EFFECT OF DIFFERENT CONCENTRATIONS OF N-3 AND N-9 FATTY ACIDS ON FATTY ACID ETHANOLAMIDE LEVELS IN RATS

by

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ABSTRACT

Dietary fatty acids are precursors of the lipid mediator group of compounds termed fatty acid ethanolamides (FAE). Prolonged intake of specific types of dietary fats has been shown to increase FAE levels. However, the short term effects of qualitative dietary fat intake on FAE levels remain understudied. Hence, the objective of this study was to identify the effect of diets containing varying concentrations of n-9 from canola oil (CO) and n-3 fatty acids from DHA rich oil (DRO) on plasma and organ FAE levels after different time points in male Sprague Dawley rats. Sixty-four rats were randomly assigned into four groups and were fed diets containing 40% as energy of either safflower, 95% CO: 5% DRO, 50% CO: 50% DRO and 5% CO: 95% DRO. These diets were consumed within a 2hr window in all groups. Circulating fatty acid and FAE levels were measured at 3, 6, 12 and 24hr within each group. At 3hr, significant differences. At 3hr, significant differences ($p<0.05$) in plasma oleoylethanolamide (OEA) levels were seen in the 95% CO group: 5% DRO group and 5% CO group: 95% DRO group as well as between 50% canola oil group: 50% DRO and 5% CO group: 95% DRO. In all dietary groups, palmitoylethanolamide (PEA) levels were not significantly different at 3, 6 and 24hr compared to 0hr, but did at 12hr where the 50% CO:50% DRO group showed significantly lower levels than seen in the 95% CO group, but PEA levels were not different from the 5% canola oil group. Linoleoylethanolamide (LEA) also failed to show any differences in plasma concentrations at all time points in the three dietary groups. Although plasma FAE levels were generally multiple times lower than observed in small intestine, liver or brain, arachidonoylethanolamide (AEA) levels were significantly lower in the 95% DRO group than in the remaining two groups. Plasma docosahexanoylethanolamide (DHEA) showed no difference across all time points except at 24hr where levels were higher ($p<0.05$) in the 95% DRO group than in the remaining two groups. No differences were seen in the small intestine across time points in any groups. In

liver at 3hr, OEA levels were higher ($p<0.05$) in the 95% CO group than the groups with lesser concentrations of oleic acid, while liver OEA levels showed no difference at any other time points across dietary groups. LEA levels were higher in 95% CO: 5% DRO group compared to the 5% CO group: 95% DRO group after 3hr of feeding. Liver DHEA levels were observed to be highest in the 5% CO group: 95% DRO group at 3 and 12, but not at 6 or 24hr. The dietary fatty acid composition affects plasma and organ fatty acid profiles in a time dependent manner and also produces time shifts in plasma and organ FAE levels. These dietary induced changes according to time points in the levels of FAEs may translate into discernible changes in energy expenditure and lipid levels which may in turn influence the risk of obesity.

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DEDICATION

I dedicate this thesis to God Almighty for giving me wisdom, understanding and most of all the strength to study and successfully complete this program.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
AEA	Arachidonoylethanolamide
ALA	α-linolenic acid
ANOVA	Analysis of variance
BF3	Boron trifluoride
BM	Body mass index
BW	Body weight
CO	Canola oil
D	Days
DFA	Dietary fatty acids
DHA	Docosahexaenoic acid
DHEA	Docosahexanoylethanolamide
DRO	DHA rich oil
EE	Energy expenditure
EPA	Eicosapentanoic acid
FAE	Fatty acid ethanolamide
FA	Fatty acid
FFM	Fat-free mass
FFA	Free fatty acids
GC	Gas chromatography
GC-FID	Gas chromatography-flame ionization detector
Hr	Hours
LA	Linoleic acid
LCFA	Long chain fatty acids

LEA	Linoleoylethanolamide
MUFA	Monounsaturated fatty acid
NAPE	N –acetylated phosphatidylethanolamine
NAPE	N –acetylated phosphatidylethanolamine- phospholipase D
NAT	N-acyl transferase
OA	Oleic acid
OEA	Oleoylethanolamide
PEA	Palmitoylethanolamide
PUFA	Polyunsaturated fatty acid
RBC	Red blood cell
SFA	Saturated fatty acid
TP	Time points
VLCFA	Very long chain fatty acids
WC	Waist circumference
WHO	World Health Organization

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CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Obesity, a condition characterized by excessive accumulation of body fat, has gradually become one of the most prominent and complex issues in the healthcare system in recent years. Obesity is also a leading preventable cause of death globally, especially with alarmingly increasing rates occurring in adults and children (1,2). More than 400 million adults worldwide are obese. About one billion more are considered overweight (3), making obesity a major contributor to the global burden of the country and the world at large, especially because it is predictive of the development of other chronic metabolic diseases ranging from cancer, hypertension, strokes, insulin resistance and type 2 diabetes (4–6). Obesity also poses some non-lethal health problems such as infertility, respiratory difficulties and chronic musculoskeletal problems (7).

In North America, a 24% increase in the prevalence of obesity occurred between 2009 and 2012 (8) with current estimates of 69% of adults falling into the overweight category and 35% classified as obese. According to a survey carried out by the Public Health Agency of Canada in 2012, between 1978 and 2004, a significant increase from 15% to 26% occurred in the number of obese and overweight Canadian children particularly among those aged 2 to17 years (9). A statement from the World Health Organization notes that between 2009 and 2011, about 32% of Canadian children aged between 5 and 17 years fell into the overweight (19.8%) or obese (11.7%) category (10). In 2011, research showed that 1 in 4 Canadian adults was obese and 8.6% of children and youth aged 6 to 17 were obese (11). An observation made in the trend of obesity in the last 20 years reveals a significant increase in obesity among United States populations (12) with more than one-third (34.9% or 78.6

million) of U.S. adults classified as obese. With these numbers, there exists an urgent need for development of different effective anti-obesity strategies to help curb this increase. In Canada, the latest data from Statistics Canada in 2008 show that 29% of all causes of deaths are attributed to obesity. Among all CVD caused deaths, 54% were due to coronary heart diseases (CHD), followed by heart failure (23%) and stroke (20%) (13). The causes of obesity are usually the presence of a combination of risk factors, including age, gender, diet, family history, inactivity, smoking and alcohol consumption. Research has linked wrong food choices to this increased prevalence of obesity (14,15). The major macronutrients that constitute our diets include carbohydrates, protein and fat, with fat significantly promoting the risk of obesity. Evidence shows that monounsaturated fatty acid (MUFA) rich diets induce greater diet-induced thermogenesis, energy expenditure, fat oxidation and weight loss compared to saturated fatty acid (SFA) diets (16). One possible explanation is that a derivative of OA termed oleoylethanolamide (OEA), an endogenous chemical signaling lipid, modulates peroxisome proliferator activated receptor (PPAR- α) activity to stimulate lipolysis, which leads to food intake reduction and weight loss (17). Dietary fatty acids (DFA) convert to these lipid mediators generally termed fatty acid ethanolamides (FAEs) which are a group of lipid signaling molecules that contain different acyl groups linked to the nitrogen atom of ethanolamine. Examples include arachidonoylethanolamide (AEA), OEA, linoleoylethanolamide (LEA), palmitoylethanolamide (PEA), and docosahexanoylethanolamide (DHEA). These fatty acid derivatives play a role in lipid signaling, fat oxidation and appetite regulation (18). Therefore, the aim of this research was to determine the impact of short term consumption of diets containing different concentrations of MUFA-rich canola oil and DHA rich algal oil on plasma and organ levels of FAE in male Sprague Dawley rats.

1.2 Fatty Acid Nomenclature

Fatty acids are one of the subgroups of lipids, and the properties of any specific lipid molecule are dependent on its fatty acid composition. Fatty acid classification is dependent on chain length, presence or absence of double bonds and the configuration of double bonds (19). These different properties affect the characteristics of fats and also influence the regulation of different aspects of metabolism, including fat oxidation (20,21). The carbon atom next to the carboxyl group is termed the alpha carbon, and the subsequent one the beta carbon. Saturated fatty acids have their carbon atoms saturated with hydrogen (22). Unsaturated fatty acids contain double bonds within the carbon chain. Fatty acids with only one double bond are monounsaturated (MUFA), while those with more than one double bond are polyunsaturated (PUFA). MUFAs and PUFAs are classified based on the location of their double bonds. The first double bond placed after the 3^{rd}, 6^{th} or 9^{th} carbon from the methyl group denotes the name n-3, n-6 or n-9 respectively with n-3 and n-6 being essential because the human body lacks the ability to synthesize these two fatty acids, hence the name essential fatty acids (23).

In unsaturated fatty acids, the orientation of the hydrogen atoms relative to the double bond can be cis (same side of the double bond) or trans (opposite side of the double bond). In terms of fatty acid chain length, short-chain fatty acids have between 4-6 carbons, medium-chain fatty acids have 6-12 carbons, the long-chain fatty acids such as DHA and EPA have 13-21 carbon atoms, and very long-chain fatty acids are 22 carbons or longer.

1.3 Obesity and Diet

Different animal and human trials have shown a link between dietary intake and obesity (24–26). Being overweight and obese has several negative impact; balancing energy intake and expenditure proves to be the most effective prevention method for obesity (27).

Also a large body evidence supports the relationship between high fat intake and elevated risk for obesity and other chronic diseases such as cardiovascular diseases and overall mortality (28,29). Of recent, controversies exist regarding the association between the quantity and quality of dietary fats and how they affect the progression of chronic diseases (25-27).

Current dietary guidelines suggest reductions in total intake of fat, trans fat and cholesterol to nothing more than 20 to 35 percent of calories (30,31). But fatty acid profile of diets is more important than total fat content for the prevention of obesity (32). The harmful effects of a high fat diet depend on the type of dietary fat consumed as the quality of our health is greatly determined by the quality of our food choices. It has been shown in several recent studies that the replacement of SFA with MUFA and PUFA can prevent up to 60% of cardiovascular events in North Americans whose major meals exceed the recommended amount of total fat intake which is less than 7% (33).

Western diets consumed by North Americans are richer in saturated fats than unsaturated fats; these diets tend to increase the risk of obesity, impair insulin sensitivity, and increase the risk of other cardiovascular related diseases (34). Also, foods high in SFA have a positive correlation with increased low density lipoprotein cholesterol (LDL-C) levels. But replacing SFA with MUFA can reduce cholesterol levels in the body, especially LDL-C levels (35). Canola oil which is one of the most widely consumed oils in North America is rich in MUFAs and has been proven to reduce plasma cholesterol levels (36) while n-6 fatty PUFA is largely found in safflower and corn oil, while fish oil is rich in n-3 PUFA such as DHA. However, no dietary recommendations exist for the optimal dietary amounts of MUFA and PUFA, including n-9, n-6, and short- and long-chain n-3 fatty acids. The most commonly used method for assessing the prevalence of obesity is the body mass index (BMI) (37), defined as the weight in kilograms divided by the square of the height in metres (kg/m^2) (38). Obesity is further classified as severe obesity (BMI 35-40 kg/m^2), morbid obesity ($\geq$35 kg/m^2

m^2), or >40-45 kg and super obesity (>50 kg/m^2) (39). The advantages of using BMI are that it is inexpensive and easy to calculate. BMI also correlates well with weight and is a good indicator of excess body fat and the risk of chronic diseases (38). Although MUFA and PUFA intake controls circulating fatty acid levels, there are still some significant knowledge gaps in understanding the effects of various fatty acid classes on risk factors for chronic diseases such as obesity. In addition, a need exists to study more closely the relationship between oleic acid and DHA in health promotion. A human trial recently conducted showed that the addition of DHA to an oleic acid rich diet represses the production of OEA to favor the production of DHEA. It is therefore important to investigate the inverse relationship observed between these two fatty acids.

1.4 Saturated Fats

Dietary saturated fats commonly termed 'the bad fats', have long been associated with cardiovascular diseases and other metabolic disorders and all-cause mortality (40). These fats are usually solid and more stable at room temperature, a physical property that makes them less prone to spoilage or oxidation compared to liquid oils. Saturated fats also do not have double bonds in between their carbon chains (41). Excessive consumption of saturated fats can increase circulating LDL levels, regarded as the bad cholesterol, thereby creating a link with atherosclerosis and heart diseases as higher levels of blood cholesterol increase the risk of heart disease (42). Foods rich in SFA include refined carbohydrates, pastries, dairy products such as egg yolk, butter, whole milk, cheese, cream, meat produce such as lard, sausages, hamburgers, tallow, pork, lamb, oils such as coconut and palm oil (43). The recommended daily intake of SFA, according to the Dietary Guidelines for North Americans, suggests not more than 10% of total energy intake of calories from SFA with the remaining 20% of recommended daily fat intake being replaced with MUFA and PUFA (44).

