with up to 9.5% of testing oil incorporated suggests similar clinical conditions including, body weight gains, and food consumption. Beside, hematology and urinalysis parameters were not affected by the DAG oil. In the same study, no serum chemistry changes indicated any toxicity of DAG oil consumption.

Genotoxicity of DAG oil was conducted [Kasamatsu et al., 2005] by using bacterial reverse mutation assay, chromosomal aberration assay in cultured Chinese hamster lung cells (CHL/IU), and a bone marrow micronucleus assay in ICR CD mice. At the end of the test, no adverse events were observed. Another study heated DAG oil to 180 ^{o}C for 8 hours per day for three consecutive days. The study results did not show any genotoxic effect of heated or unheated DAG oil. DAG oil also exerts no effect on the fat soluble vitamins [Watanabe et al, 2001].

As most studies in animals suggest DAG oil is safe for short-term and long-term consumption, researchers have moved to the clinical level to further assess the safety of DAG oil consumption by humans. A human trail done by Yasunaga et al [2004] administered a dosage of 0.5g/kg/d of either DAG or TAG oil to 42 men and 39 women for 12 weeks. The subjects tolerated the test oils well. No adverse effects were reported, and no significant differences in the occurrence of clinical signs and physical complaints related to the testing oil were observed.

In summary, DAG oil consumption did not cause adverse effects in most studies. It should be noted that both investigated beneficial and harmful effects might be from the constituents of the diets, side chain fatty acids, or the combination of different factors other than the structure of DAG oil alone.

Metabolism of DAG oil

The energetic value and digestibility of DAG and TAG oils with similar side chain fatty acids measured by bomb calorimetry are not different [Taguchi et al, 2001].

The energetic value of DAG and TAG are 38.9 kJ/g, 39.6kJ/g, respectively, without difference in digestibility (96.3±0.4% and 96.3±0.3%, respectively). Watanabe et al [1997] reported that DAG oil consumption decreased body fat accumulation after 3 or 4 weeks. Therefore, DAG oil would be metabolized differently if it introduces weight control effect on human health.

Although the mechanism of action of DAG oil remains to be determined, two hypotheses are generated from previous results. The first is that more nonesterified fatty acids (NEFA) are transported by the portal vein directly to the liver where they undergo fat oxidation with consumption of DAG oil [Maki et al., 2002; Kamphuis et al., 2003]. The second one is that DAG oil delays the lymphatic transport of TAG, therefore the entry of TAG-rich chylomicrons into blood circulation is delayed. In the late postprandial phase, decreased insulin levels cause a decrease in the activity of lipoprotein lipases [Williams et al., 1996]. The delay in TAG entry into the systemic circulation increases the utilization of lipids for energy and therefore, less lipids are stored in the body in adipose tissue [Yanagita et al., 2004].

It has been suggested by researchers [Kondo et al., 2003; Osaki et al., 2005] that TAG is digested to 1, 2-DAG, 2-monoacylglycerol (MAG) , and FFA, whereas, 1, 3-DAG is digested to 1(3)-MAG and FFA [Kondo et al, 2003; Osaki et al., 2005]. In addition, the metabolism of DAG oil also seems to be affected by the side chain fatty acids. When linoleic acid is incorporated with 1, 3-DAG, the mucosal synthesis of TAG is significantly decreased in rats in comparison with TAG [Kondo et al., 2003]. Results from this study indicate that 1, 3-DAG production required the presence of

1(3)-MAG to be acylated and 1, 3-DAG and is only minimally utilized in TAG synthesis [Kondo et al., 2003].

Taguchi et al [2002] suggest that DAG oil consumption introduces less increasing in serum TG compared with TAG oil. The results also suggest that the activity of hepatic MTP, which involves in the biosynthesis and lipid loading of apolipoprotein B, is down-regulated by DAG oil consumption. Beside, down-regulating of fatty acid synthetase, glucose 6-phosphate dehydrogenase and malic enzyme was also observed with the consumption of DAG oil. On the other hand, DAG oil consumption up-regulates enzymes involving fat oxidation reaction including carnitine palmitoyltransferase, acyl-CoA dehydrogenase, acyl-CoA oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, 2, 4-dienoyl-CoA reductase and delta 3, delta 2-enoyl-CoA isomerase [Murata et al. 1997]. The analyses of gene expression suggest that the treatment of DAG oil down-regulates phosphoenol pyruvate carboxylase gene in liver and up-regulates peroxisome proliferator –activated receptor alpha, lipoprotein lipase, and uncoupling proteins 2 and 3 gene in skeleton muscle [Saito et al, 2007].

DAG oil consumption on energy expenditure and fat oxidation

DAG oil consumption may increase total energy expenditure (EE), fat oxidation and thermogenesis. Murata et al [1997] looked at the activities of enzymes for lipid synthesis and fat oxidation after the ingestion of DAG oil. They found that the activities of fatty acid synthesis enzymes including fatty acid synthetase, glucose 6-phosphate dehydrogenase and malic enzyme were down-regulated and the enzymes

involves in mitochondrial and peroxisomal oxidation of palmitoyl-CoA in liver were up-regulated by the consumption of DAG oil, in comparison with TAG oil with similar side chains. In liver, the enzymes involving in the beta-oxidation pathway including carnitine palmitoyltransferase, acyl-CoA dehydrogenase, acyl-CoA oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase , 2,4-dienoyl-CoA reductase and delta 3, delta 2-enoyl-CoA isomerase are up-regulated. This study indicates a potential for DAG oil to increase fat oxidation, which may be helpful in decreasing the storage of fat in the body. The value from direct measurement of respiratory quotient (RQ) shows that animals consumed DAG oil at 20g /100g body weight (BW) for two weeks increased fat oxidation. After 23 weeks, animals consumed DAG oil had lower body adiposity compared with animals consumed TAG oil [Osaki et al, 2008]. Similarly, Kimura et al [2006] compared RQ values after administering 10g/kg BW of either DAG or TAG oil with similar fatty acid side chains to 12 male Wistar rats, and found lower postprandial RQ values which indicates higher fat oxidation rate with the DAG oil administration.

In humans, short-term (less than 1 week) DAG oil consumption increased fat oxidation in female [Kamphuis et al, 2003] and male [Saito et al, 2006] subjects. However, increased total EE has only been observed with male subjects so far [Saito et al, 2006]. Contradictory study results have shown that DAG oil consumption does not alter total EE and fat oxidation in pre-menopausal females [Hibi et al., 2008]. However, in the same study, subgroup analysis shows that DAG oil consumption

increases fat oxidation in subjects with a higher percentage of body fat [Hibi et al., 2008].

All three human studies indicate the potential of DAG oil consumption to increase fat oxidation in human [Kamphuis et al, 2003; Saito et al, 2006; Hibi et al., 2008]. And it is indicated that the DAG oil consumption burns four grams more fat everyday, which makes long term significance of 1460g extra fat been burnt for 1 year [Kamphuis et al, 2003]. However, all three studies assessed only short term effects of DAG oil consumption on total EE and fat oxidation. Studies to assess long term DAG oil consumption on total EE and fat oxidation are necessary.

Action of DAG oil consumption on lipid profiles

Blood lipid profile is usually measured to assess risks associated with CVD and diabetes. Elevated low density lipoprotein cholesterol (LDL-C), total cholesterol (TC), serum triacylglycerol (TG) and decreased high density lipoprotein cholesterol (HDL-C) are associated with higher risk of CVD and stroke. Many previous studies suggest DAG oil as an effective agent to reduce total serum LDL-C, TG, as well as to increase HDL-C. However, the observations are inconsistent. Usually, fasting and postprandial lipid profiles are used to assess the impact of DAG oil consumption on lipid profiles. The following section will discuss the effects of DAG oil consumption on lipid profile of animal and human subjects with different physiological status.

The impacts of DAG oil consumption on lipid profiles by animals with insulin resistance/glucose intolerance

Ijiri et al [2006] have suggested that DAG rich high fat diets suppress LDL-C

level and exerts antithrombotic and anti-antherogenic effects compared to TAG rich diets. A mice model of high-fat diet-induced insulin resistance and obesity was used to investigate the effect of DAG oil on lipid profile modification [Saito et al. 2007]. The results suggest that DAG oil consumption for seven weeks does not alter fasting TG or postprandial TG levels, or hepatic TG content, or the rate of secretion of TG from liver. However, DAG oil consumption exerted preventative effects on insulin resistance and body adiposity in the study. Saito et al [2007] suggests that the reason that no beneficial effects of the DAG oil consumption on postprandial lipemia is that all mice consumed a complete Western type diet (WTD) for 8 weeks before being randomized to either DAG-WTD or TAG-WTD.

The impacts of DAG oil consumption on lipid profiles by healthy animals

Kimura et al [2006] suggest lower postprandial serum TG levels after a loading of DAG oil compared with the TAG oil with similar side chain fatty acids in 12 male Wistar rats. Similar results were observed when DAG and TAG oil consumed by four pigs twice a week at a dosage of 10% BW for three weeks. [Kristensen et al., 2006]. However, the major limitation for the two animal studies is their small sample sizes.

The impacts of DAG oil consumption on lipid profiles by human subjects with lipoprotein lipase deletion, insulin resistance and glucose intolerance

Individuals with insulin resistance usually develop post-prandial hypertriglyceridemia. The effect of DAG oil was investigated in a man homozygous for complete liproprotein lipase (LPL) deletion [Yamamoto et al., 2005]. The results of the study suggest no correlation between the long term ingestion of DAG oil and

serum TG. But DAG oil consumption decreased acute serum TG in the study. However, a small sample size was the major limitation of the study.

To further verify the effect of DAG oil consumption on lipid profiles, short term studies with larger sample sizes have been conducted. A single loading cross-over design study with 45g of either DAG or TAG oil was conducted in 18 male subjects with insulin resistance [Takase et al., 2005]. Results show that DAG oil consumption introduces lower postprandial increments of serum TG and remnant lipoprotein cholesterol (RLP –C). Another single loading, double blind and cross-over study conducted over a 2-week interval by Tada et al [2005] looked at the effects of DAG oil consumption on postprandial lipids for diabetic patients. With the consumption of test oils at a dosage of 30g fat/m^2 body surface, DAG oil successfully suppressed the increasing of postprandial serum TG. It is suggested that DAG oil consumption may decrease serum TG up to 39.4% after 12 weeks for subjects with type 2 diabetes [Yamamoto et al., 2001]. Lower serum concentrations of TG, RLP-TG and RLP-C were observed after a loading dose of 17g/m^2 body surface DAG in 25 male subjects with impaired glucose tolerance [Ai et al, 2007]. However, no significant difference was observed for the 11 subjects with normal glucose tolerance. The study concluded that DAG oil has a protective effect in individuals with impaired glucose tolerance. In summary, DAG oil consumption successfully suppresses postprandial increasing of serum lipids compared with TAG in short term human studies.