1.5 Monounsaturated Fats

Monounsaturated fatty acids, also known as MUFA have a single double bond between their carbon chains. Unlike saturated fats which are termed the bad fats, MUFA as a result of their already established health benefits are referred to as good or healthy fats (45). MUFA, found in plants and animal products, are liquid at room temperature and more prone to oxidation than are saturated fats (46). In 2000, the American Heart Association suggests that MUFA intake should make up as much as 15% of total energy content (47). Average MUFA intake in U.S. adults is 13% to 14% of total energy while SFA intake is in excess at 11-12% of energy. Current dietary guidelines based on consistent evidence from clinical trials suggest replacing 5% of saturated fats with monounsaturated fats can help reduce the risk of obesity by up to 20%-40% (48). This recommendation is based on findings from animal and clinical trials whose data show an inverse association between monounsaturated fat intake and LDL cholesterol levels, weight control, insulin sensitivity, blood triglycerides and increased HDL-C levels in the body (49,50). Oleic acid is the most abundant monounsaturated fatty acid and also the most important representative of MUFA in diet. Adherence to a traditional Mediterranean diet, characterized by its high MUFA content as a result of its dietary fat source and olive oil, the most abundant source of oleic acid, has been greatly associated with reduced risk of CVD events such as obesity (51). A 12-month randomized control trial showed that the Mediterranean diet effectively helped obese and overweight subjects lose weight (52). Data showed an inverse relationship in obesity between people consuming a Mediterranean diet and those consuming a Western diet (52). Canola oil which is the second most abundant source of oleic acid (>61%) in North America has also been shown in different randomized control trials to have cardio protective properties by increasing HDL-C and reducing LDL-C levels (36,53,54). Major dietary sources of MUFA include olive oil, canola oils, nuts and avocados. In a weight maintenance study, a 4wk

controlled feeding of high MUFA diet tended to favorably shift adipose tissue from the android to gynoid region compared to an ALA rich diet. A review by Gillingham et al. 2011 provided a critical assessment of the studies surrounding efficacy of dietary MUFA for reduction of obesity. This review suggested that the metabolism and preferential oxidation of dietary MUFA can play a role in influencing body composition and decrease the risk of obesity (55).

1.6 Polyunsaturated Fats

One major characteristic unique to the PUFA is the presence of 2 or more double bonds on their carbon chain, hence the name 'Poly'. Foods high in PUFA are liquid at room temperature. Long chain PUFAs have chain lengths between 18–20 carbons or more and can be divided into n-3, n-6 and n-7 PUFA depending on the location of the first double bond from the methyl group. The most studied and recognized are the n-3 and n-6 DFA (56). The human body lacks the delta 12 and delta 15 desaturase enzymes needed for PUFA synthesis and therefore is not capable of synthesizing PUFA (57), which implies that these fatty acids should be obtained from the diet to avoid deficiency, hence the name 'essential fatty acid' (58). Major dietary sources of PUFA include leafy vegetables, corn oil, safflower oil, linseed soya bean, sunflower oil, cottonseed oil, walnuts (59). Eating foods high in PUFA as replacements for saturated fat rich diet can help lower blood-cholesterol thereby lowering the risk of heart disease. It can also help to reduce the risk of diabetes (60,61). Data from clinical trials found that eating 13% to 21% energy from PUFA decreased total plasma cholesterol by 13% to 15%, and CHD events by 25% to 43% (62). Recently, it was suggested by the American and European Heart Associations to have sufficient intake of about 1g/dy of EPA and DHA n-3 PUFAs for preventing cardiovascular events (62).

1.6.1 N-6 PUFA

Linoleic acid (LA), an essential fatty acid that belongs to the PUFA group, exists as the parent fatty acid of the n-6 family and serves as a precursor for other fatty acid metabolites such as arachidonic acid (AA), dihomo-λ-linolenic acid (DGLA), leukotrienes (LT), thromboxane (TXA2) and prostaglandins (PGE2) (63). Linoleic acid accounts for 84%-90% of n-6 PUFA intake (64). The conversion from LA to AA is catalyzed by the enzyme Δ6 desaturase enzyme which is also responsible for the conversion of n-3 ALA to EPA (65,66) . Since n-6 and n-9 fatty acids compete for the same conversion enzyme, there is a possibility of deficiencies in one of these essential fatty acids. n-6 PUFA deficiency is very rare as it is the predominant class of fatty acid found in diet mainly consumed by Americans (67). A 5% replacement of SFA with omega-6 PUFA has been shown to reduce cardiovascular disease risk by 13% (68). Also, data from a randomized control trial conducted in 2007 report that replacement of SFA with n-6 PUFA in women/men lowered total and LDL-C levels (35). The Food and Nutrition U.S. Institute of Medicine Board suggests a daily intake of 17g (male) and 12g (female) of n-6 PUFAs while the World Health Organization recommends 4% of total energy for optimum health (69).

1.6.2 N-3 PUFA

Major types of n-3 fatty acids include plant derived ALA, (C18:3n-3) and marine derived EPA, DPA, and DHA (70). Docosapentaenoic acid (DPA) is a n-3 PUFA formed in a process of elongation and desaturation of EPA (71). EPA and DHA are the primary n-3 fatty acids with proven benefits and they are derivatives of ALA which has little or no evidence for improving cardiovascular health compared to the former. Data from epidemiological, observational studies and clinical controlled trials show the beneficial effects of n-3 PUFA from fish on CHD risk (72,73). Various human and animal trials have shown positive roles for n-3 fatty acids in infant development, including cancer, cardiovascular diseases and more

recently in depression, attention-deficit hyperactivity disorder and dementia (74–76). Since discovery of the beneficial properties of DHA, this FA has been used as a supplement in different food products. DHA and AA are essential components of the brain and retina (59). Although ALA is the precursor of EPA, the conversion of ALA in humans is extremely variable, ranging from 0.2 to 21% for EPA and 0 to 9% for DHA (66,77).

1.7 Fatty Acid Ethanolamides

Fatty acid ethanolamides (FAEs), are a group of lipid signaling molecules that contain several types of acyl groups linked to the nitrogen atom of ethanolamine (78). FAEs are formed in vivo from N–acetylated phosphatidylethanolamine (NAPE) derivatives, where phosphatidylcholine and ethanolamine undergo transesterification by the action of N-acyltransferase (NAT). N-acylphosphatidylethanolamine-phospholipase D (NAPE-PLD) cleaves to NAPE to yield FAE and ethanolamine. Degradation of FAEs is carried out by the action of two enzymes named N-acylethanolamine-hydrolyzing acid amidase (NAAA) and fatty acid amide hydrolase (FAAH) (79). These regulators, widely distributed in plants and animal tissues, have many biological and physiological roles that range from lipid signaling and regulation of food intake to energy metabolism (80). Levels of FAEs seem to be dependent on the concentration of their fatty acid precursors (81). For example, oleic acid is the precursor of OEA, while arachidonic acid is the precursor of AEA also known as anandamide, palmitic acid is for PEA, linoleic acid is the precursor for LEA, stearoylethanolamide (SEA) from stearic acid, while DHA is the precursor for DHEA.

1.7.1 Arachidonoylethanolamide (AEA)

Anandamide also known as arachidonoylethanolamide (AEA**)** was the first discovered FAE and is widely distributed in the brain and neurons (82). This arachidonic acid derivative has multiple pharmacological properties which include appetite, inflammatory process and

cell proliferation (82,83). These actions are carried out by its binding to the cannabinoid receptors (CB1 and CB2). These receptors alongside the G-protein-coupled receptors makes up the endocannabinoid system (84). The endocannabinoid system (ECS) regulates various physiological activities in the body such as inflammation, hunger, pain modulation, energy balance and mood (85). It was recently discovered that the activation of the ECS shifts energy balance toward energy storage. A reduction in food intake was seen in food-deprived lean CB1 receptor knock out animals and in *ad lib*-fed obese animals (83,86,87). AA serves as a substrate for the synthesis of anandamide and other endocannabinoids (88).

1.7.2 Oleoylethanolamide (OEA)

Oleoylethanolamide is a derivative of the 18-carbon oleic acid and also the most abundant FAE found in biological tissues, including the intestine and brain (79). Aside from the small intestines, this FAE can also be found in small quantities (about 2µg) in some edible food products such as oatmeal, nuts and cocoa. OEA has a similar structure to AEA but biologically different in function (89). OEA has attracted attention as a lipid mediator involved in peripheral appetite regulation, body weight and lipid metabolism (90). OEA stimulates hepatic lipolysis, decreases body weight gain, and lowers hepatic and adipose tissue hyperlipidemia in obese rats (91).

Oleoylethanolamide acts opposite to anandamide because in contrast to anandamide that increases appetite, OEA suppresses appetite. Previous studies have shown that intestinal OEA levels decrease during starvation and both intraperitoneal injection and oral administration of OEA decrease food intake in rodents (81,91,92). Unlike AEA which carries out its actions by activating the cannabinoid receptors, OEA regulates weight gain and feeding by activating peroxisome proliferator-activated receptor alpha (PPAR-α) (91,93), a ligand-activated transcription factor that regulates several pathways of lipid metabolism. The

PPAR-α is a nuclear receptor involved in the control of metabolism. This means that when OEA binds to PPAR-α, energy expenditure increases.

1.7.3 Palmitoylethanolamide (PEA)

This FAE is another family of the N-acylethanolamides with very similar structure to AEA but with a weaker affinity to the cannabinoid receptors. PEA has the ability to act as an analgesic, anticonvulsant and it also inhibits inflammation (93,94).

1.7.4 Docosahexanoylethanolamide

Docosahexaenoylethanolamide (DHEA), an n-3 PUFA ethanolamide, is found in abundance in brain and retina. DHEA is the derivative ethanolamine of DHA. DHEA was found to be about 9.5 times higher in pig brains fed a diet supplemented with DHA compared to the groups fed a diet without DHA. Like AEA, DHEA weakly activates the cannabinoid receptor, CB_1 located in brain (95). Previous studies show that OEA levels were significantly lower in canola diet rich in DHA compared to a high-OA canola oil diet despite their similar OA content. This implies that the elevation of DHEA in response to the dietary DHA interferes with the function of OEA.

1.8 Study Rationale

Examining the health benefits of the combination of different concentrations of n-3 and n-9 fatty acids is important in strengthening our understanding of the role of these specific DFA in human health and disease prevention. Since previous studies have focused on long term dietary fat feeding, this present research therefore investigated the effect of short term feeding of various ratios of n-9 MUFAs and n-3 PUFAs created from consumption of diets rich in different concentrations of canola oil versus DHA rich algal oils, in contrast to diets rich in n-6 fatty acids. Findings from previous studies show that the addition of DHA to an oleic acid rich diet blunts the effects and benefits of an n-9 rich diet, while n-6 rich oil was

chosen as the control over a saturated fat rich oil since the effect of saturated fat has been established in previous studies. Thus, the overarching goal of this thesis is to elucidate how short dietary fat feeding affects FAE levels in a rat model by mainly focusing on the n-9 and n-3 fatty acids. Animals used for this study were trained to eat within a 2hr window to ensure that an adequate amount of fatty acids, enough for conversion to fatty acid ethanolamides, was consumed by each animal.

1.8.1 Hypothesis

The study hypothesized that fatty acid ethanolamide levels in plasma, small intestine, liver and brain will not increase within 3hr and 6hr but will possibly increase within 12hr and 24hr of consumption of dietary fat in a manner that is affected by the type of fat consumed.

1.9 Objectives of the Research

The overall aim of the study is to examine the effect of a single meal fat feeding with varying concentrations of n-3 and n-9 fatty acids on short term changes in fatty acid and FAE levels in a rat model.

The specific objectives of the research are to:

a) determine the effect of different ratios of dietary oils given as a single meal on fatty acid concentrations in the plasma, small intestine, liver and brain; and
b) examine the effect of different ratios of dietary oils given as a single meal on plasma, small intestine, liver and brain FAE levels in an acute condition over a period of 3, 6, 12 and 24hr.

BRIDGE TO CHAPTER 2

The next chapter contains a manuscript which focuses on the relationship between dietary fatty acids and plasma and organ fatty acids. Most importantly, the paper focuses on the elevation of plasma and organ fatty acids within a short time frame. This chapter serves as the background for the conversion of fatty acids to lipid mediators called fatty acid ethanolamides.

CHAPTER 2

Time dependent effects of different concentrations of n-3 and n-9 fatty acids on plasma, liver, small intestine and brain fatty acid levels in rats

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Running Head: Acute dietary fat feeding on fatty acid levels

Abstract

Because circulating levels of plasma fatty acids associate with disease risk, current dietary guidelines suggests replacement of saturated fats with other classes of fatty acids such as n-9 monounsaturated (MUFA) and n-3 polyunsaturated fatty acids (PUFA). However, significant knowledge gaps remain in understanding how fatty acid metabolism occurs across these various classes of fats in terms of timelines of relative absorption rates of FA into the circulating compartment. The main objective of this study was therefore to examine effects of consuming meals varying in concentrations of n-9 and n-3 fatty acids on short-term changes in plasma and organ fatty acid levels. Sixty-four male Sprague Dawley rats were randomly

assigned into four dietary groups. Animals were fed diets containing 40% energy given as either safflower oil that was identified as a control oil, or canola oil together with docosahexaenoic acid (DHA) rich algae oil (DRO) at ratios of 95% CO: 5% DRO, 50% CO: 50% DRO, and 5% CO: 95% DRO. Rats were trained for 2wk to consume their entire daily calories as a single treatment meal within a 2hr window, after which sub-groups of animals were sacrificed at 3, 6, 12 and 24hr. Blood, small intestine, liver and brain samples were collected at all time points. Body weight and food intake failed to show any significant differences after feeding the experimental diets. In plasma and small intestine, uptake of fatty acid began 3hr after ingestion of the meal, while in the liver, n-9 and n-3 fatty acid levels were observed to be the highest at 24hr and 12hr time points, respectively, in all treatment groups. In contrast, brain fatty acid levels at all time points in all treatment groups failed to show any differences from the control group. These data suggest that the introduction of n-9 and n-3 dietary fatty acids into tissue and plasma fatty acids begins almost immediately after ingestion and exhibits FA specific trajectories in the Sprague Dawley rat model.