Reyes et al [2008] looked at the impact of five weeks DAG oil consumption in 25 non-diabetic but insulin-resistance subjects (7 males, 18 females). Postprandial TG

introduced by DAG oil consumption does not differ from TAG oil. This study also indicates similar fasting TG between DAG and TAG oil consumption. According to the authors, high fat background diets and high baseline serum TG may have been responsible for the absence of hypolipidemic effect of DAG oil consumption in the study.

The long term (three months) effects of DAG oil consumption on lipid profiles were assessed in 25 subjects with type 2 diabetes by Yamamoto et al [2006]. In this study, subjects replaced their daily use oil with DAG oil for three months. Decreased serum TG was associated with an increased concentration of HDL-C and apolipoprotein A1 with DAG consumption. The particle size of LDL-C was increased with the DAG ingestion. The study also suggested that plasma plasminogen activator inhibitor-1 concentrations, which play important role to degrade blood clots, were significantly lower with the DAG oil administration.

However, limitations for previous studies including absence of a cross-over design [Yamamoto et al., 2006, Tada et al., 2005], small sample size [Yamamoto et al., 2005], and the short term nature of the intervention [Tada et al., 2005; Takase et al., 2005] compromise their abilities to assess the potential of DAG oil consumption on lipid lowering.

In summary, DAG oil exerts potential protective capacity in subjects with glucose intolerance and diabetes including decreasing serum TG, RLP-C, RLP-TG, and increasing HDL-C and apolipoprotein A1, concentration of plasminogen activator inhibitor-1, and the particle size of LDL-C. Results from Yamamoto et al [2006]

suggest that the consumption of DAG oil for a longer period of time such as three months may be necessary to elicit a serum lipids lowering effect. However, the inconsistency of these results may be due to differences in background diets, baseline lipid profile, dosage of test oil, and subjects' degree of insulin resistance. Therefore, long term studies with different ethnic groups, physiological situations are needed to assess the beneficial effects of DAG oil consumption.

The impact of DAG oil consumption on lipid profiles by healthy human

Tomonobu et al [2006] conducted a lipid loading test on 43 Japanese subjects with 10g of DAG or TAG oil. The results suggest that the 29 subjects with fasting TG greater than 1.13mmol/L showed smaller incremental areas under the response curve for TG and RLP-C after the loading of DAG oil.

Similarly, Yanai et al [2008] conducted a study to look at the potential postprandial modification effect of 40g DAG oil consumption on seven lean Japanese students. The study results show lower postprandial VLDL-C and insulin level associated with the ingestion of DAG oil. Also, higher plasma serotonin level, which associates with higher EE, was observed with the ingestion of DAG oil.

For the two studies on healthy subjects, single loading of DAG oil exerted a beneficial impact on lipid profiles. We conclude that DAG oil may be more effective for hypertrglyceridemic (>1.13 mmol/L) but healthy individuals.

Antithrombotic effect of DAG oil consumption in animals

Atherosclerosis is a condition defined as slowly blocking of blood vessels for the

long term elevated lipidemia and triglyceridemia [Fuji et al, 2007]. ApoE deficient mice, which develop accelerated atherosclerosis, were treated with DAG oil for 20 weeks. Lower TG and TC levels were observed within larger TG-rich lipoproteins with 1, 3-DAG oil consumption. The extent of atherosclerotic lesions in the aortic arch and thoracic aorta decreased by37 and 44%, respectively, with the consumption of 1, 3-DAG compared to TAG.

Metabolic syndrome, diabetes and CVD are associated with elevated LDL-C, TG, and decreased HDL-C levels. DAG oil can be an effective agent to modify the lipid profiles, to further decrease the risk of many long term complications. However, as side chain fatty acids also affect the effectiveness of the DAG oil consumption on the modification effects of lipid profiles, more effective DAG oil can be generated by incorporating lipids lowering agents such as plant sterols [Meguro et al, 2003; Saito et al., 2006].

DAG oil consumption and body composition

DAG oil consumption and body composition in animals

DAG oil is a potential weight control agent [Rudkowska et al., 2006]. In previous animal studies, reduced body weight gain [Murase et al., 2001; Murase et al., 2002; Meng et al., 2004] and visceral weight gain [Murase et al., 2001] were observed with DAG oil consumption. A mouse model of high fat diet induced insulin resistance and obesity was used to investigate the efficacy of DAG oil [Saito et al, 2007]. The seven-week trial using 8-week old mice suggested that DAG-WTD introduces less

weight gain and less body fat accumulation compared with the TAG-WTD. However, DAG oil exerts no effects on body weight and composition in other studies [Taguchi et al., 2002; Sugimoto et al., 2003; Sugimoto et al., 2003].

DAG oil consumption and body composition in humans

Previous studies have suggested that long-term ingestion of DAG oil modifies body composition by reducing visceral fat/total body fat [Yanagisawa et al., 2003, Nagao et al. 2000], body weight [Nagao et al, 2000], BMI [Taramoto et al, 2004], waist circumference [Nagao et al., 2000], subcutaneous area fat [Nagao et al., 2000], hepatic fat content [Nagao et al., 2000] and body weight gain [Maki et al., 2001] in humans. However, contradictory studies suggest DAG oil consumption has no effect on body weight and body composition [Yamamoto et al., 2001, Yasunaga et al., 2004]. When daily use conventional oil was replaced by DAG oil, total and subcutaneous fat of 11 male and female obese children decreased significantly after 5 months [Matsuyama et al, 2006]. The body composition modification effect of DAG oil is more often observed with relatively higher dosage and longer treatment period. In general, DAG oil shows beneficial effects in subjects with different physiological conditions such as patients who underwent hemodialysis [Teramoto et al., 2004], children [Matsuyama et al., 2006], diabetic patients [Yamamoto et al., 2001], dietary induced obesity mice [Saito et al., 2007], etc. Although it can be disputed whether a small amount body weight reduction is clinically significant, a weight reduction and body composition modification for 1% may have substantial effects on an individual's weight over several years.

Conclusion

Collective data available suggest that 1, 3-DAG may be a safe and effective agent for the battle against obesity and related complications. The potential capacities of DAG oil consumption include increasing energy expenditure, fat oxidation, modifying body composition and lipid profiles. However, more understanding on the potential unique metabolism of DAG oil is still in need for future investigation.

Part 2. Study rationale

1. Limitations of previous research

The initial studies were limited by three factors including small sample sizes, less than rigorous scientific designs and indirect measures of major outcome variables.

2. The importance of this study

The study was cross over design and all the subjects were randomized to eliminate the inter-individual response. All data such as energy expenditure was measured directly. To eliminate the limitations of previous studies, sample size of 26 was decided.

Part 3. Hypotheses

Hypothesis 1 (Body Weight & Composition): DAG oil will produce greater loss in body weight, fat and regional body fat versus TAG oil in overweight, hypertriglyceridemic women.

Hypothesis 2 (Energy Balance): DAG oil will increase total energy expenditure and fat oxidation versus TAG oil in overweight, hypertriglyceridemic women.

Hypothesis 3 (Lipids): DAG oil will favorably alter lipid profiles in overweight, hypertriglyceridemic women versus TAG oil. This will be due, in part, to decreased triglyceride-fatty acid (TGFA) synthesis and enhanced fat oxidation.

Part 4. Sample size calculation

The sample size is determined by the following formula:

$$N=2T^2 (V^2)/(p^2)$$

Whereas N=sample size, V=variance, p=significance level [Morris., 1999]

Compared with other factors, body weight may be the least sensitive parameter which may require larger sample size to reach significance. Therefore we chose the variance of body weight for the calculation of sample size. Based on previous studies, a variance value of 9.01was decided. We decided value 2 for T, as it is used by most studies and 5 for p, as 5% is the significant value for the present study.

Part 5: Paper 1

Diacylglycerol oil fails to increase total energy expenditure or fat oxidation but reduces body fat in overweight and mild hypertriglyceridemic women

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Abstract

BACKGROUND: Diacylglycerol (DAG) may control body weight by altering energy expenditure (EE), fat oxidation and body fat accumulation. OBJECTIVE: Our objective was to examine the efficacy of DAG oil (Enova oil^{TM}) on energy expenditure and body composition in comparison with a control oil composed of sunflower, safflower and rapeseed oil. DESIGN: Twenty-six overweight and mild hypertriglyceridemic women consumed two treatment oils for 28 days separated by a four weeks washout period using a randomized crossover design. Forty grams of either DAG or control oil were consumed daily by study subjects. RESULTS: The baseline EE and fat oxidation were not different between DAG and control oil. At endpoint, DAG oil failed to alter postprandial total energy expenditure (EE) or fat oxidation compared with control. DAG did not alter total body lean mass, or lean mass in trunk, android or gynoid areas, but did reduce the accumulation of total body fat, at trunk ($p<0.05$), android ($p<0.05$), and gynoid ($p<0.05$) areas. CONCLUSION: We conclude that DAG oil possesses potential as an effective weight control agent; however, the effects may not come from increasing of lean mass, postprandial EE or fat oxidation.

Key Words: DAG oil. TAG oil. mild hypertriglyceridemic. DEXA. indirect calorimetry; total energy expenditure; body composition; lean mass; fat oxidation

Introduction

Health Canada reports that almost two out of three Canadian adults are either overweight or obese [Health Canada and Public Health Agency of Canada, 2006]. It is well known that obesity is closely related to numerous long term complications including cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM). Maintaining body weight and composition within healthy ranges represents an effective way to decrease the onset of CVD and T2DM. The disequilibrium between energy intake and energy expenditure contributes to the onset of obesity, by which surplus energy is stored in the body as fat. Therefore, dietary components that possess the capacity to tilt the equilibrium between energy intake and expenditure to favor the latter will result in a decline in body fat which is desirable in overweight and obese individuals.