Keywords

n-9 fatty acids, n-3 fatty acids, canola oil, DHA rich oil

Background

The fatty acid composition of dietary oils varies greatly. This notable difference in composition is believed to substantially influence health (96,97), implying that the harmful effects of a high fat diet are dependent on the type of dietary fat consumed, not the total fat consumed (69,98–100). For example, results from previous clinical trials have shown that consumption of n-9 and n-3 classes of fatty acids promotes overall health by reducing the risk of heart disease, especially when replacing saturated fats (24,34,101,102). Numerous clinical controlled trials and animal studies have been conducted to explain how different types of

fatty acids modulate plasma and organ fatty acid concentrations (103–105). However, only a few of these trials have acutely looked at the uptake rate of these fatty acids into different organs (6). The Dietary Guidelines for Americans recommend that the daily intake of saturated fatty acids (SFA) should not exceed 10% of total energy intake, with the remaining 20% of recommended daily fat intake being replaced with MUFA and PUFA (30,102).

Although considerable literature has investigated the health benefits and effects of various fatty acid classes, limited studies have thoroughly evaluated the time course of uptake and interconversion of dietary fatty acids into plasma and organ compartments. In addition, better knowledge is needed in understanding the effects of various fatty acids, especially the relationship between oleic acid and DHA. Results from a human trial conducted by Pu et al showed that the addition of DHA to an oleic acid rich meal suppressed circulating levels of OEA (106). The overall goal of this study was, therefore, to examine effects of meals varying in concentrations of n-3 and n-9 fatty acids on acute shifts in plasma, liver and small intestine FA levels in a rat model.

Materials and Methods

This study was conducted at the animal facility of the Richardson Centre for Functional Foods and Nutraceuticals. The protocol was reviewed by the Animal Ethics Board of the University of Manitoba and conducted under the guidelines of the Canadian Animal Care act.

Animals and Diets

Sixty-four male Sprague Dawley rats weighing between 200 and 250g, purchased from Charles River Laboratories, were housed in individual plastic cages with lighting provided on a 12hr light, 12hr dark cycle in a temperature controlled room (25 degrees celcius). All rats were allowed free access to typical rat chow and water for the first 5 d

following their arrival. After acclimatization, rats were randomly assigned into 4 different dietary groups, with the first group of 4 animals serving as control and 3 treatment groups having 20 rats each. Rats were systematically randomized into groups of almost identical mean body weights. Rats were fed a nutritionally adequate diet that contained 100% safflower oil (Jedwards Int., United States) as the oil source for 2wk during which they were trained to consume their food within a single daily 2hr meal window. The restricted window feeding was achieved by gradually restricting the animal's food intake first to an 8hr, then a 4hr and finally to a 2hr window from 7am-9am followed by a 22hr fast. On the 14th day of the 2hr food training, the diet of the 3 treatment groups was changed to a single meal containing different ratios of MUFA-rich canola oil and a DHA rich algal oil. Treatment oils were safflower oil (SO), or three canola oil/DHA rich algae oil blends given in a ratio of either 95% CO to 5% DRO (group 1), 50% CO to 50% DRO (group 2) or 5% CO to 95% DRO (group 3). Canola and DHA oils were purchased from local stores in Canada and DSM, United States, respectively. Experimental diets contained 39.9%, 17.8% and 42.3% energy as fat, protein, and carbohydrate, respectively. Oils were weighed to the nearest g and added to other ingredients. Four rats in the control group were sacrificed at time point zero (0) to serve as a baseline measurement. After 3, 6, 12 and 24hr of feeding, five rats from each of the three dietary treatment groups were sacrificed simultaneously to amount to a total of 15 rats per time point.

Sample Collection and Analysis

At the end of the feeding period, rats were euthanized by decapitation and blood samples were collected into anticoagulated heparin bottles, centrifuged and stored at -80 ºC for fatty acid analyses. Small intestine, brain, and liver were removed, rinsed in saline and stored for additional analyses.

Fatty Acid Extraction and Methylation

Fatty acids were extracted from plasma, small intestine, brain, and liver using a direct one step fatty acid methylation procedure with heptadecanoic acid (C17:0) added as internal standard (109). The composition of fatty acid methyl esters of dietary oils, plasma and tissues were determined using a gas-liquid chromatography equipped with a 30 m x 0.25 mm, internal diameter 0.25um column (Agilent, CA) and flame ionization detectors (FID). Fatty acid methyl esters were identified by comparing their retention times with authenticated standards. All fatty acid levels were calculated as percentage values of total identified fatty acid from the GC-FID measures.

Statistical Analysis

Results were analyzed using the SPSS version 9.4. Effects of treatment at different time points across groups were compared using one-way ANOVA method. A post hoc analysis using the Tukey's method was used to test the present hypotheses. Differences in results were deemed to be statistically significant at $p<0.05$.

Results and Discussion

Food Intake and Body Weight

After 2wk of consuming the background diets, no significant differences were observed in body weight or food intake across groups. All animals were healthy and none reacted adversely to the diets throughout the feeding period. Animals also adapted well to the 2hr food restriction. **(Figure 2 and 3; Food Intake and Body Weight)**

Fatty Acid Profiles

The fatty acid time course profiles in plasma, small intestine, liver and brain are summarized in **Table 2** and **Figure**s **4-11**. Time dependent changes in the concentrations of

certain specific plasma, small intestine, liver and brain fatty acids were observed, reflecting dietary intakes after feeding the single meal to the rats. According to the dietary groups, n-9 levels in plasma were higher ($p<0.05$) in the 95% CO group than the other two dietary groups after 3hr of consuming the meal. No differences across the groups were seen at 6hr, but were seen at 12hr and 24hr where the 95% CO had higher ($p<0.05$) n-9 levels than the 50% CO, 5% CO group in plasma **(Figure 4)**. At 3hr, n-3 levels in plasma showed a statistically significant difference among all groups while at 6hr, the 95% CO group differed ($p<0.05$) from the other two groups. At 12hr, plasma n-3 levels in all groups differed ($p<0.05$) from each other. At 24hr, the 5% CO group differed in n-3 FA levels from the 95% CO: 5% DRO and 50% CO: 50% DRO groups **(Figure 5).** In small intestine, n-9 levels between groups showed no difference at all time points while n-3 levels at all time points differed from each other. At 3hr, the 95% canola oil group and 50% canola oil groups did not differ from each other but differed from the 5% CO: 95% CO group **(Figure 6-7)**. Overall time points examined, n-3 and n-9 DFA levels showed no significant differences in liver and brain between groups **(Figure 8-11)**.

According to the dietary groups, n-9 levels in plasma were higher ($p<0.05$) in the 95% canola oil group than the other two dietary groups after 3hr of consuming the meal. No differences across the groups were seen at 6hr but difference was seen at 12hr and 24hr where the 95% CO had higher ($p<0.05$) n-9 levels than the 50% CO: 50% DRO and 5% CO: 95% DRO group in plasma. At 3hr, n-3 levels in plasma showed a statistically significant difference among all groups while at 6hr, the 95% CO group differed ($p<0.05$) from the remaining two groups. At 12hr, all groups differed ($p<0.05$) from each other. At 24hr, the 5% CO: 95% DRO group differed from the 95% CO and 50% CO: 50% DRO groups.

In small intestine, n-9 levels groups showed no difference at all time points while n-3 levels at 3hr, 6hr 12hr and 24hr differed from each other. But at 3hr, 95% CO: 5% DRO

group and 50% CO: 50% DRO groups did not differ from each other but differed from the 5% CO: 95% DRO group. However, n-3 and n-9 FA levels showed no significant differences in liver and brain between groups at all time points.

Discussion

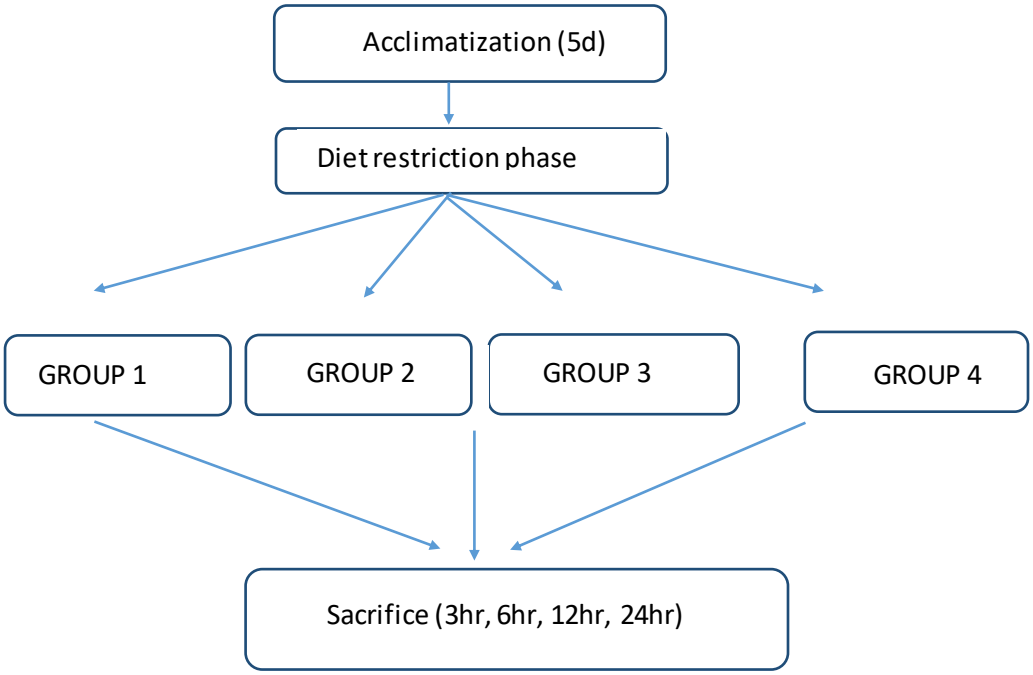
The major finding of this study was the rapid deployment of intestinally derived fatty acids to peripheral tissues swiftly following ingestion of a single meal. Although many studies have shown that levels of fatty acids in plasma and tissue reflect the dietary fatty acid consumption, only very limited data are available looking at the time course of fatty acid change immediately after ingestion of a single meal (18,107). The elevation of plasma and tissue fatty acids seen 3hr after consuming the experimental meal was decreased back to baseline after the 22hr fast. However, the initial elevation seen suggests swift incorporation of the dietary fat into plasma, as seen in a study by Stulnig et al where elevation of plasma free fatty acid levels was seen in mouse after a 3hr feeding (108). However, the present work contradicts results from a study done by Reeves et al where no major significant differences were seen in plasma fatty acid levels after a one-year dietary feeding in healthy individuals (109). The rapid uptake in plasma seen in our study could have been due to the effect of the enzyme lipase which breaks down dietary fat into fatty acids and glycerol. BuEko et al (110) noted an increase in lipase levels in rats fed a high n-6 FA-rich oil for 28 d. Also Deschodt-Lanckman et al showed an increase in lipase levels in rats when they were changed from a low fat diet to a high fat diet (111). Our inability to see any increase in n-3 levels in plasma, small intestine, liver and brain, especially in animals fed the 95% CO and the intermediate diet, could be a result of the competition between ALA and LA for the desaturase enzyme which converts them to EPA, DHA and AA, respectively (112–116). Also, the increase in n-3 levels seen in small intestine and plasma levels at 95% DRO diet implies that an increased

absorption of n-3 FA occurs in the small intestine only when a substantial amount is fed. It could also be that the 2hr feeding of DHA was too short to produce significant effects. Different studies have shown that the time it takes for fatty acids to be taken up into the liver is dependent on the type of dietary fat from which the triacylglycerol are derived. Lambert et al showed that olive oil, which is similar to canola oil in its n-9 fatty acid content, is more slowly taken up by the liver, which could be why presently slower fatty acid uptake was observed in this organ (117–119). Our inability to see differences in brain FA levels is consistent with works done by Xiao et al where a 1wk feeding of DHA to mice failed to result in any significant increase in brain DHA levels (120). Most studies that showed effects of DHA supplementation on brain DHA fatty acids levels were long term compared to our single 2hr feeding design. A faster uptake seen in n-9 levels in plasma and small intestine establishes the fact that when dietary fat is ingested, it transits from small intestine to plasma before the remnants are taken up by the liver where an increase was not seen until after 12hr (121). A substantial dose dependent increase in DHA levels occurred in plasma, small intestine and brain after DHA supplementation and therefore decreased the levels of OA which is in line with other reports (122,123).

Conclusion

In conclusion, the present study contributes to the limited research that exists detailing the time course of uptake of fatty acid levels in plasma, small intestine, liver, and brain after a single meal containing different concentrations of n-9 and n-3 fatty acids. This acute animal trial revealed that the uptake of some major fatty acids, most especially the MUFAs, into plasma, small intestine and liver occurs almost immediately after consumption of just a single meal, establishing that dietary fatty acids have a swift definitive effect on plasma and organ fatty acid concentrations which could consequently impact the risk of chronic diseases and overall health.

Figure 2-1: Study Design



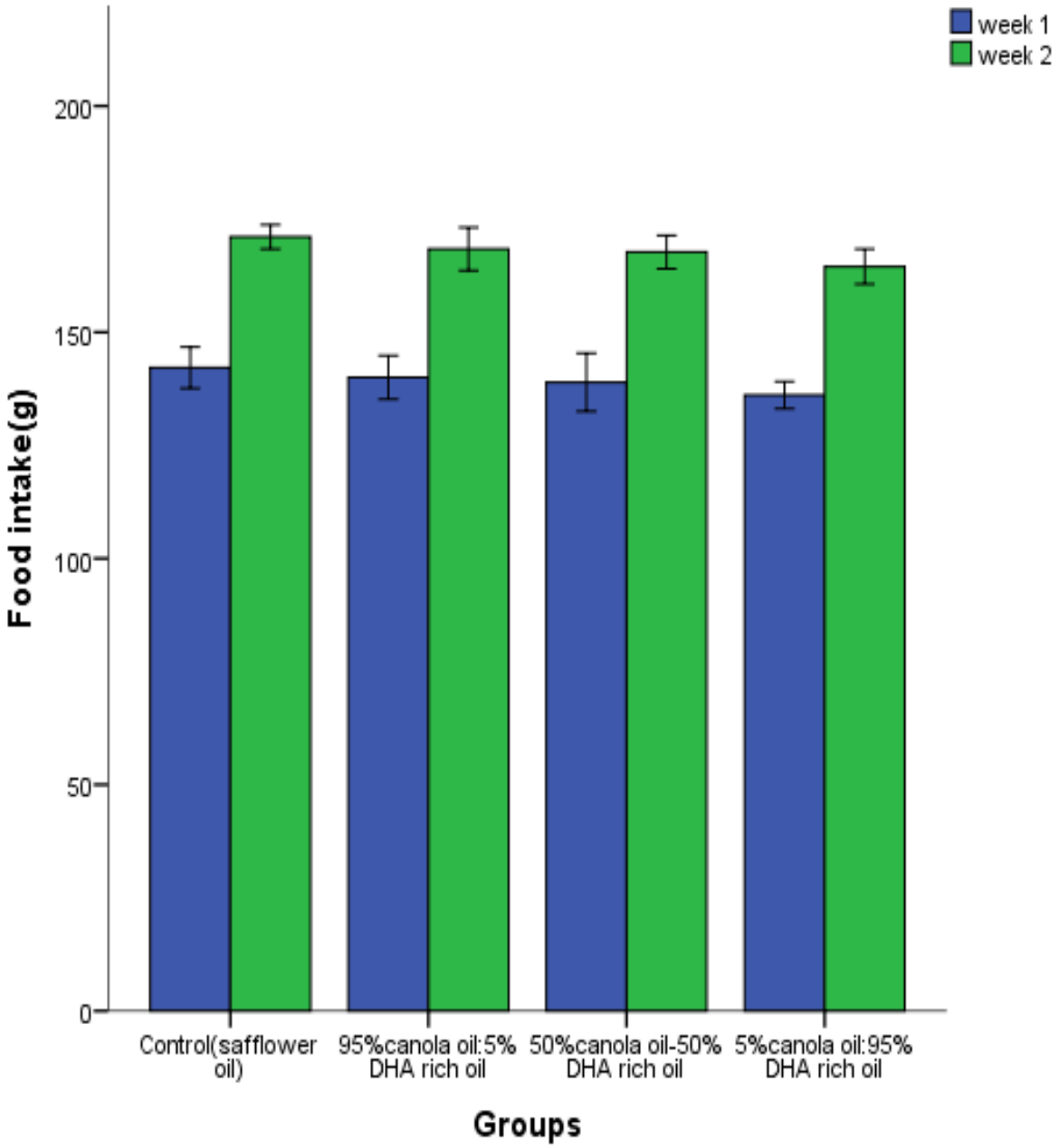


Figure 2-2: Total food intake of rats after 2 wks of feeding n=20

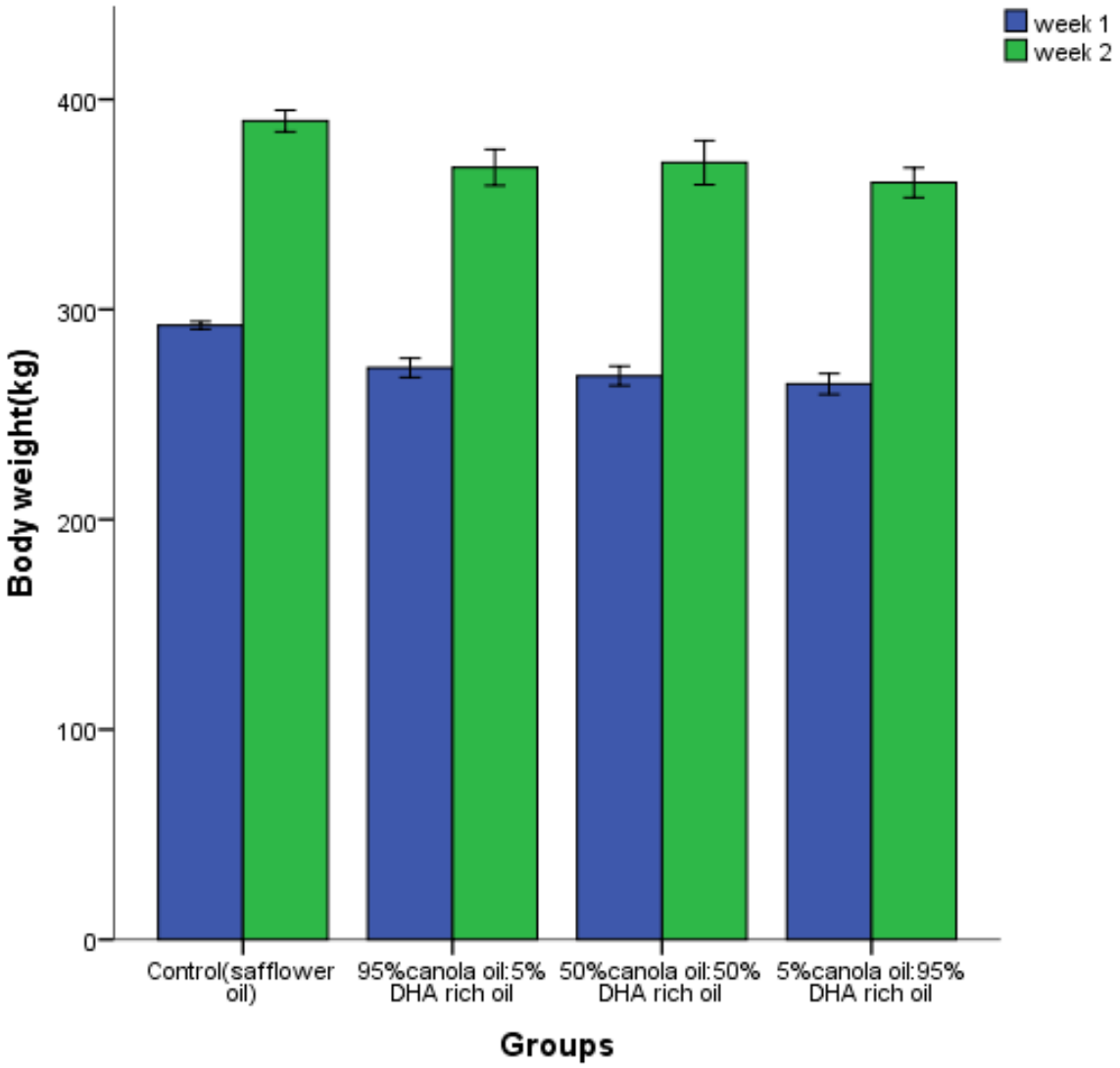


Figure 2-3: Body weight of rats after 2 wks of feeding n=20

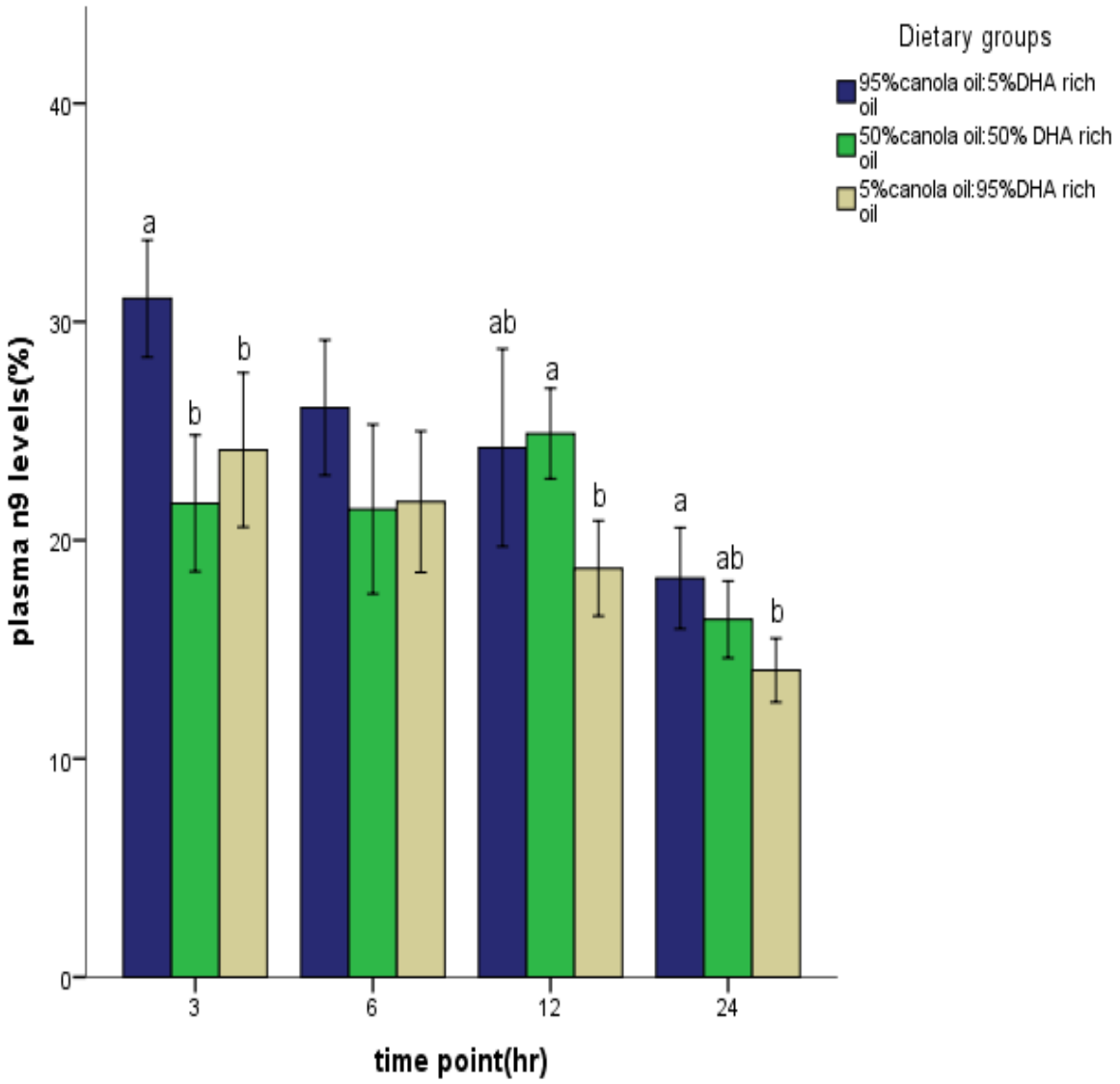


Figure 2-4: Comparison between n-9 plasma fatty acid levels across all dietary groups at each time point. All data are expressed as mean ± SEM (n=5). Values with different superscripts were significantly different at $p \leq 0.05$

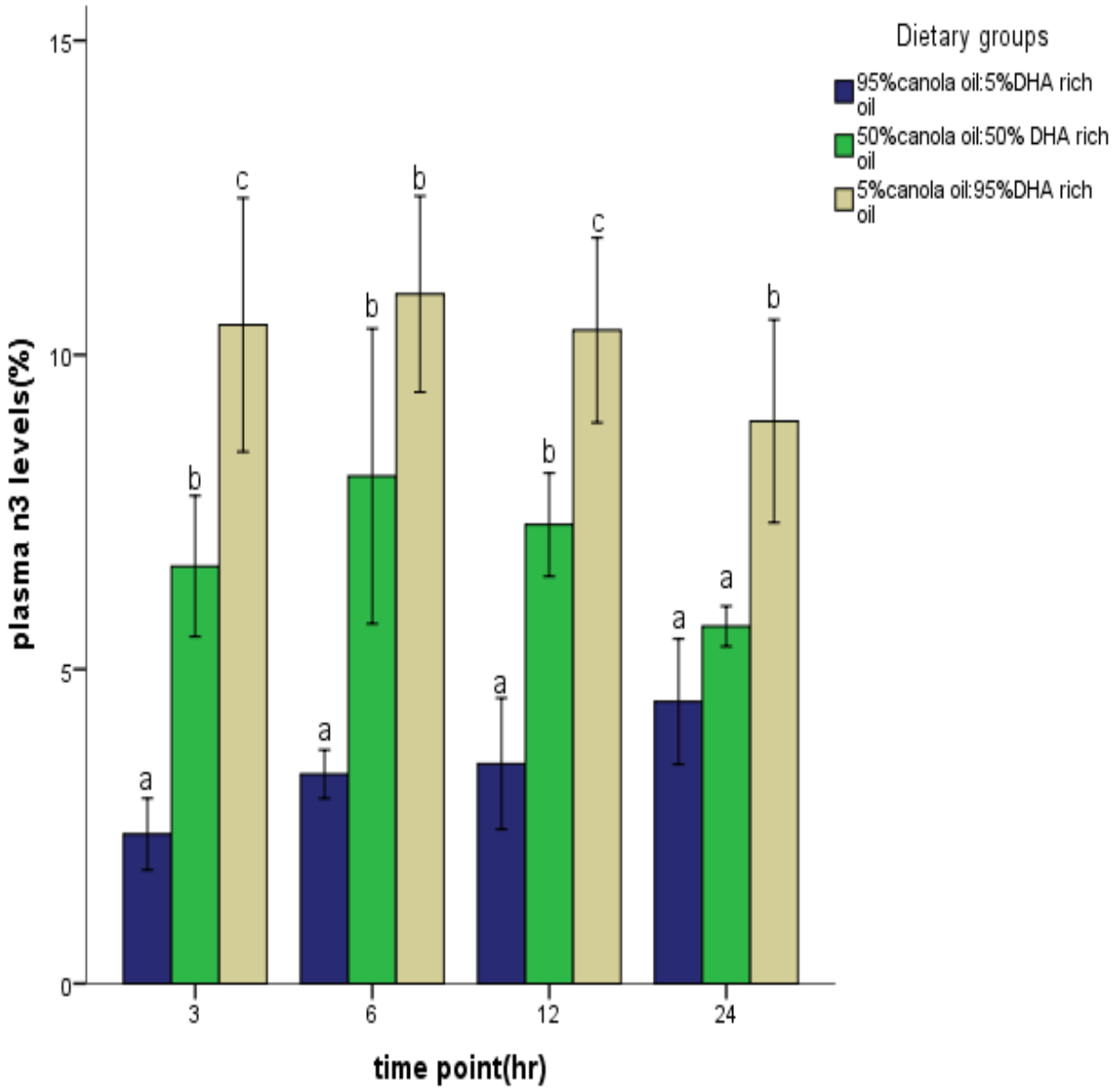


Figure 2-5: Comparison between n-3 plasma fatty acid levels across all dietary groups at each time point. All data are expressed as mean ± SEM (n=5). Values with different superscripts were significantly different at $p \leq 0.05$