Diacylglycerol (DAG) oil is a minor component that naturally appears in different edible oils ranging from 0.8% in rapeseed oil to 9.5% in cottonseed oil [Flickinger et al., 2003]. Removal of one fatty acid from the glycerol group of triacylglycerol (TAG), produces DAG either as 1(3), 2-DAG or 1, 3-DAG. DAG is also a digestive intermediate of the lipid digestion [Matsuo et al., 2001] in which TAG is broken down by lipase to produce 1, 2-DAG.

It has been hypothesized that DAG oil is metabolized differently than TAG that instead of being transported to the lymphatic system and repacked into chylomicrons, DAG enters the liver directly via portal vein, and undergoes rapid oxidation for energy [Maki et al, 2002; Kamphuis, et al, 2003]. Compared with TAG oil, less fat is

stored in the body at the adipose tissue after the consumption of DAG [Yanagita et al., 2004]. DAG oil has since become available as a functional food to control body adiposity retailed within the United States and Japan.

Although several studies indicate that DAG oil consumption increases total energy expenditure [Saito et al., 2006], fat oxidation [Kamphuis et al., 2003; Kimura et al., 2006; Saito et al., 2006; Osaki et al., 2008] and effectively modifies body weights [Osaki et al., 2008] and composition [Nagao et al., 2000; Maki et al., 2002; Yanagisawa et al., 2003; Teramoto et al., 2004], not all data are in support of a positive metabolic action of DAG oil. DAG oil consumption failed to increase total energy expenditure [Kamphuis et al., 2003; Hibi et al., 2008], or fat oxidation [Hibi et al., 2008] or modify body weight [Yamamoto et al., 2001., Sugimoto et al., 2003; Yasunaga et al., 2004., Sugimoto et al, 2007] and composition [Sugimoto et al., 2003]. To date, human trials have yet to examine the effects of medium and long term DAG oil consumption on total energy expenditure and fat oxidation. The objective of present study was, therefore, to assess the actions of acute and long-term DAG oil consumption on postprandial total EE, fat oxidation, body weight and composition. We tested the hypothesis that DAG oil consumption increases postprandial total EE, fat oxidation, thermic effect of food, and favorably modifies body weight and body composition in overweight and mild hypertriglyceridemic female subjects who consumed either DAG or conventional oil mixed control for four weeks.

Methods

Subjects

Twenty-nine (twenty premenopausal and six postmenopausal) non-smoking, female subjects free from lipid lowering medication, aged between 18-65 years, with a body mass index (BMI) between 24.5 to 36 kg/m^2, serum triacylglycerol greater than 1.0 mmol/L, were recruited by advertising through a local Winnipeg radio station. Subjects were excluded if they were diagnosed with diabetes mellitus, kidney or liver disease. Exclusion criteria also included chronic alcohol consumption, use of laxatives, concentrated fiber, fish oil, or plant sterols. Fasting blood samples were collected to screen for normal biochemical and hematological parameters. Subsequent physical examination by a research physician was carried out. The study protocol was reviewed and approved by the Human Ethical Review Committee of the University of Manitoba. All subjects received explanations about the protocol and written consent forms were obtained from each participant.

Study design

The study was a randomized, single-blind, crossover design consisting of two independent phases of four weeks during which subjects consumed either DAG oil or a control oil, consisting of sunflower, safflower and rapeseed oil at a ratio of 1:1:1. A washout period of four weeks separated the two study phases. A typical North American breakfast was provided by the metabolic kitchen at the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN) of the University of Manitoba. A total of 40g/d of test oil was consumed by each study subject during each study intervention. On each day, 20g of testing oil was incorporated into their breakfast, which was consumed under supervision, while 20g was given to the subjects to be

consumed with their other meals of the day. The caloric intake of food was not controlled during the study period. Maintaining a consistent physical activity level was strongly recommended by study coordinators during the two interventions. The caloric values of the energy expenditure meals were calculated using the Mifflin [1990] equation.

Energy expenditure protocol

Energy expenditure (EE) was measured using indirect calorimetry (Viasys Vmax Encore 29N, Summit technology, CA). On a daily basis, the metabolic monitor was calibrated using gas containing 95% O_2 and 5% CO_2 under ambient pressure. Subjects were required to undergo a 12 hr overnight fasting period prior to each measurement period for assessment of basal metabolic rate (BMR) and resting metabolic rate (RMR). During the 6.5 hr test, subjects were studied under standardized physical rested conditions in a thermo-neutral (23°C, relative humidity 80%) environment in a recumbent position. BMR was assessed 30 min prior to breakfast. Subjects remained motionless during the EE measurement; however, watching movies and reading books were permitted. The measurement of thermic effect of food lasted for 6 hours after breakfast and was divided into six separate sessions with 30 minutes testing for each and 30 min rest in between.

Substrate oxidation was determined from the respiratory quotient (RQ) on a per minute basis. Protein oxidation was calculated from the lean mass of subjects and assumed to be constant for each measurement [Elia et al., 1992]. All lean mass data were obtained from the DEXA data. The trapezoid method was used to calculate the

thermic effect of food (TEF). Metabolic rates were plotted against time under the 6 h curve minus the projected BMR over the 6 h period.

Dual energy x-ray absorptometry (DEXA) protocol

Body weights were recorded daily. Whole body DEXA scans were performed on subjects at the RCFFN once at the beginning and the end of each experimental phase. The DEXA system assesses body composition by measuring differential absorption of x-rays at two frequencies. Subjects were asked to lie down with palms down and remain as still as possible during the scan. Calibration was conducted daily to maintain the quality of measurement. The DEXA unit used a GE lunar BX-1 L-8743 scanner. The software used to analyze body composition was Encore 2005 produced by GE Healthcare. The program yielded total tissue volume for the whole body. The whole body lean and adipose mass were divided into trunk, android, gynoid, arms and legs by adjusting the lines surrounding each part. Total tissue mass, tissue mass at trunk, android, and gynoid areas were used for analysis.

Statistics

All data were expressed as means and standard errors. End-points and percentage change for total energy expenditure, fat oxidation, body weight, fat mass, and lean mass were compared using paired student t tests. For energy expenditure, fat oxidation and TEF, the first week EE test of each study period was used as baseline, and the EE test on the last week was used as an end point. For body weight and composition analysis, day1 data were taken as the initial value, and day 29 as the end

point. A value of $p<0.05$ was used to determine significance. JMP statistical software, student edition, (SAS Institute, Cary NC) was used to carry out the analysis.

Results

Twenty-nine overweight and mild hypertriglyceridemic females were recruited, of whom, 26 (34.3 ± 2.6 years of age, 78.3 ± 3.6 kg BW, BMI 30 ± 0.69 kg/m^2 (mean ± SEM)) completed the study. None of the subjects was taking any lipid lowering medication. Drop-out reasons included moving away from the city (n=1), commitment to daily attendance at the RCFFN for meals (n=1), and no reason (n=1). All subjects tolerated the testing oils well. Only one subject reported a case of stomach flu, which was not considered to link with testing oils.

Energy Expenditure

Table 1 lists the effects of DAG oil consumption on total EE and fat oxidation throughout the two study phases. Our short-term and long-term data shows that with similar BMR across two study phases, DAG oil consumption failed to increase total EE or fat oxidation 6 hrs after. Similarly, total EE, fat oxidation and TEF were not different between the consumption of DAG and control oil across the study period. CHO oxidation was consistent on two study phases. The percentage changes were not different cross the study period. DAG oil did not introduce higher thermo-genesis than the control oil.

Body composition

Baseline body weights were not different between DAG and control oil phases.

At end-point, body weight was lower ($p<0.05$) with DAG oil consumption. Percentage changes in body weight values (0.2 ± 0.25 % and 0.6 ± 0.33 % respectively) were not different between two treatments. Table 2 and 3 summarize the changes observed in adipose tissue and muscle mass for both DAG and control oil groups respectively. At the end-point, DAG oil reduced ($p<0.05$) total body fat at trunk, android and gynoid areas (Table 2) without changing lean mass (Table 3). None of the percentage changes were significant different between diets. In addition, no change of lipid profile parameters was observed in present study.

Discussion

The novel findings of this study are that DAG oil consumption for a 4-week period does not increase postprandial total EE or fat oxidation, but effectively reduces body weight and total body fat at the trunk, android and gynoid areas. Moreover, the body weight and composition effects from DAG oil consumption appear not to be due to postprandial total EE and fat oxidation.

The importance of energy balance on body weight and composition has been well-established. However, to our knowledge, the present study is the first study in humans to examine medium-term effects of DAG oil consumption on postprandial total EE and fat oxidation. We observed no change in acute and long-term postprandial total EE and fat oxidation with the consumption of DAG oil, which is consistent with the results suggested by Hibi et al [2008]. Contradictory studies have suggested that DAG oil consumption increases short term total EE [Saito et al., 2006] and fat oxidation [Kamphuis et al., 2003; Saito et al., 2006]; however, in those studies

[Saito et al., 2006; Kamphuis et al., 2003], the subjects were not restricted in a recumbent position as was the case in the present study. Therefore, the body weight and composition modification effects observed in the present study may be due to DAG oil consumption combined with physical activity increased the total EE and fat oxidation. Maki et al [2002] found that the consumption of DAG oil enhanced loss of body weight and fat in comparison to TAG oil consumption. Future studies are warranted to investigate the impacts of DAG oil consumption on total EE and fat oxidation with restricted energy intake and/or increased physical activity. Hibi et al [2008] suggested that DAG oil consumption may increase fat oxidation in people with high adiposity. However, this conclusion was made based on a small sample size. DAG oil consumption failed to affect fat oxidation in present study, even though our subjects had higher BMI values than reported in other studies. Therefore, the lack of response in postprandial total EE and fat oxidation in the present study may be due to our subjects being less sensitive to DAG oil than people with higher lean mass, as people with high adiposity are more prone to store energy as fat in their bodies.