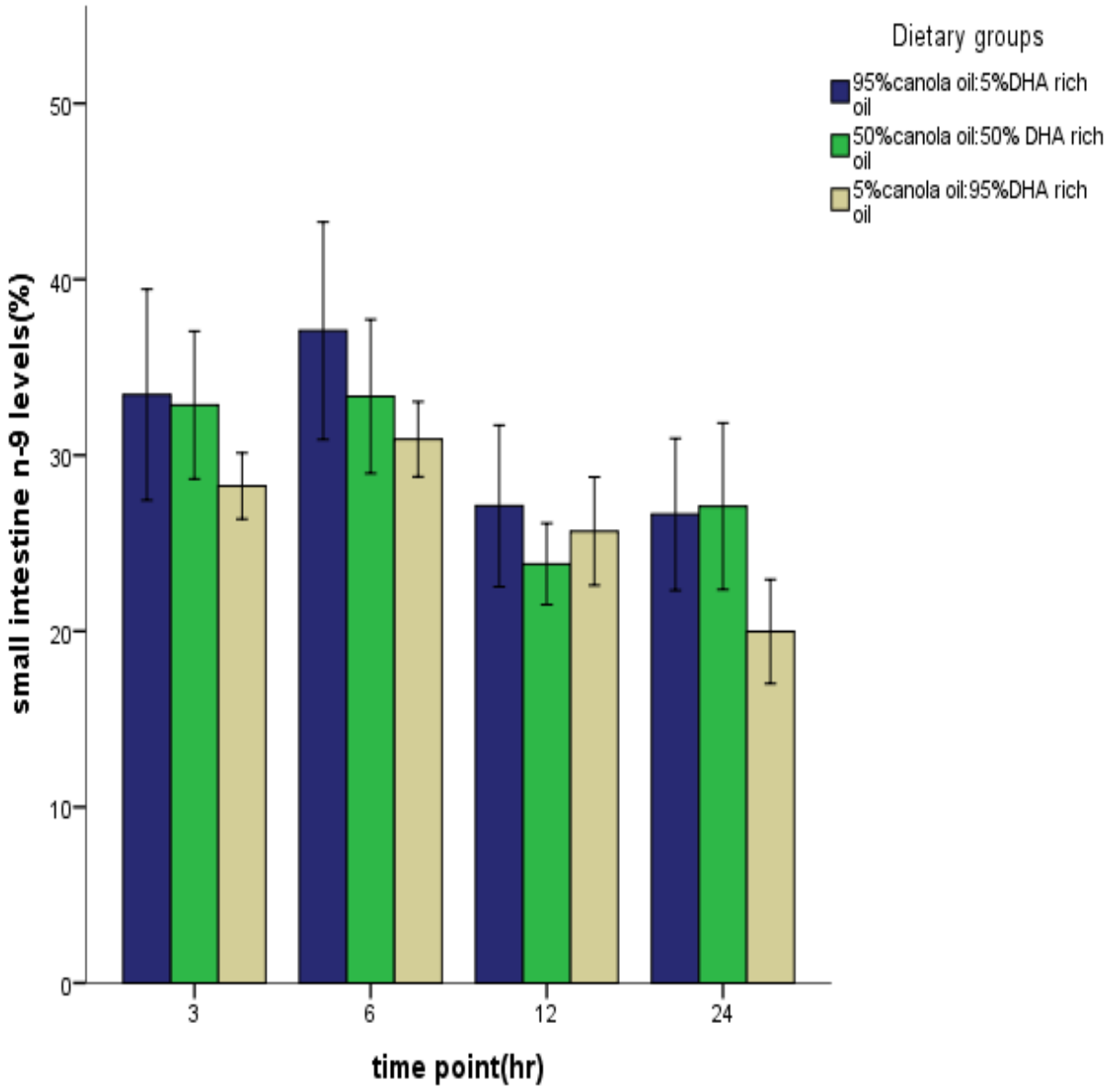


Figure 2-6: Comparison between n-9 small intestine fatty acid levels across all dietary groups at each time point. All data are expressed as mean ± SEM (n=5). Values with different superscripts were significantly different at $p \leq 0.05$

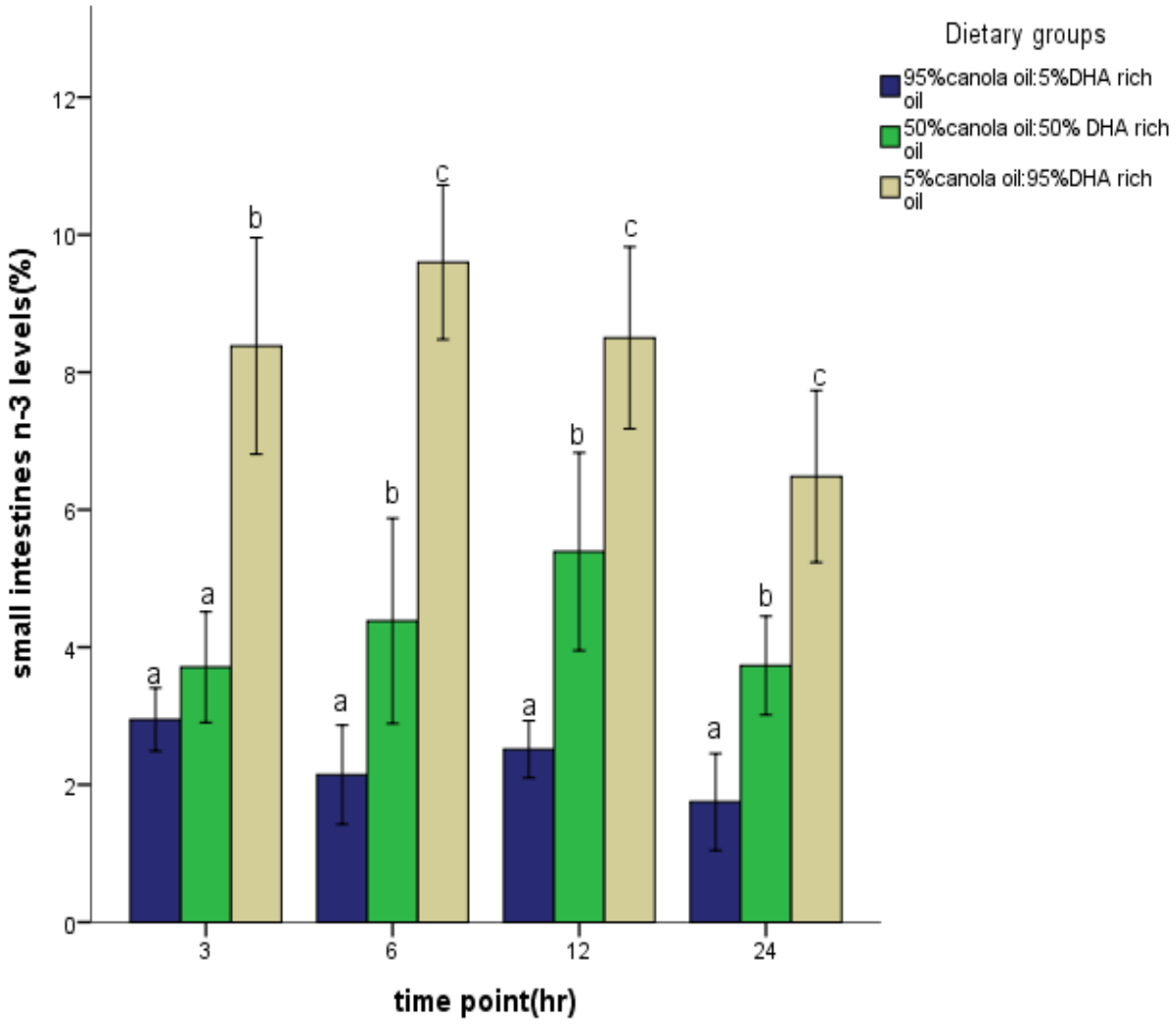


Figure 2-7: Comparison between n-3 small intestine fatty acid levels across all dietary groups at each time point. All data are expressed as mean ± SEM (n=5). Values with different superscripts were significantly different at $p \leq 0.05$

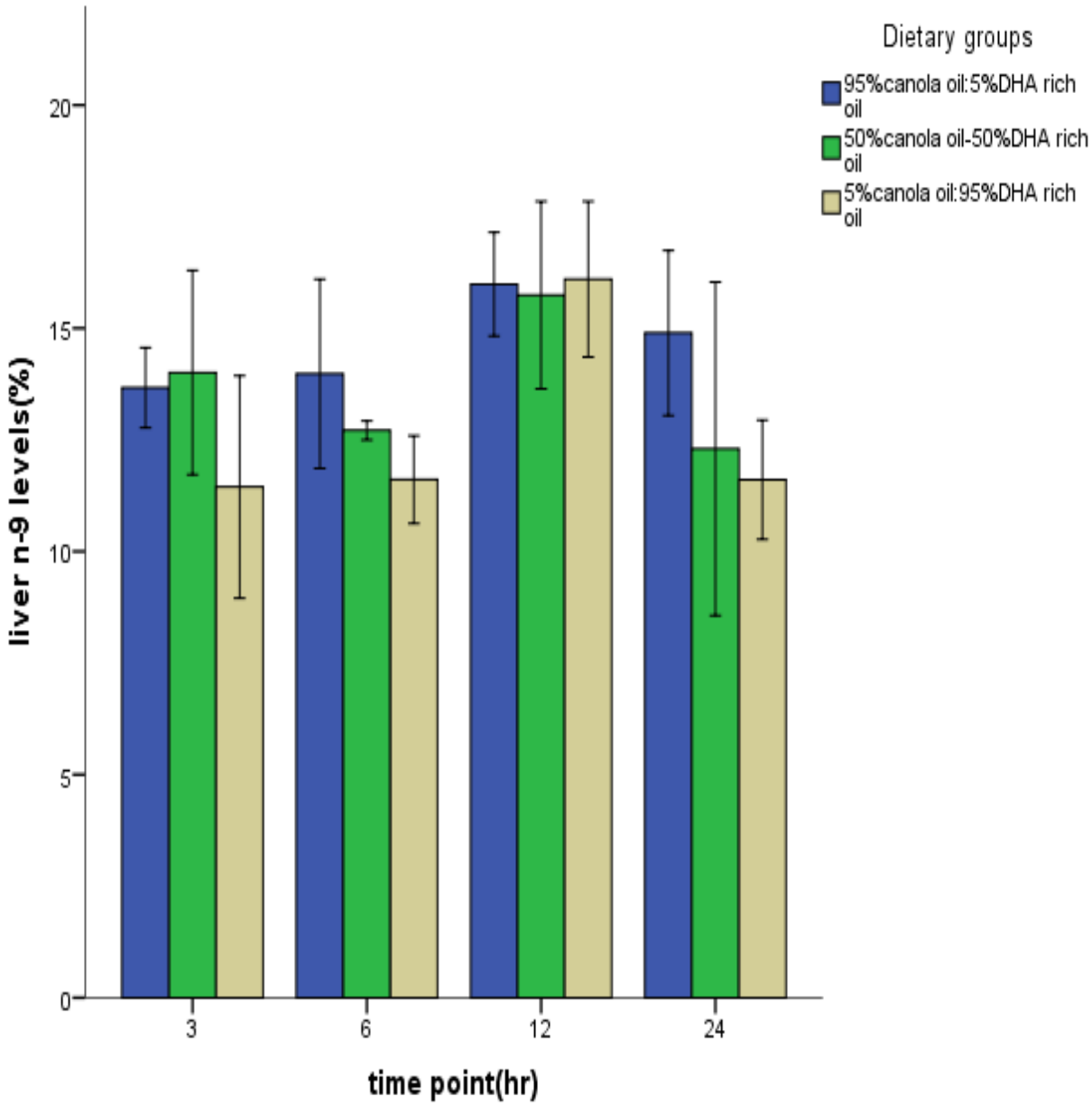


Figure 2-8: Comparison between n-9 liver fatty acid levels across all dietary groups at each time point. All data are expressed as mean ± SEM (n=5). Values with different superscripts were significantly different at $p \leq 0.05$

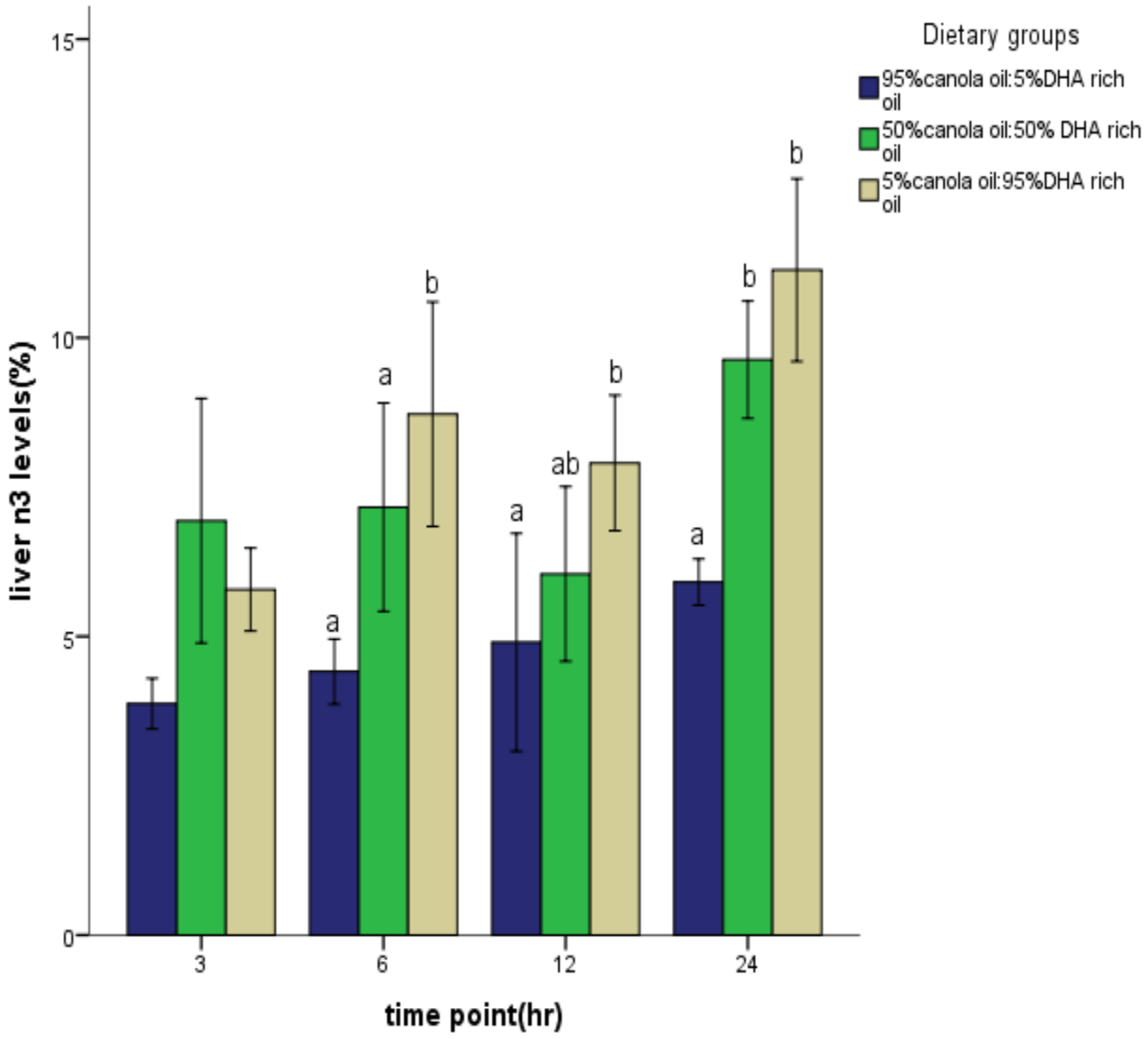


Figure 2-9: Comparison between n-3 liver fatty acid levels across all dietary groups at each time point. All data are expressed as mean ± SEM (n=5). Values with different superscripts were significantly different at $p \leq 0.05$

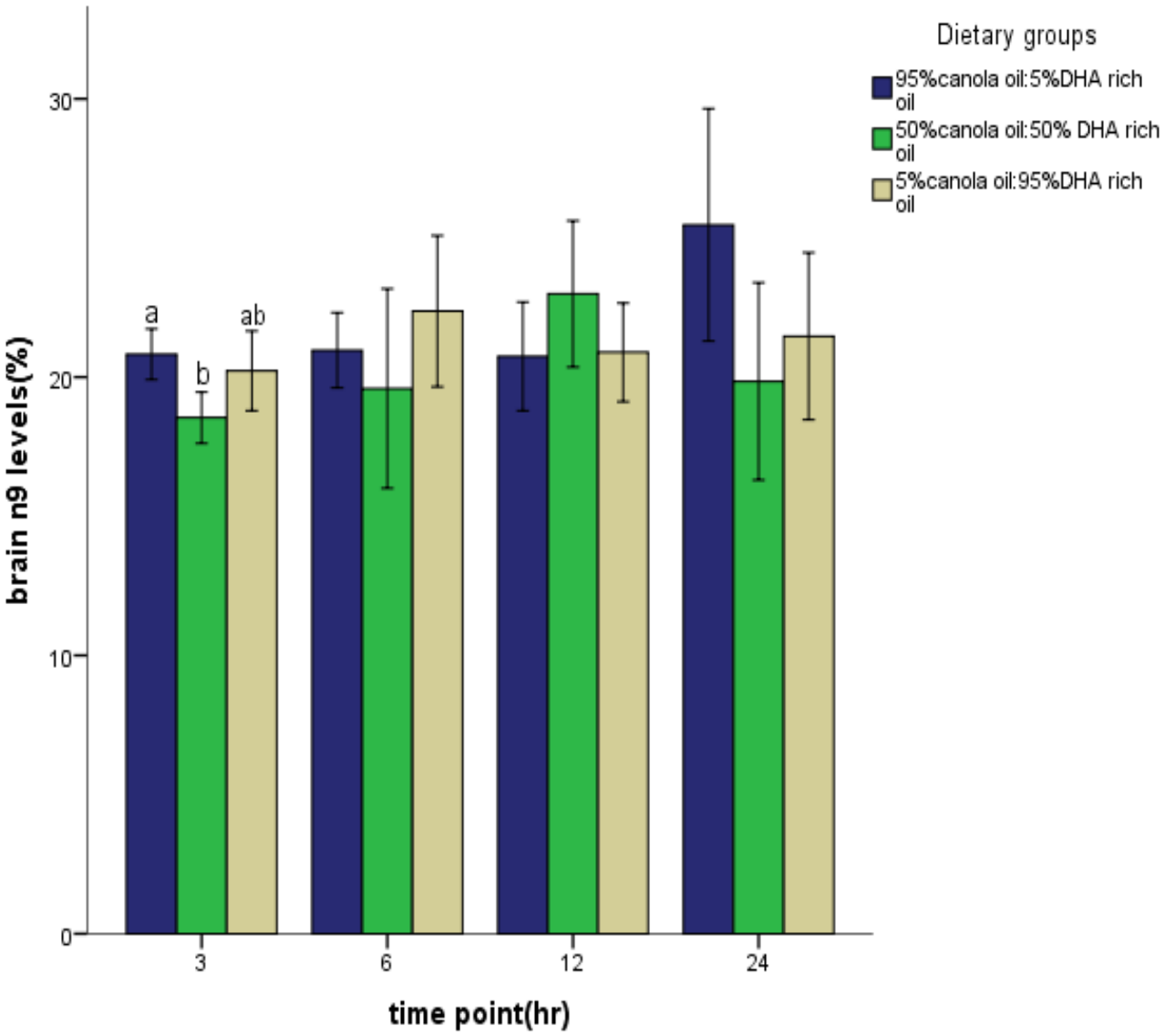


Figure 2-10: Comparison between n-9 brain fatty acid levels across all dietary groups at each time point. All data are expressed as mean ± SEM (n=5). Values with different superscripts were significantly different at $p \leq 0.05$

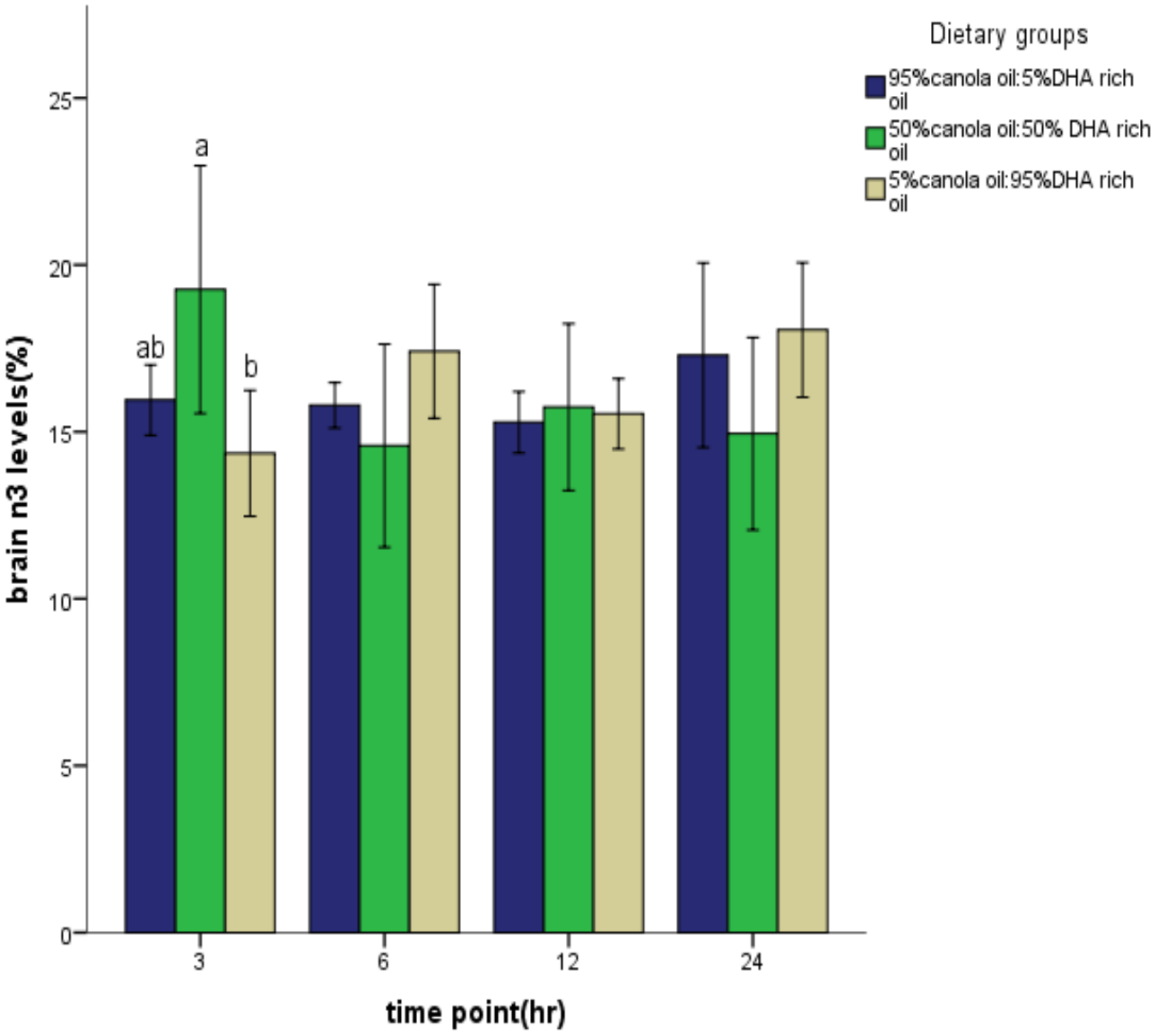


Figure 2-11: Comparison between n-3 brain fatty acid levels across all dietary groups at each time point. All data are expressed as mean ± SEM (n=5). Values with different superscripts were significantly different at $p \leq 0.05$.