We observed that the lower body weights associated with DAG oil feeding resulted in reductions in total adiposity as well as those in trunk, android and gynoid areas with the consumption of DAG oil, which is consistent with previous studies [Osaki et al., 2008; Nagao et al., 2000; Maki et al., 2002; Yanagisawa et al, 2003; Teramoto et al, 2004]. Contradictory studies show that DAG oil exerts no effect on body weight and composition [Yamamoto et al., 2001; Yasunaga et al., 2004]. However, these studies were not of a crossover design; therefore, their ability to

measure the impact of DAG oil on body weight/composition may have been compromised by inter-individual variation. Nagao et al [2000] suggested a weight loss of 1.5 kg over 4 months attributable to consumption of DAG oil. Our results suggest DAG oil consumption induced an average of 0.3 kg more weight reduction than conventional oil over a 4-week period, which is similar to results observed by Nagao et al [2000]. Kamphuis et al [2003] suggested that DAG oil consumption causes 4g/d of more fat to be shunted for oxidation than TAG oil with similar side chains, resulting in 112g of fat loss for 4 weeks. Our result suggests a 188g of more reduction of body weight. Therefore, increased fat oxidation of DAG oil consumption is not the sole source of body weight reduction. Besides observing up regulation of fat oxidation enzymes such as carnitine palmitoyltransferase, acyl-CoA dehydrogenase, acyl-CoA oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, 2,4-dienoyl-CoA reductase and delta 3, delta 2-enoyl-CoA isomerase with DAG oil consumption, Murata et al [1997] also suggested down regulation of fatty acid synthesis enzymes including fatty acid synthetase, glucose 6-phosphate dehydrogenase and malic enzyme. DAG oil consumption decreases fatty acid synthesis enzymes on gene expression including lipoprotein lipase and uncoupling proteins 2 and 3 [Saito et al., 2007]. Collectively, the weight reduction and body composition modification effect of DAG oil consumption observed in the present study may also be a result of down-regulated fatty acid synthesis.

Side chain fatty acids are also factors affecting our results. Short chain fatty acids (SCFA) and medium chain fatty acids (MCFA) are metabolized differently than fatty

acids with longer chains: SCFA and MCFA get transported directly via the portal vein to the liver to get oxidized for energy, while fatty acids with longer chains are packed into the chylomicron and re-synthesized into TAG to be stored in adipose tissue. However, the side chain fatty acids were not modified in the present study, which may have compromised our ability to measure the impact of DAG oil on total EE and body composition.

Although the postprandial total EE and fat oxidation measured in the recent study failed to explain the body weight and body composition modification effects of the DAG oil, appetite-related effects [Kamphuis et al., 2003] of DAG oil may exist as another reason explaining the observation of the current study. Other factors attributed to the different results observed in the present study in terms of total energy expenditure, fat oxidation, body weight and body composition include use of a different population, men versus women, lean versus overweight, and different doses of test oils.

In conclusion, although similar postprandial total EE and fat oxidation were associated with the consumption of DAG oil and TAG oil in the present study, incorporation of DAG oil in daily diets for four weeks effectively reduced body weight and adiposity compared with conventional oil consumption. Therefore, DAG oil can be considered as a useful agent for the battle against obesity.

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Table 1: Acute and long-term energy expenditure and fat oxidation per kilogram fat free mass of overweight women after consumption of DAG and control oil diets

Table 1						
Per kg FFM	DAG oil		Control oil		Diet effect p value	Change from acute p value
	Acute	Long-term	Acute	Long-term		
At rest						
RMR (kcal/min)	0.0179±0.0007	0.017±0.00006	0.0193±0.0006	0.0177±0.0008	0.51	0.39
Fat oxidation (g/min)	0.0011±0.0001	0.0009±0.0001	0.0009±0.0002	0.002±0.001	0.28	0.26
1.5 h post meal						
EE (kcal/min)	0.0277±0.0013	0.0264±0.0011	0.0275±0.0007	0.027±0.0018	0.76	0.92
Fat oxidation (g/min)	0.0001±0.0001	-0.0002±0.0001	-0.0001±0.0002	-0.0005±0.0004	0.59	0.9
2.5 h post meal						
EE (kcal/min)	0.0244±0.0009	0.0239±0.0009	0.0246±0.0006	0.0232±0.0011	0.53	0.35

Fat oxidation (g/min)	0.006±0.0001	0.0005±0.0001	0.0007±0.0002	0.0008±0.0001	0.12	0.52
3.5 h post meal						
EE (kcal/min)	0.0237±0.0009	0.0219±0.0001	0.0231±0.0006	0.0219±0.0011	1	0.65
Fat oxidation (g/min)	0.0009±0.0002	0.0011±0.0004	0.0007±0.0002	0.0008±0.0001	0.45	.78
4.5 h post meal						
EE (kcal/min)	0.0217±0.0008	0.02±0.001	0.0229±0.0007	0.0207±0.0011	0.68	0.53
Fat oxidation (g/min)	0.001±0.0001	0.0007±0.0001	0.0008±0.0002	0.0009±0.0001	0.45	0.26
5.5 h post meal						

EE (kcal/min)	0.0219±0.0013	0.0189±0.0007	0.0205±0.0006	0.0192±0.001	0.8	0.23
Fat oxidation (g/min)	0.0012±0.0002	0.0012±0.0002	0.0011±0.0001	0.0015±0.0003	0.42	0.39
6.5 h post meal						
EE (kcal/min)	0.0215±0.0012	0.0183±0.0007	0.0193±0.0006	0.0183±0.0008	0.97	0.22
Fat oxidation (g/min)	0.0013±0.0001	0.0017±0.0005	0.0017±0.0003	0.0012±0.0001	0.37	0.19
Total						
EE (kcal/min)	0.0224±0.0008	0.0207±0.0007	0.0221±0.0004	0.0209±0.001	0.82	0.79
Fat oxidation (g/min)	0.001±0.0003	0.0024±0.0013	0.0024±0.0011	0.001±0.0003	0.32	0.24
TEF	-2.3186±2.2	8.7672±1.45	-2.2922±1.8	6.9293±1.10	0.32	0.49

(kcal/day)	196	46	344	29		

Table 2: Change in adipose tissue of overweight women after 4-week of consumption of DAG and control oil diets

	Baseline (kg)			Endpoint (kg)			
	Control oil	DAG oil	Δ Diet *p* value	Control oil	DAG oil	Δ Diet *p* value	Δ Diet for change *p* value
Adipose tissue							
Total	35.04±1.77	35.13±1.85	1	35.8±1.68	34.84±1.57	0.049	0.13
Trunk	16.92±1.07	17.38±1.07	0.61	18.01±1	17.3±1	0.03	0.17
Android	2.96±0.2	3.03±0.17	0.66	3.3±0.2	3.05±0.17	0.003	0.1
Gynoid	6.38±0.3	6.46±0.32	0.7	6.67±0.35	6.46±0.32	0.049	0.07

Table 3: Change in lean tissue of overweight women after 4-week consumption of DAG or control oil diets

	Baseline (kg)			Endpoint (kg)			
	Control oil	DAG oil	Δ Diet *p* value	Control oil	DAG oil	Δ Diet *p* value	Δ Diet for change *p* value
Lean mass							
Total	41.78±0.96	41.57±0.88	0.67	41.32±0.8	41.5±0.85	0.55	0.53
Trunk	19.37±0.62	18.97±0.52	0.33	19.04±0.47	19.17±0.56	0.5	0.32
Android	2.84±0.08	2.84±0.11	0.59	2.89±0.09	2.85±0.11	0.7	0.67
Gynoid	6.31±0.15	6.19±0.19	0.34	6.3±0.17	6.31±0.2	0.82	0.27

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Part 6. Bridge

In the present study, reduction of body weight and adiposity does not seem from increased postprandial total EE and fat oxidation. DAG oil consumption has the potential to alter hepatic lipogenesis and exert positive impacts on blood lipid profiles. The second paper will discuss the impacts of DAG oil consumption on hepatic lipogenesis and blood lipid profiles.

Part 7. Paper 2

Our material is original, and has not been previously published before and has not been submitted for publication elsewhere while under consideration for the *European Journal of Clinical Nutrition*.

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Diacylglycerol oil fails to alter the blood lipid profiles or hepatic lipogenesis in overweight and mild hypertriglyceridemic women

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Abstract

Background: Diacylglycerol (DAG) oil is a natural component of various edible oils as well as an intermediate of the lipid digestion process. The capacity for DAG oil to lower serum triacylglycerol and hepatic lipogenesis appears to be controversial. **Objectives:** The objective of this study was to determine the effect of DAG oil consumption on blood lipid profiles and hepatic lipogenesis in mild hypertriglyceridemic women. **Subjects/Methods**: The study included DAG oil or conventional oil phases separated by a four weeks washout period, using a randomized crossover design. Twenty-six overweight and mild hypertriglyceridemic female subjects consumed 40g of either DAG oil or a mixture of sunflower, safflower and rapeseed oil at a ratio of 1:1:1 for 28 days. **Results**: Study results showed that both DAG and the control oil altered serum triacylglycerol by -3±7 % and -8.7±6 %, respectively, although no difference between two treatments was suggested by our results. At the end point, no significant differences between these two dietary interventions for total cholesterol (TC), triacylglycerol (TG), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) levels, or hepatic lipogenesis were observed. **Conclusions**: Our results suggest that consumption of DAG oil for four weeks does not affect serum lipid profiles or hepatic lipogenesis compared with conventional oil in overweight and hypertriglyceridemic women.

Key Words: DAG oil. TAG oil. serum TG. lipid profiles. mild hypertriglyceridemic. hepatic lipogenesis

Introduction

Cardiovascular disease (CVD) is common in North America, affecting nearly one out of every three adults in the United States [American Heart Association 2008]. The American Heart Association estimates that CVD contributes 36% of the total mortality every year making it the number one cause of death in United States [American Heart Association 2008]. In the mean time, 2005 data from the National Diabetes Association suggested that 7% of the American population is affected by diabetes [National Diabetes Statistics 2005].

Dyslipidemia, defined as elevated circulating TC, LDL-C and TG with sub-optimal HDL-C, is a common symptom to the development of both CVD and diabetes. Chronic dyslipidemia causes atherosclerotic and thrombotic effects to the circulatory system, therefore the blood lipid profiles are important biomarkers to assess the risks associated with CVD and diabetes. High dietary lipid concentrations are positively correlated [Expert et al., 2001] with serum TG, and therefore, adversely impact blood lipid profiles [Akoh et al., 1995].

Diacylglycerol (DAG) oil is a minor component naturally present in different edible oils ranging from 0.8% in rapeseed oil to 9.5% in cottonseed oil [Flickinger et al., 2003]. DAG is also a digestion intermediate of the lipid digestion process in that dietary TAG are hydrolyzed by lipase to form 1, 2-DAG or 2, 3-DAG [Matsuo et al., 2001; Osaki et al., 2005]. Previous studies suggest that consumption of DAG oil modifies blood lipid profiles [Yamamoto et al., 2001; Tada et al., 2005; Takase et al., 2005; Ijiri et al., 2006; Kimura et al., 2006; Kristensen et al., 2006; Yamamoto et al.,

2006; Ai et al., 2007, Yanai et al., 2008] by decreasing serum TG. More over, DAG oil exerts similar physical properties as TAG [Yasunaga et al., 2004]. Therefore, replacing TAG oil with DAG may exist as an effective strategy to improve health status by decreasing risks associated with CVD and diabetes. However, contradictory studies suggest that DAG has no impacts on lipid profiles [Saito et al., 2007; Reyes et al., 2008].

The objective of this study was therefore to determine whether DAG oil synthesized from soybean oil and rapeseed oil impacts blood lipid profile and hepatic lipogenesis. Therefore, we tested the hypothesis that DAG oil consumption over a 4-week period alters fasting serum TG and the hepatic synthesis of TG compared with conventional oil, in overweight and mild hypertriglyceridemic female subjects.

Subjects and methods

Subjects

Twenty-nine nonsmoking, female subjects free from lipid lowering medication, between 18-65 years of age, with a body mass index (BMI) between 24.5 to 36 kg/m^2, serum TG greater than 1.0 mmol/L, were recruited by advertising through a local radio station in Winnipeg area. Subjects were excluded if they were diagnosed with diabetes mellitus, kidney or liver diseases. Exclusion criteria also included additional factors that may affect lipid profile parameters such as chronic alcohol consumption, use of laxatives, concentrated fiber, fish oil, or plant sterols. The study protocol was described in full to the subjects and written consent forms were obtained from each

participant. Fasting blood samples were collected to screen for normal biochemical and hematological parameters and subjects were deemed otherwise healthy through a physical examination by a qualified physician. The study protocol was reviewed and approved by the Human Ethical Review Committee of the University of Manitoba.

Study design

The study was a randomized, single-blind, crossover design with two independent phases separated by a 4-week time during which subjects consumed either the DAG oil or the control oil, a mixture of sunflower, safflower and rapeseed oil at a ratio of 1:1:1. A typical North American breakfast was provided by the metabolic kitchen at the Richardson Centre for Functional Foods and Nutraceuticals of University of Manitoba. A total of 40g of testing oil was consumed by each study subject everyday during the study period. On each day, 20g of testing oil was incorporated into their breakfasts, which was consumed under supervision, and 20g was given to the subjects to be consumed with their other meals of the day. The energy of the food vehicle for each participant was calculated using the Mifflin equation [Mifflin et al., 1990] for healthy female adults as the carrier of 20g of testing oil. A light to moderate activity factor of 1.5 was used to estimate the daily energy expenditure. Caloric intake was not controlled during the study period. A consistent physical activity level was strongly recommended by the study coordinators for the two experimental periods.

Composition of the diacylglycerol oil

The DAG oil used in the present study (Archer Daniels Midland Company,

Decatur, USA) contained more than 80% of DAG oil, mostly 1, 3-DAG. The DAG oil used in the study was prepared by using soybean oil and rapeseed oil in the presence of an immobilized lipase according to the method reported by Watanabe et al [Watanabe et al., 2003].

Serum analyses

Serum samples were collected into vacutainer tubes after a 12 hr fast and a 24 hr alcohol exemption period on day 1, 2, 28 and 29 of each phase. Blood samples were centrifuged at 1500 g at 4°C for 20 min. Serum was immediately separated from red blood cells (RBCs) into 0.5- to 1-mL aliquots and stored at −80°C until future analysis. TC, HDL-C, and TG concentrations were measured by automated methods on the multianalyzer Dimension RxL Max utilizing enzymatic reagents Flex (Ortho-Vitros 350, New York, USA). LDL-C levels were calculated using the Friedewald [1972] equation as all the subjects had their TG levels < 4.5 mmol/L. All samples were analyzed in duplicate and day 1 and day 29 samples were used as initial and end-point data.

Hepatic lipogenesis measurement by deuterium incorporation

Before samples were analyzed for deuterium enrichment, the detection limits for palmitate was determined. Palmitate standard (methyl palmitate) was purchased from Sigma-Aldrich. Each standard was mixed with 1 ml hexane with HPLC grade to make a standard solution.

Subjects received an oral dose of 0.7 g/kg deuterium oxide of the estimated body

water on day 28 of each study phase. Blood was collected by vacutainers on day 28 (hour 0) and 29 (hour 24). Plasma was separated from RBCs by centrifugation at 15,000 rpm for 20 min and stored at -80 ^{o}C until further analysis. Human triacylglycerol fatty acid (TG-FA) synthesis rates were determined by the incorporation of deuterium from the plasma water pool *in vivo* into TG-FA over 24 hrs. The fraction of newly synthesized TG-FA was taken as the enrichment of the baseline (day 28) pool, relative to the peak level of achievable enrichment (day 29). The lipid fraction of plasma was isolated using the modified fatty acid extraction (Folch et al., 1956) procedure. Extracted lipids were analyzed using gas chromatography isotope ratio mass spectrometry (GC-IRMS, ThermoFinnigan, Bremen, Germany). Separation was achieved using a 6890 N Agilent GC fused capillary column (SAC-5; 30 m × 0·25 mm internal diameter, 0·25 μm film thickness) from Sigma-Aldrich. Isolated triacylglycerol was directed to the pyrolysis reactor to release hydrogen gas into the mass spectrometer analyzed for deuterium abundance by isotope ratio mass spectrometry. The isotope ratio mass spectrometer was calibrated each time before use using three water standards. Samples for each subject were analyzed in the same day. Standard Mean Ocean Water (SMOW) was the reference standard. The injector temperature was set at 300°C and initial oven temperature was 160°C. The oven temperature was increased to 245°C at 15°C/min and held for 4 min. The temperature was again increased to 280°C at 15°C/min. After 4 min, a final temperature of 305°C was reached at a rate of 40°C/min and held for 11 min [Marinangeli et al., 2007]. The carrier gas used was helium [Scott & Perry, 1998].

Lean mass is determined by DEXA and the whole body water pool estimated by multiplying LBM by a factor of 0.73 [Wang et al., 1999]. Deuterium enrichment of the plasma TG was estimated by the following equation:

Del (‰) TG max=del (‰) plasma *0.87 D/C* 51C/93H

The correlation factor 0.87 D/C was suggested by Jungas [1968] that 0.87 g-atoms ^3H per g-atom C incorporated into adipose fatty acids. The ratio 51C/93H represents three monounsaturated 17-carbon fatty acids attaches to a glycerol group in a typical triacylglycerol [Leitch and Jones, 1991].

The FSR, which represents the fraction synthesis rate over free fatty acid pool that is synthesized per day, was calculated as

FSR (% day^{-1}) = ‰ fatty acid/ ‰plasma

‰ refers to deuterium enrichment of free fatty acid above baseline 24 hrs.

Statistics

All data were expressed as means ± SEM. End points and percentage of change for TC, TG, HDL-C, LDL-C, FSR, and body weight, were compared using paired student t tests. Day1 body weights and lipid profiles were taken as the initial value, and day 29 as the end point. Spearman's rank correlation was used to determine the interactions between FSR and serum TG. A value of $p<0.05$ was used to determine significance. JMP statistical software, student edition, (SAS Institute, Cary NC) was used to carry out the analyses.

Results

Subjects

Of the 29 over weight and mild hypertriglyceridemic females that were initially recruited, twenty premenopausal and six postmenopausal subjects (34.3 ± 2.6 years of age, 78.3 ± 3.6 kg BW, BMI 30 ± 0.69 kg/m^2) completed the study. Drop-out reasons included moving away from the city (n=1), commitment to daily attendance at the RCFFN for meals (n=1), and no reason (n=1). All subjects tolerated testing breakfast, as well as the take home oil well. Only one reported case of stomach flu, which was not considered to be directly related to the testing diets or oils.

Body weight and composition

The body weights of the subjects at baseline (day1) were not statistically different between the two groups (Table 1). DAG oil introduced less body weight gain compared with the control oil at the endpoint. Baseline body composition was not different between the treatment groups. At the end point, DAG oil consumption reduced total body fat ($P<0.05$, n=19) at trunk ($P<0.05$, n=19), android ($P<0.05$, n=26) and gynoid ($P<0.05$, n=26) areas.

Blood lipid profiles

Serum TC, HDL-C, TG, and LDL-C baseline and endpoint values and their percentage differences are listed in Table1 and Table 2 respectively. The baseline parameters were not different with DAG and TAG groups. At the end point, lipid profile parameters after DAG oil feeding were not different than that after the TAG oil consumption (Table 1). After four weeks of DAG oil consumption, serum TG

decreased by 3 ± 7 %, and control oil introduced 8.7 ± 6 % decrease of serum TG, however, these effects failed to reach statistical difference. The two test oils exerted a similar impact on lipid profiles.

Hepatic lipogenesis as a result of the 4-week consumption of DAG oil

Figure 1 shows the estimated enriched body water. Body water was enriched by 1 ± 0.02 ‰ and 1 ± 0.02 ‰ in DAG and control phases respectively and was not different in two treatment phases. The FSR values over 24 hours with DAG and control treatments were 16.4 ± 1.5 % and 13.8 ± 2.2 % respectively. Serum TG was positively correlated to TG-FA in TAG oil group (r^2=0.5 $p<0.0001$), however, no correlation was observed in DAG oil group (r^2=0.16 $p=0.05$).

Discussion

Our results suggest that incorporation of DAG oil into daily diets for a period of four weeks does not alter fasting blood lipid profiles or hepatic lipogenesis compared with conventional oil mixed control in overweight and mild hypertriglyceridemic female subjects. Therefore, DAG oil may not be an effective agent to control dyslipidemia associated with CVD and diabetes.