Table 1: Dietary composition of treatment meal

Diet (%w/w)	**Safflower oil (g)**	**95% CO: 5% DRO (g)**	**50% CO: 50% DRO (g)**	**5% CO: 95% DRO (g)**
Casein	200	200	200	200
Cellulose	50	50	50	50
Cornstarch	195.5	195.5	195.5	195.5
Sucrose	300	300	300	300
Mineral Mix	35	35	35	35
Vitamin Mix	10	10	10	10
Cholesterol	2.5	2.5	2.5	2.5
BHT	0.02	0.02	0.02	0.02
DL-Methionine	5	5	5	5
Choline Bitrate	2	2	2	2
Beef Tallow	120	120	120	120
Oil	80 (safflower oil)	80 (76g CO and 4g of DRO)	80 (40g CO and 40g of DRO)	80 (4g CO and 76g of DRO)

BHT- Butylatedhydroxytoluene, CO- canola oil, DRO- DHA rich oil

Table 2: Fatty acid composition of dietary oils

Fatty acids (%)	Safflower oil	Canola oil	DHA rich algae oil
ΣSFA	10.45	5.84	27.59
C14:0	0.44	0.06	7.32
C16:0	6.79	4.05	17.77
C 18:0	2.24	1.32	0.88
ΣMUFA	15.35	64.83	32.84
C16:1n-9	0.08	0.19	0.36
C18:1n-9	14.52	64.49	16.78
C20:1n-9	0.51	0.05	0.51
C24:1n-9	ND	0.01	14.33
Σn-6 PUFA	73.88	19.90	2.87
C 18:2n-6	73.88	19.85	1.54
C 18:3n-6	ND	ND	0.33
C20:2n-6	ND	0.45	0.09
C20:3n-6	ND	0.45	ND
C20:4n-6	ND	ND	0.74
Σn-3 PUFA	0.13	8.78	36.69
C18:3n-3	0.13	8.71	0.14
C20:3n-3	ND	0.05	0.39
C20:5n-3	ND	ND	35
C22:5n-3	ND	ND	0.19
C22:6n-3	ND	ND	35.97

ΣSFA, Total saturated fatty acids, ΣMUFA, Monounsaturated fatty acids, ND-not detected, PUFA-Polyunsaturated fatty acids. The values are percentages (%) of identified total fatty acids

BRIDGE TO CHAPTER 3

We were able to demonstrate in chapter 2 the relationship between dietary fat and levels of fatty acids in plasma, small intestine, liver and brain over the short term after 2hr of meal consumption. Since previous studies show that long term consumption of these fats can elevate fatty acid levels which in turn impacts the levels of fatty acid ethanolamides, this chapter investigated whether the fatty acid levels obtained from the short term feeding are enough to be converted to fatty acid ethanolamides. Data in this chapter show that although not all FAE levels were elevated in response to the short term feeding, an increasing trend was seen, especially in OEA where a 95% canola oil diet was fed.

CHAPTER 3

Relationship between circulating fatty acids and fatty acid ethanolamide levels after a single 2hr dietary fat feeding in male Sprague Dawley rats

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Running Head: Time based effects of dietary fat on fatty acid ethanolamide levels

Conflict of Interest: None of the authors declared interests or potential conflicts of interest

Abstract

Only few studies have investigated short term effects of dietary fat feeding on fatty acid ethanolamide (FAE) levels. We explored the effect of acute feeding of varying concentrations of n-9 and n-3 fatty acids on plasma and organ levels of FAE specifically the relationship

between oleoylethanolamide (OEA) and docosahexanoylethanolamide (DHEA). Sixty-four rats were assigned to four groups containing 40% of energy as either safflower oil (control), canola oil (CO), or DHA rich oil (DRO) consumed within a 2hr window. FAE levels were measured at 3, 6, 12 and 24hr. At 3hr, plasma and liver OEA levels were higher ($p<0.05$) in the 95% CO: 5% DRO group than in other groups. At 12hr, plasma PEA levels were significantly lower in the intermediate group compared to the 95% CO group. Plasma DHEA levels showed a significant increase ($p<0.05$) only after 24hr of feeding. All 4 dietary groups increased DHEA in a dose dependent manner. Data demonstrate that a single meal feeding of diets with different ratios of fat types impacts tissue levels of FAE within a short time frame which could further impact the already established physiological roles of FAE such as appetite regulation and energy expenditure.

Abbreviations

AEA-anandamide/arachidonoylethanolamide, **CO**- canola oil, **DHA**-docosahexaenoic acid, **DHEA**-docosahexaenoylethanolamide, **DRO**-DHA rich oil, **FAE**- Fatty acid ethanolamide, **LA**-linoleic acid; **LEA**-linoleoylethanolamide, **OEA**-oleoylethanolamide, **PEA**-palmitoylethanolamide, **PPAR**-α-peroxisome proliferator-activated receptor α

Keywords

Fatty acid ethanolamides, canola oil, DHA, energy expenditure, fat oxidation

Introduction

With the increasing global prevalence of obesity, it is necessary to develop effective anti-obesity strategies to help curb this increase. Derivatives of fatty acids, fatty acid ethanolamides (FAE), are found in plant and animal tissues and have been proven to actively play many biological, pharmacological and physiological roles, including lipid signaling, regulation of depression, anxiety, food intake and energy metabolism (124,125). Levels of

FAE in plasma and tissues depend on the concentration of their fatty acid precursors (81). OEA has attracted attention due to its ability to regulate food intake as well as enhance energy expenditure (81,126). Levels of OEA increase after consumption of a high fat diet and decrease during starvation (81,91,127). OEA acts by activating peroxisome proliferator-activated receptor alpha (PPAR- α), a ligand-activated transcription factor that regulates the pathway of lipid metabolism (128). The activation of PPAR-α by OEA increases energy expenditure, triggers lipolysis, decreases body weight gain, and reduces fat accumulation in obese rats (127) . Another FAE, arachidonoylethanolamide (AEA), which belongs to the cannabinoid family, acts by binding to CB1 receptors of the endocannabinoid system (92,128). Increasing AEA levels, which is the ethanolamine derivative of arachidonic acid (AA), have been associated with increasing body weight (129). A study showed that newborn piglets fed with a diet containing 1.0% energy of arachidonic acid for 18 d had an increase in brain arachidonoylethanolamide levels (130). Palmitoylethanolamide (PEA) and linoylethanolamide (LEA) derivatives of palmitic and linoleic acid have also proven to be agonists of PPAR- α just like OEA, although with a lower affinity(127). Significant knowledge gaps remain in understanding the effects of various fatty acid classes on FAE levels, in particular oleic acid and DHA. A study conducted by Pu et al (131) showed that the addition of docosahexaenoic acid (DHA) to an oleic acid (OA) rich diet suppresses the production of OEA to favor DHEA, which implies that DHEA could have an inhibitory effect on OEA function by blunting its production. In addition, limited amounts of animal trial data exist regarding whether dietary fatty acids are converted to FAE immediately or whether this process takes a longer time. Accordingly, the present research was designed to test the null hypothesis that FAE levels in the plasma, small intestine, liver and brain will not increase over 24hr after consumption of dietary fat in a manner that is affected by the type of fat consumed. The overall goal of this study was to examine the effect of a high fat diet with

varying concentrations of n-3 and n-9 fatty acids on short term changes in FAE levels in a rat model, with canola oil being high in n-9 FA and DHA rich oil being high in n-3 FA.

Materials and Methods

The study protocol was reviewed and approved by the Animal Ethics Board of the University of Manitoba and followed guidelines of the Canadian Council on Animal Care. Sixty-four adult male Sprague Dawley rats from Charles River Laboratories (Quebec, Canada) were initially allowed to acclimatize to their new environment for 5 d during which they were fed a typical laboratory chow and water ad libitum. This period was followed by randomization into 4 groups, where animals were fed the AIN 93G diet from Dyets Inc, USA containing safflower oil for 2wk after which the diet was changed to the experimental single meal which was fed over a 2hr period. The four dietary groups contained 40% of energy as i) safflower oil (control), ii) 95% canola oil 5% DHA rich oil, iii) 50% CO:50% DRO, iv) 5% CO:95% DRO with canola oil containing an OA content of 64.3%, safflower with an OA content of 14.9% and DHA with an OA content of 16.8%. Animals were sacrificed by decapitation. Blood as well as tissue from small intestine, liver and brain were collected, weighed, and immediately frozen in liquid nitrogen and stored at -80ºC for further analysis. Four rats in the control group were sacrificed at 0hr to serve as a baseline measurement. After 3, 6, 12 and 24hr of feeding, 5 rats from each of the three dietary treatment groups were sacrificed at each time point.

Fatty Acid Ethanolamide Extraction

Fatty acid ethanolamides were extracted from plasma and organs using solid phase and liquid phase extraction procedures, respectively. The FAE levels of plasma and tissues were determined using an ultra-performance liquid chromatography technique equipped with a Kinetex XB-C18 column (100 x 2.1 mm, 1.7 µm, Phenomenex, Torrance, CA). All data

were processed by Mass Lynx 4.1 (Waters Corporation, Milford, MA). Brain, liver and small intestine samples were placed into glass tubes followed by the addition of OEA-d4, PEA-d4, LEA-d4, AEA-d8, DHEA-d8. Cold acetone from Fischer scientific, USA was added, followed by homogenization. Samples were vortexed for 1 min and centrifuged at 2000 g for 15 min at 4°C. The top layer of the centrifuged sample was transferred into fresh tubes and evaporated under nitrogen gas. Methanol-chloroform (1:2, v/v) and deionized water were then added to the dried samples. Samples were vortexed for 30 sec and centrifuged at 2000 g for 15 min at 4°C. The upper layer of the mixture was discarded and the lower layer was transferred into clean tubes. This procedure was followed by drying the sample completely under nitrogen gas. Acetonitrile (150 ul) was added to the dried sample and transferred into GC vials for UPLC-MS/MS analysis. Fatty acids were extracted from plasma, small intestines, liver and brain using a direct one step fatty acid methylation procedure with heptadecanoic acid (C17:0) added as internal standard.

Statistical Analysis

Results are presented as mean and standard error of the mean. SPSS version 9.4 was used for data analysis. Statistical significance was determined using a one-way ANOVA and Tukey's post hoc test was used for multiple comparisons to test the effect of treatment at different time points. Pearson's correlation was used to investigate the relationship between fatty acid and FAE levels. Significance was set at $p<0.05$ unless otherwise mentioned.

Results

Fatty Acid Ethanolamide Levels in Plasma, Small Intestine, Liver and Brain at Different Time Points

Levels of OEA, AEA, DHEA, PEA, LEA, in plasma, small intestine, liver and brain of rats at different time points and dietary groups are shown in **Figures 1-4,** respectively.

Concentrations of FAE were generally lower in plasma and brain than in the small intestine and liver **(Figures 1-4)**. The 95% canola oil diet increased ($p<0.05$) levels of OEA in all tissues compared to animals fed diets with lower concentrations of canola oil. In the 95% CO:5% DRO group, a difference in plasma OEA and LEA levels was seen at 12hr compared to 3hr and 6hr, although this higher level was observed to drop back to baseline by 24hr **(Figure 1)**. No significant difference was seen in all plasma FAE across all time points in the 50% CO: 50% DRO group (**Figure 1)**. In the 5% CO:95% DRO group, OEA levels were significantly higher at 12 and 24hr compared to 3hr but not between 3 and 6 hr. Also, a difference ($p<0.05$) was seen in PEA and AEA levels at 12hr. Levels of LEA failed to change across time, while DHEA was significantly higher at 24hr compared to 3, 6 and 12hr **(Figure 1)**. In the small intestine, the 95% CO: 5% DRO group FAE levels showed no significant differences over time, except for LEA which differed ($p<0.05$) at 6hr from 3, 12 and 24hr **(Figure 2)**. In the 50% CO:50% DRO, no differences were seen in OEA and AEA levels, while PEA level was higher ($p<0.05$) at 12hr compared to 3hr. DHEA showed significant differences between 3 and 6hr **(Figure 2)**. In the 95% CO group, none of the FAE examined showed differences, except liver PEA levels where a difference ($p<0.05$) was seen at 12hr compared to 3hr, and a drop below baseline was seen at 24hr **(Figure 2)**. No significant differences in liver FAE concentrations were observed across any of the diets **(Figure 3)**. In the brain, animals fed the 95% CO: 5% DRO diet showed no significant difference across time. The intermediate group showed no difference in OEA and AEA levels, while PEA levels were higher ($p<0.05$) at 6, 12, 24hr compared to 3hr. Significant differences were seen in LEA and DHEA levels between 6 and 12hr in brain. In the 5% CO:95% DRO group, none of the FAE showed significant differences in brain, except OEA levels which at 3hr significantly differed from 12hr **(Figure 4)**.

Correlations between Fatty Acid Ethanolamide Levels and Their Precursor Fatty Acids in Plasma, Small Intestine, Liver and Brain

Tables 1 - 4 summarize correlations between fatty acid levels and their FAE derivatives across all dietary groups in the tissues examined. In plasma, OA and linoleic acid (LA) were correlated ($p<0.05$ $r=0.314$, $r=0.289$) positively with their respective FAE, OEA, and LEA, while no relationships were seen between levels of PEA, AEA, DHEA and their corresponding fatty acid precursors. In small intestine tissue, none of the FAE examined, except LEA, correlated with their corresponding precursor fatty acids. Here, LEA showed a trend to a negative correlation with LA. In liver tissue, a positive ($p<0.05$, $r=0.283$) correlation was seen between LEA and DHEA with their corresponding fatty acid precursors, while no correlations were noted between OEA, PEA and AEA and their precursors. In brain tissue, all FAE showed a positive relationship with their precursor FA except for AEA which showed a negative correlation with its precursor FA.

Discussion

This study shows for the first time the interactive effects of dietary fatty acid composition and the immediate time course of change of FAE levels in the circulation and tissues following bolus meal consumption in rats. Present data reveal immediate elevations in certain but not all FAE, following specific dietary fat intakes after a single meal was provided to rats. Particularly, presumably due to the high levels of AA in plasma, small intestine and liver, high AEA concentrations occurred in plasma, small intestine and brain most especially at 6 and 12hr which implies an efficient conversion of LA to AA and eventually to AEA (132). High OEA levels seen in all tissues with the 95% CO: 5% DRO diet compared to the other groups suggests a dose-dependent increase in OEA levels, driven by the higher

concentrations of dietary OA. However, at 24hr, levels of OEA had dropped back to baseline irrespective of the diet fed, which could be due to the upregulation of the degradative enzymes which act on FAE to break them down to ethanolamide and fatty acids. Although not a direct comparison to this due to the difference in study duration, this increase in OEA levels does agree with the work by Lin et al where HOCO-fed hamsters significantly increased plasma OEA levels over a period of 28 d compared to baseline implying that the increase in OEA levels does not require a longer term feeding duration to be achieved. The high OEA levels were not only observed in small intestine but also in liver which implies that OEA is not produced only in the small intestines but also in the liver (132). This finding is not surprising since N-acyltransferase (NAT), the enzyme involved in the synthesis of FAEs is also located in the liver. However, it will be useful to see if the formation of OEA in the liver occurs through a different process than it does in the small intestine.