The link between dyslipidemia to CVD and diabetes has been well established. To our knowledge, the present study is the first study to investigate the impacts of DAG oil consumption in non-Japanese overweight women without glucose intolerance/insulin resistance. In the present study, consumption of DAG oil for a period of 4-week showed moderate weight modification and composition effects,

however, moderate body weight and composition modification effects does not seem to alter fast lipid profiles, which is also suggested by Santosa et al, [2007]. Our results are consistent with previous studies [Nagao et al., 2000; Maki et al., 2001; Kamphuis et al., 2004; Teramoto et al., 2004; Yasunaga et al., 2004; Takase et al., 2005; Yamamoto et al, 2006; Saito et al., 2007; Reyes et al, 2008]. However, contradictory studies suggest that DAG oil consumption modifies lipid profiles [Taguchi et al., 2002; Tada et al., 2001; Yanagisawa et al., 2003]. Reyes et al (2008) conclude that differences in dietary fat intake between Japanese and North American populations may account for the lack of response to the DAG oil consumption [Yoneyama et al., 2007; Akoh et al., 1995]. Similarly, a high fat background diet may be one the reasons for the lack of response to DAG oil consumption in the present study. Tomonobu et al [2006] have suggested that DAG oil consumption modifies serum TG in the healthy subjects with elevated (>1.13mmol/L) serum TG but not the population with lower baseline TG. Therefore, lower baseline fasting TG (>1.0 mmol/L) in the present study may be the other reason for the subjects failing to respond to DAG oil consumption. Further studies in healthy subjects with higher baseline TG are necessary to assess the long term impact of DAG oil consumption on fasting TG.

To our knowledge, this is the first study in women to examine effects of DAG oil consumption on FSR of TG-FA using deuterium incorporation. DAG oil consumption for a period of four weeks failed to alter hepatic lipogenesis in the present study. Our observation is consistent with Saito et al [2007]. However, Taguchi et al [2002] observed a decreased hepatic TG concentration with DAG oil consumption. However,

the study only assessed short term DAG oil consumption and used ^{14}C instead of deuterium incorporation which may attribute to the differences in comparison to the present study. In addition, we observed a positive correlation between FSR and fasting serum TG with TAG oil consumption, and no significant correlation was observed between FSR and serum TG with DAG oil consumption. However, we cannot provide an explanation for this observation.

The lack of change in the lipid profiles with the consumption of DAG oil observed in the present study in comparison to previous studies may also be related to the different type of population used, men versus women, normolipidemic versus hypertriglyceridemic, normal weight versus overweight, insulin resistance versus insulin sensitive. Different doses of DAG and control oil consumed and the length of the treatment also attribute to the impacts on lipid profiles. A longer time period of consumption may promote the efficacy of DAG oil in reducing serum TG concentrations as suggested by Yamamoto et al (2006), who observed a decreasing of serum TG in female subjects with T2DM who replaced their daily cooking oil with DAG oil for 3 months. Different side chain fatty acids exert different impacts on the blood lipid profile. For example, linoleic acid exerts a blood cholesterol lowering impact, while stearic acid exerts a neutral effect on total and LDL cholesterol, and lauric, myristic and palmitic acids have been associated with increasing of blood cholesterol [Bonanome et al., 1998]. The combination of the DAG structure with different side chain fatty acids may further strengthen or diminish the impacts of DAG oil consumption on lipid profiles. The side chain fatty acids were not modified

in the present study, which may compromise our ability to measure the structure of dietary lipids in modification of lipid profile parameters and hepatic lipogenesis. On the other hand, the health benefits of DAG may be enhanced by modifying side chain fatty acids. The incorporation of plant sterols with DAG oil effectively lowered TC and modified atherosclerosis compared with TAG oil in a 14-week rabbit model [Saito et al, 2006].

In conclusion, DAG oil consumption failed to show any modification effects on fasting serum lipid profiles or hepatic lipogenesis in comparison to a conventional mixed control oil. As such, DAG oil consumption cannot be recommended as an effective agent to manage dyslipidemia associated with CVD and diabetes. However, the beneficial effects of DAG oil may go beyond the modification of lipid profile and hepatic lipogenesis.

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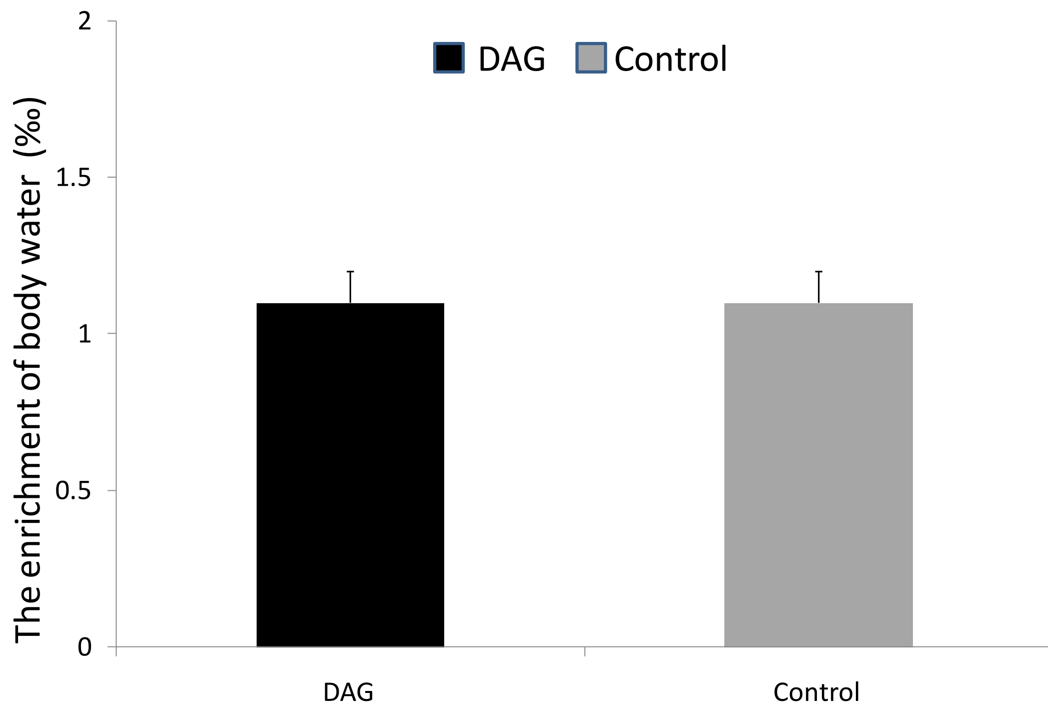

Figure1: The enrichment of body water in two treatment phases (p>0.05)

Table 1

Changes in blood lipids in overweight and mild hypertriglyceridemic women after 4 weeks of consumption of DAG or Control oil

	DAG oil		Control oil		Between group p	
	Baseline±SEM	Endpoint±SEM	Baseline±SEM	Endpoint±SEM	Baseline p	Endpoint p
Weight (kg)						
Average	81.24±2.52	81.43±2.53	81.86±2.5	82.38±2.54	0.09	0.04*
Difference		0.189±1.015		0.52±1.36		0.22
TC (mmol/L)						
Average	5.2±0.17	5.11±0.18	5.18±0.21	5.04±0.18	0.89	0.46
Difference		-0.09±0.09		-0.13±0.12		0.8
LDL-C (mmol/L)						
Average	3.43±0.15	3.45±0.15	3.52±0.19	3.41±0.17	0.43	0.64
Differences		0.02±0.09		-0.11±0.1		0.4
HDL-C (mmol/L)						
Average	1.39±0.08	1.32±0.07	1.29±0.07	1.31±0.06	0.06	0.63
Difference		-0.07±0.04		0.01±0.04		0.08
TG (mmol/L)						
Average	1.87±0.16	1.66±0.14	1.81±0.13	1.59±0.13	0.7	0.49
Difference		-0.21±0.15		-0.22±0.1		0.91

Table 2

Percentage changes of lipid profile parameters after 4-week test oil consumption

	DAG percent change %	Control percent change %
TC	-1.5±1.7	-1.8±2
LDL-C	2.1±3	-1.3±3
HDL-C	-3.2±2	2.6±3
TG	-3±7	-8.7±6

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Part 8. Summary and conclusions

DAG oil was first conceived of by the Japanese and has been considered as a functional oil for its potential beneficial effects on body weight and adiposity management. The potential beneficial effects of DAG oil consumption include increasing EE and fat oxidation, modification of body weight, adiposity and lipid profiles. It has been hypothesized that DAG oil has a unique metabolic fate than TAG oil that DAG oil is less efficiently incorporated into chylomicrom to be re-synthesized into triacylglycerol. Instead, it is shunt directly to the liver to be oxidized for energy. However, results obtained from previous studies are inconsistent and the metabolism of DAG oil remains ambiguous. The present study tested the capacities of DAG oil in 26 over-weight and mild hypertriglyceridemic women and proves the efficacy of DAG oil in modification of body weight and adiposity. However, the body weight and adiposity management effects of DAG oil consumption do not seem to be resulting from shifts in postprandial energy expenditure or fat oxidation in the present study. Results of hepatic lipogenesis measurement failed to support the hypothesis that DAG oil is less efficient to be re-synthesized into TAG. Therefore, we conclude that DAG oil does not exert any higher thermic effect to modify body weight and body composition, and that DAG oil consumption does not necessarily alter hepatic lipogenesis to modify fast lipid profiles. Furthermore, moderate loss of body weight and adiposity does not necessarily modify fast lipid profiles.

Nonetheless, other factors such as appetite control cannot be discounted as to why the direct measurement of total energy expenditure and fat oxidation failed to explain

weight loss and body adiposity modification effects observed with the ingestion of DAG oil. First, the constitutional diet may contribute to the loss of body weight. Subjects may have chosen different diets with different caloric values on the two study phases. Second, seasonal effect may have compromised our ability to verify the body weight and adiposity control effects by the consumption of DAG oil. People usually do different types and amounts of physical activity and take different types and amounts of food during different climatic conditions. With a period of seven months, it may not be scientifically accurate to assume similar lifestyle, especially for the subjects took more than one menstruation cycle washout. Last, DAG oil consumption may exert more beneficial impact on human health when it is combined with energy intake restriction or physical activities, which is not adequately assessed in the present study. In summary, DAG oil exerts potential as an effective agent for the battle against obesity, and its mechanism is still in need for future investigation.

Part 9. Directions for future research

Future research looking at the impact of DAG oil consumption on human health should be pursued to further validate its long-term effect. Studies should focus on evaluating the efficacy of DAG oil consumption combined with physical activity and/or restricted caloric intake. Furthermore, the combined side chain fatty acids may strengthen or diminish the healthy impacts of structure of DAG oil. Modification of side chain fatty acids may optimize the beneficial effects. As observed postprandial total energy expenditure and fat oxidation failed to explain the modification effects of body weight and body composition, further studies with of DAG oil on appetite control are necessary. Finally, as beneficial effects of DAG oil are more commonly observed in the Japanese population, studies should examine population differences between North Americans and Japanese. Such studies should focus on the genotype variability concerning differences in the enzymes involving lipids metabolism.

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Part 11. Appendix

Calibration methods

Energy expenditure test

1. The energy expenditure machine should be warmed for at least 30 minutes each time before use.
2. Turn on the computer screen.
3. Select “M” from the desktop.
4. Select “Flow Sensor Calibration”.

a) F1

b) Connect syringe

c) 2 strokes, then space bar

d) 5 strokes (1st stroke does not count)-one stroke in each yellow space

e) Verify 5 strokes (1st stroke does not count)-with at least 1 stroke between each dashed red line

f) Save calibration - F3

g) Disconnect syringe.

5. Select “New Study”.

a) Enter data:

i. ID = Subject code

ii. Last name = Day of the study

iii. First name = Phase #, Study name

iv. Gender

v. Birthday

vi. Height

vii. Weight

viii. Save data – F3

6. Select “Find Patient”.

a) In ‘ID’ enter first 2 letters of patient ID

b) F1

c) F3

7. Select “Gas Calibration”

a) Turn on gas cylinders by opening the tanks on each side of the desk.

b) Select “Exercise & Metabolic Test”.

c) Select “Start Test”.

d) Press F1 and wait until the gas calibration is over.

e) Save calibration – F3.

8. Turn on fan on the unit.

9. Connect subjects to the hood.

10. Select “F8” to start.

Dual energy x-ray absorptiometry

1. Open “Lunar Prodigy”.
2. Select “Quality Assurance F5”.
3. Select “Start”.
4. Place the “Quality Assurance” block below the top right line of the DEXA bed close to the middle line.
5. Adjusting laser “+” from scanning arm to the “+” on calibration block.

Autoanalyzer

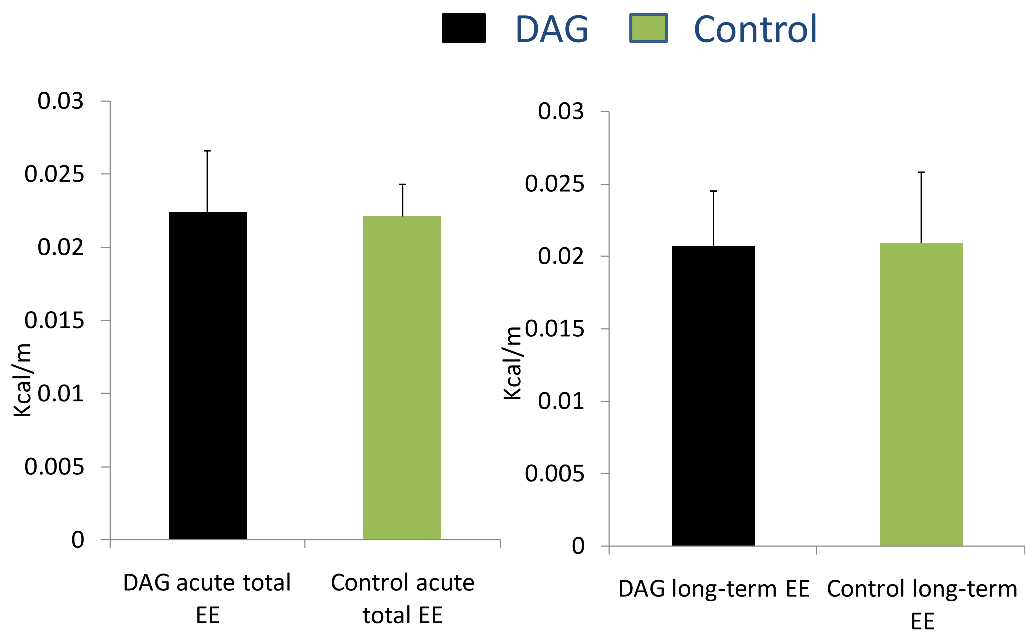
1. In main menu, select “Calibration Program”.
2. Type the name of the calibration group, then press enter. If it is a new group, press “Y” and enter.
3. Select “Calibration By Kit”. Triacylglycerol and total cholesterol are calibrated by using calibration kit 2. HDL is calibrated by using calibration kit 25.
4. Chose the chemistries to calibrate and select their lot numbers.
5. Select “Return”.
6. Select “Load Group”.
7. Prepare the tray according to the screen. Put caps on the cups. Put the tray on the track indicated on the screen.
8. Select “Group is Loaded”.
9. Select “Sample On”.
10. The calibration kits’ samples are tested by test slides and measured by spectrophotometer.

Gas chromatography isotope ratio mass spectrometer

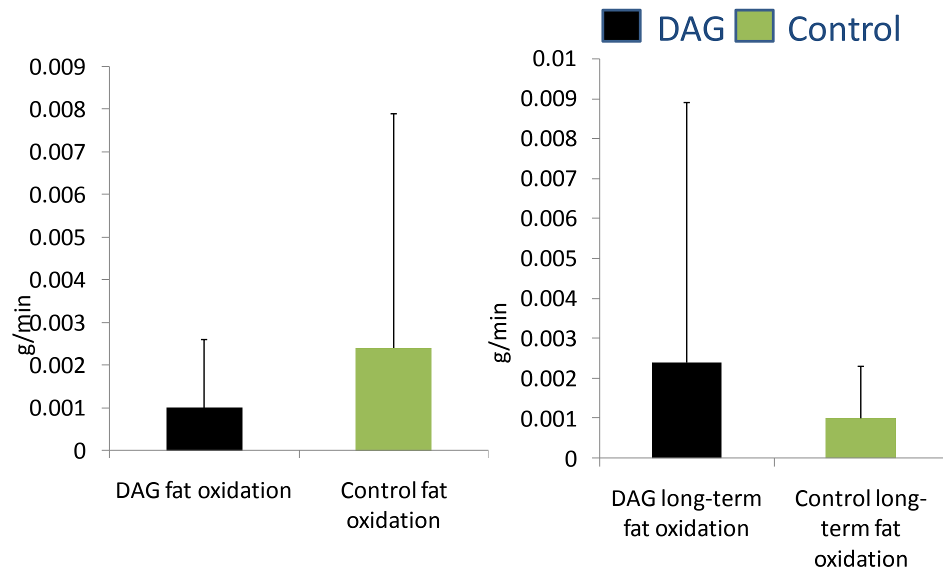
Calibration is conducted to determine and score the important relation between magnet current and selected mass.

1. In Isodat 2.5’s Instrument Control, choose “Slow Magnet” on the right side of the “Scan” window.
2. In the pulldown menu left to it, “Magnetic Field” will then be displayed automatically.
3. The “Calibration” button in the left pane of the “Scan” window becomes active.
4. Calibration can be done automatically by pressing the button.

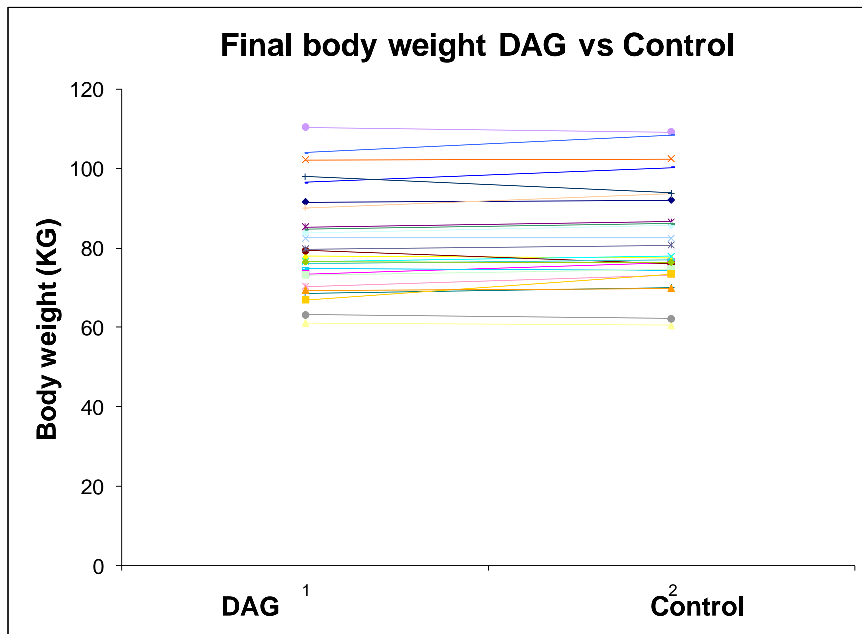
Graphs



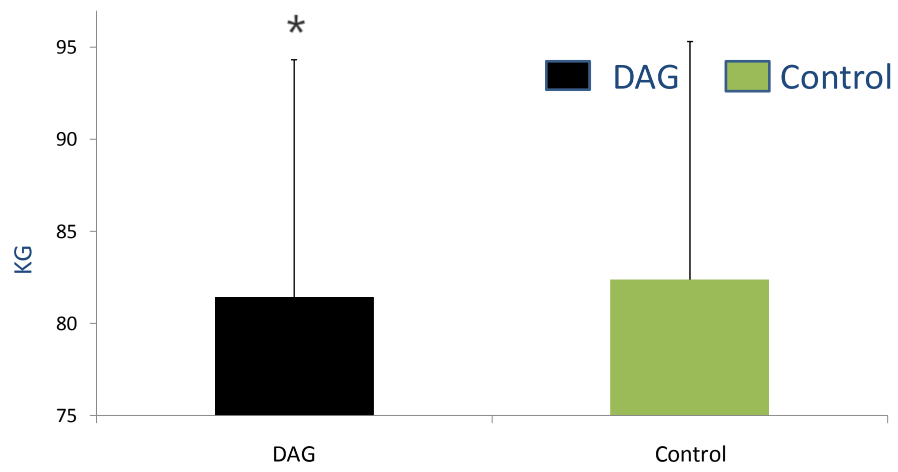
Appendix Figure 1: Total energy expenditure introduced by two test oils, $p > 0.05$



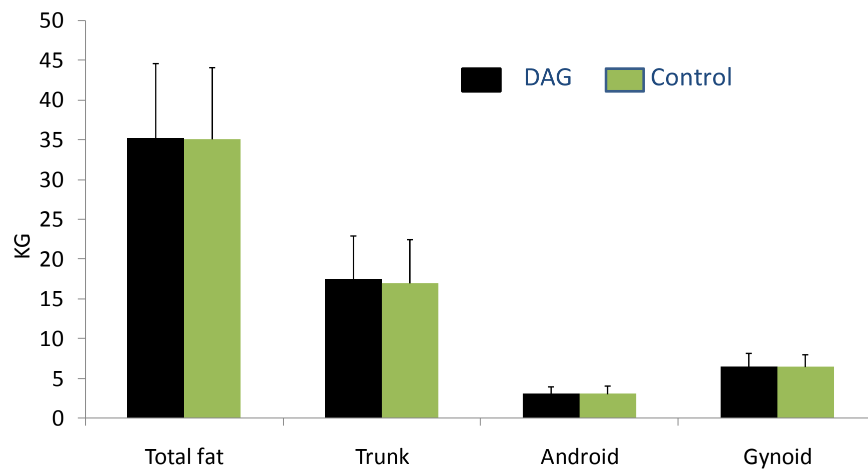
Appendix Figure 2: Beginning and endpoint fat oxidation introduced by two test oils, $p > 0.05$



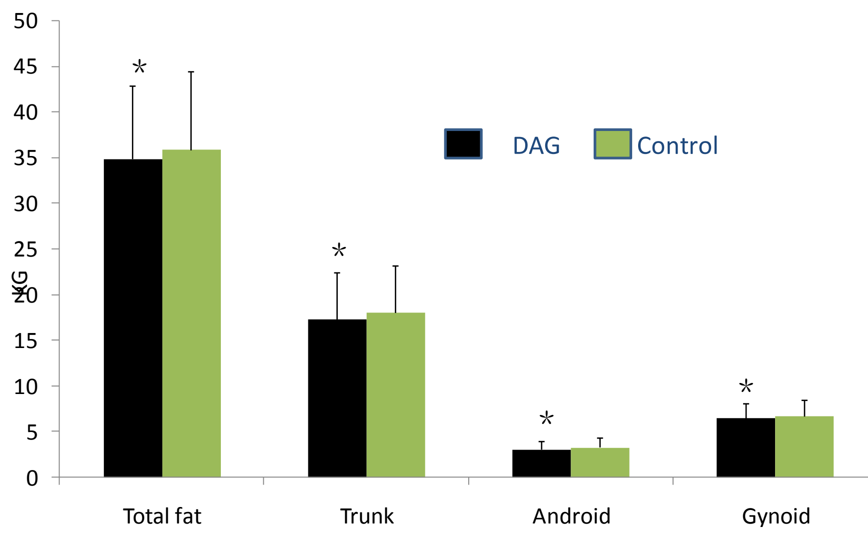
Appendix Figure 3: Endpoint body weight in two study phases, p >0.05



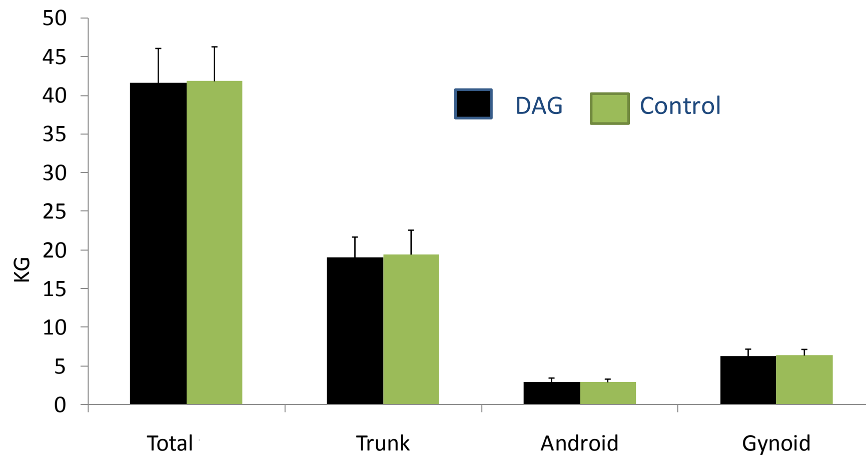
Appendix Figure 4: Endpoint body weight in two study phases, p <0.05



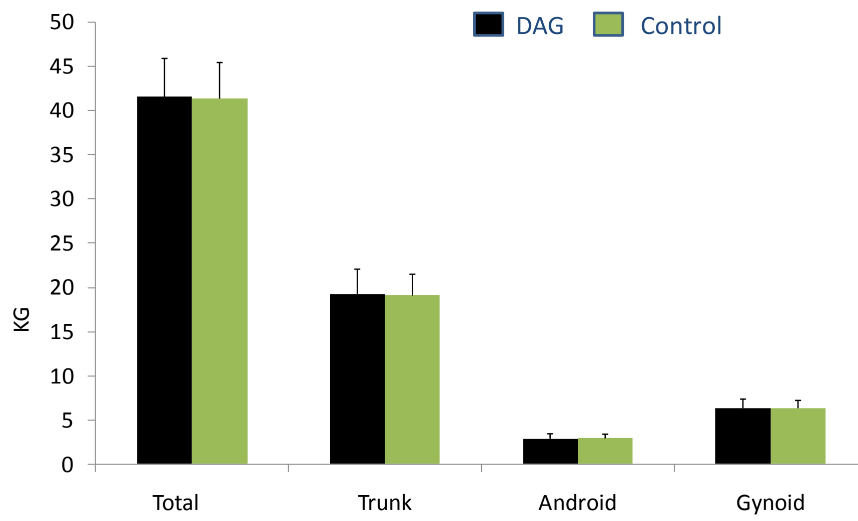
Appendix Figure 5: Baseline body fat of two study phases, p >0.05



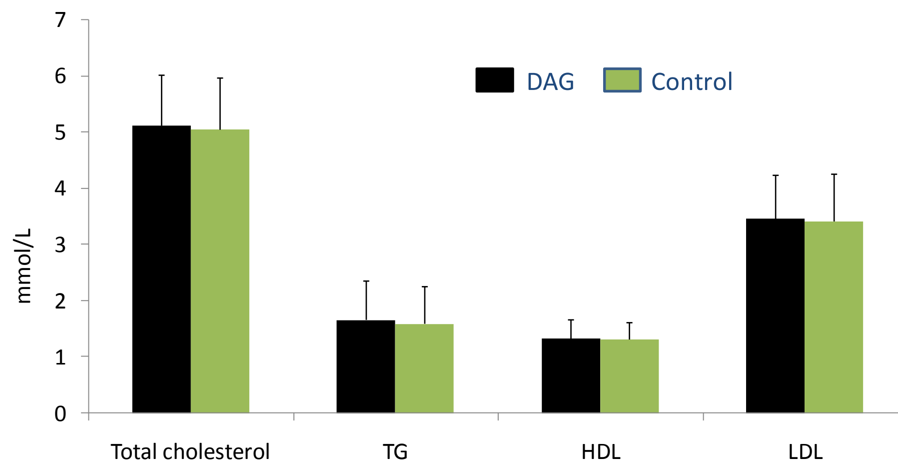
Appendix Figure 6: Endpoint body fat of two study phases, p <0.05



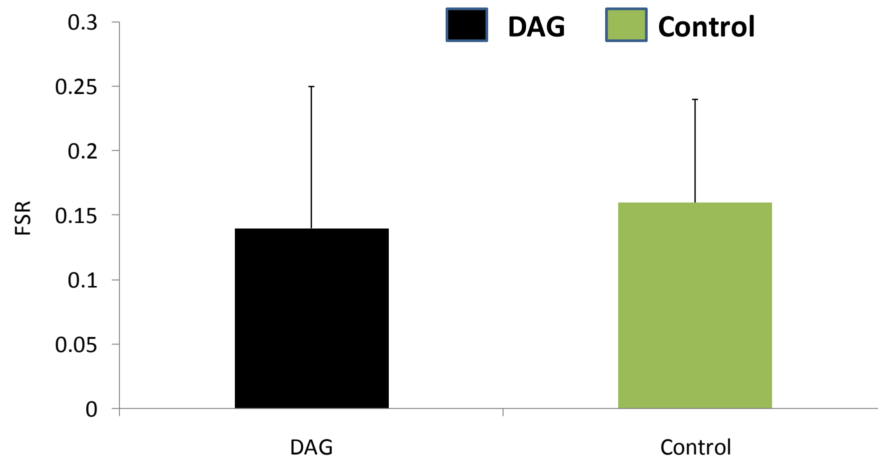
Appendix Figure 7: Baseline lean mass of two study phases, $p > 0.05$



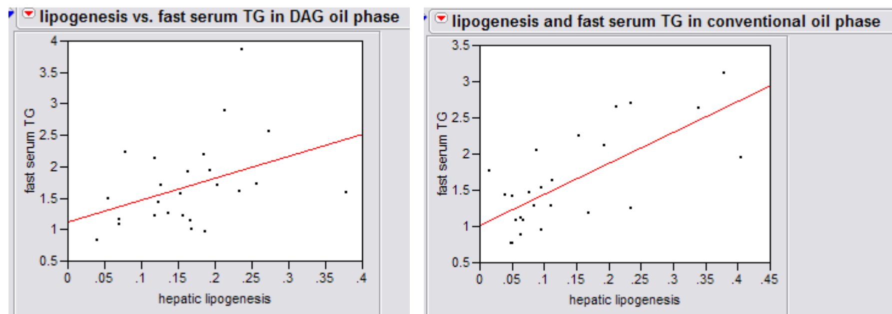
Appendix Figure 8: Endpoint lean mass of two study phases, p >0.05



Appendix Figure 9: Endpoint lipid profiles of two study phases, $p > 0.05$



Appendix Figure 10: Fractional synthesis rate of triacylglycerol over 24 hours, $p>0.05$



Appendix Figure 11: Correlation between hepatic lipogenesis and fast serum TG, $p > 0.05$