Although this is the first animal trial looking at shifts in FAE levels within a short time frame, i.e., 3hr post meal, the high levels of OEA seen after 3hr affirm that feeding is actively involved in the mobilization of OEA especially in intestinal tissue (91). This finding also implies that OEA levels increase during re-feeding but remain low during starvation, a result that is in line with previous experiments conducted by Rodriguez de Fonseca (92) where rats were divided into fed and deprived subgroups after which their FAE levels were measured. In that study, food deprived rats manifested a lower level of OEA than was seen in ad libitum fed rats. It would be interesting to further investigate if levels produced within this short term feeding are sufficiently adequate to activate the PPAR-α system.

Also, Menella et al showed that the oleic acid content of a meal affects levels of circulating FAE in plasma within 24hr (133). Our inability to see any significant changes in LEA levels is also similar to study by Menella et al (133) where 15 volunteers were fed diets containing three oils that differed in fatty acid composition over a period of 2hr. An

explanation for a lack of changes in LEA could be due to the fact that the LA content of our background diet was the same across all dietary groups. Menella et al saw reduced concentrations of plasma PEA and AEA levels after feeding, similar to the reductions in our trial in terms of postprandial plasma PEA and AEA levels (133). Low AEA levels seen in the 95% DRO group are consistent with previous findings that DHA supplementation lowers endocannabinoids levels (134), although this trend was not seen in our trial in small intestine, but was seen in liver tissue at 3 and 12hr (134). The delayed conversion of DHA to DHEA could be due to the amount of DHA in our diet being insufficient to become converted to FAE. This result could also be due to the need for a more prolonged time period than our study window allowed for to enable the conversion of DHA to DHEA, since a significant difference was detected at 24hr.

Our results show no significant differences in small intestine or brain DHEA levels, especially in the 95% CO:5% DRO and 5% CO:95% DRO groups. These findings are consistent with work done by Artmann et al (81) where even a 1wk DHA supplementation failed to increase brain DHA and DHEA levels in rats. Meanwhile, we saw a significant increase in plasma DHEA levels after 24hr of consuming the dietary fat consistent with findings from Wood et al (134), where 2wk of DHA supplementation increased plasma and brain DHA and DHEA levels in mice. Apart from the fact that the species of animals used differed from those used in our study, the disparity in results could mean that longer term feeding is needed to elevate DHEA levels in the brain. Also, a human study conducted by Pu et al showed that plasma DHEA levels, elevated in response to DHA intake, tended to be associated with lower plasma OEA levels (131). One major objective of our study was to investigate if the addition of DHA to an oleic acid rich diet will also affect the production of OEA. Although there was a decreased trend in OEA levels when the high DHA diet was fed, the gradual increase in concentration of DHA added to our oleic acid rich diet makes it

difficult to determine if the decrease in OEA levels to favor DHEA across diets was a result of the added DHA; it could also be that. Future studies investigating the relationship between oleic acid and DHA should be conducted and unlike our study which altered both the concentration of oleic acid and DHA, the concentration of oleic acid to which DHA will be added should be kept constant. Such studies will provide us with a stronger conclusion as to whether the addition of DHA to an oleic acid rich diet suppresses the production of OEA to favor the production of DHEA.

In summary, this study demonstrates that post prandial levels of FAE in rat plasma and other tissues are elevated almost immediately after consumption of the dietary fat. Therefore, our results suggest that plasma and organ FAE levels are in most part swiftly and highly sensitive to the type of dietary fatty acid consumed. This in turn implies that dietary fatty acids after a single feeding are capable of modulating FAE levels which in turn can impact appetite regulation, obesity and energy expenditure.

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Figure Legend

Figure 3-1: Plasma FAE levels at different time points across all dietary groups. All data are expressed as mean ± SEM (n=64).

Figure 3-2: Small intestine FAE levels at different time points across all dietary groups. All data are expressed as mean ± SEM (n=64).

Figure 3-3: Liver FAE levels at different time points across all dietary groups. All data are expressed as mean ± SEM (n=64).

Figure 3-4: Brain FAE levels at different time points across all dietary groups. All data are expressed as mean ± SEM (n=64).

Table 3: Correlations between plasma fatty acid ethanolamide and their plasma fatty acids levels

Fatty acids	Fatty acid ethanolamide	Correlation coefficients	P-value
OA	OEA	0.314	P<0.05
PA	PEA	0.018	0.545
LA	LEA	0.289	P<0.05
AA	AEA	0.067	0.601
DHA	DHEA	0.206	0.102

Table 4: Correlations between liver fatty acid ethanolamide and liver fatty acids levels

Fatty acids	Fatty acid ethanolamide	Correlation coefficients	P-value
OA	OEA	0.182	0.150
PA	PEA	0.045	0.726
LA	LEA	0.263	<0.05
AA	AEA	0.040	0.754
DHA	DHEA	0.283	<0.05

Table 5: Correlations between small intestine fatty acid ethanolamide and small intestine fatty acids levels

Fatty acids	Fatty acid ethanolamide	Correlation coefficients	P-value
OA	OEA	0.042	0.744
PA	PEA	0.037	0.773
LA	LEA	-0.274	<0.05
AA	AEA	0.083	0.516
DHA	DHEA	0.233	0.064

Table 6: Correlations between brain fatty acid ethanolamide and their precursor fatty acids levels

Fatty acids	Fatty acid ethanolamide	Correlation coefficients	P-value
OA	OEA	0.002	0.989
PA	PEA	0.046	0.719
LA	LEA	0.009	0.947
AA	AEA	-0.311	<0.05
DHA	DHEA	0.182	0.150

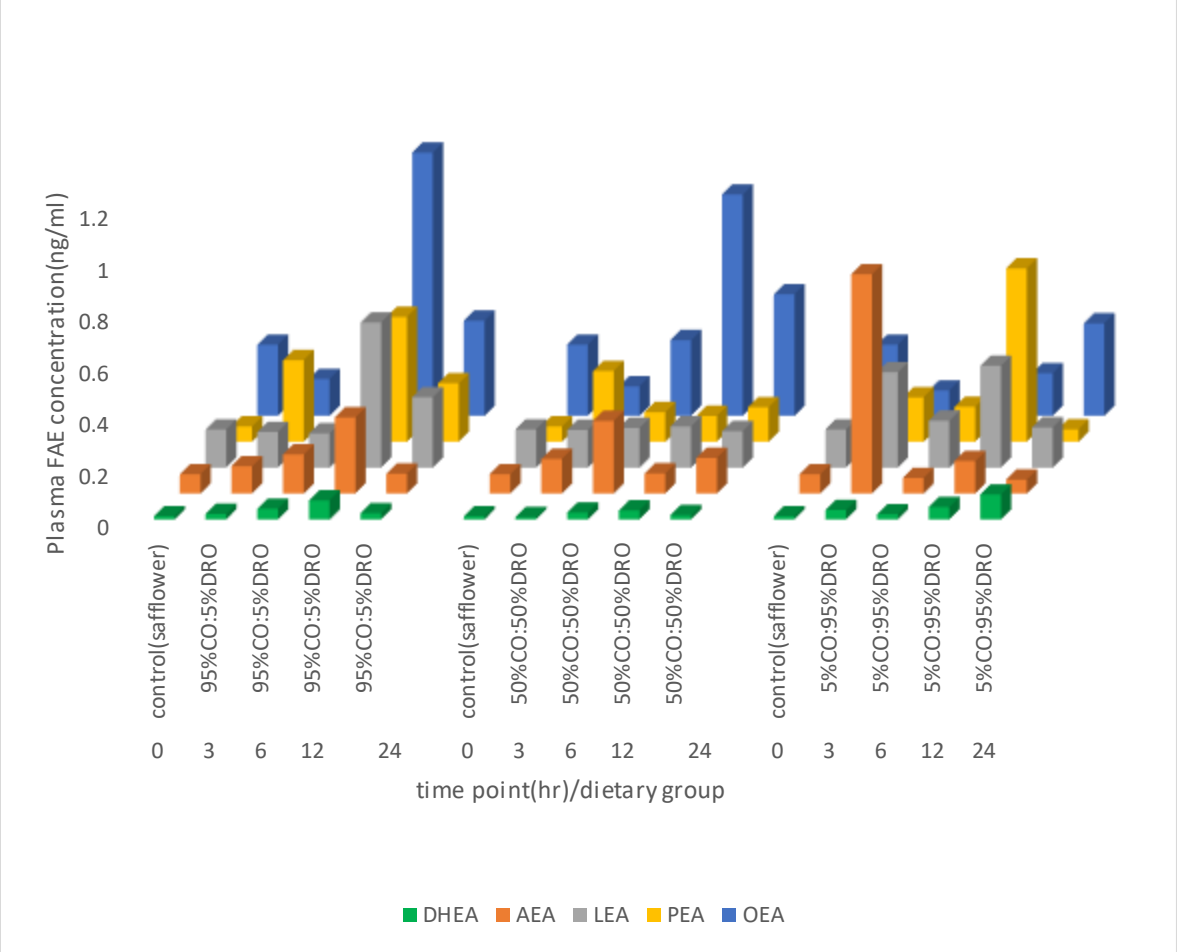

Figure 3-1: Plasma FAE levels at different time points across all dietary groups All data are expressed as mean ± SEM (n=64).

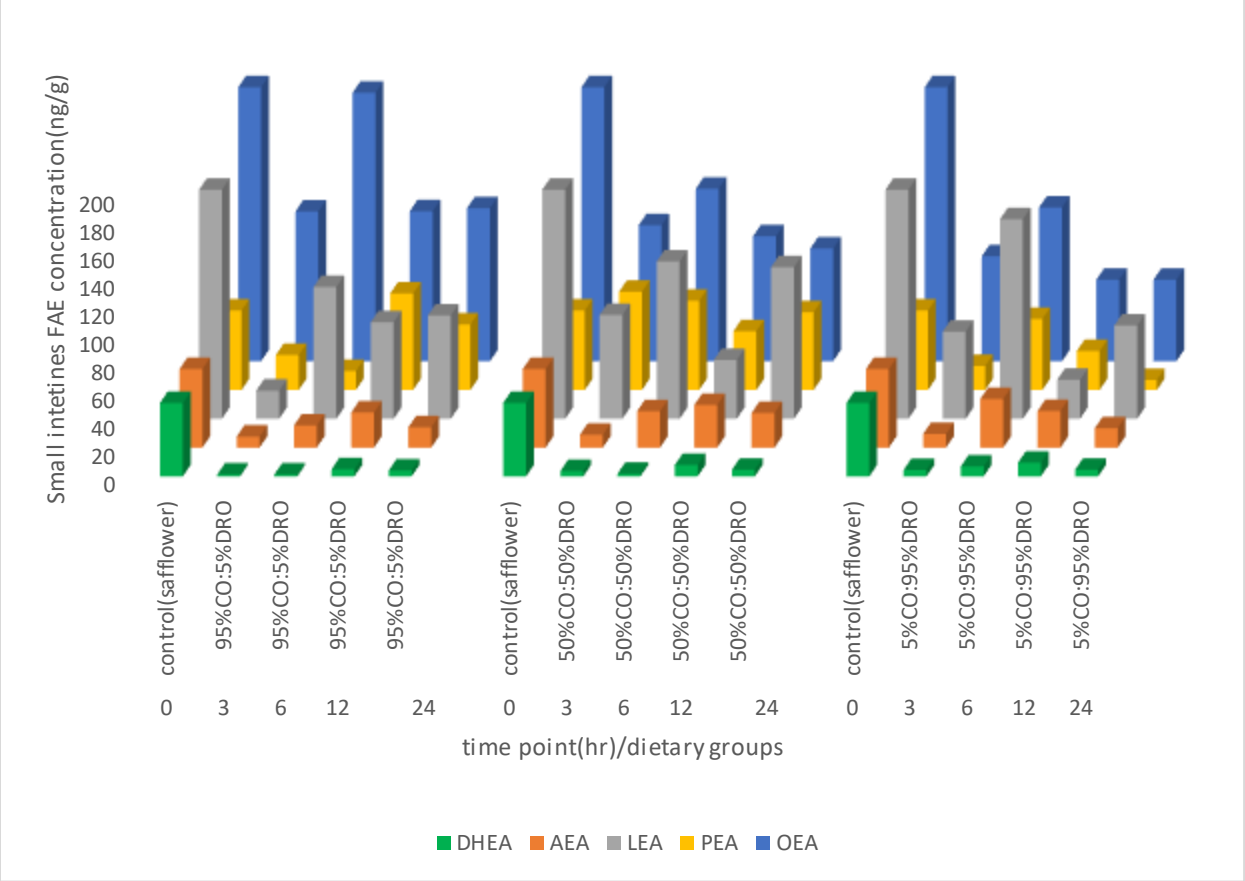

Figure 3-2: Small intestine FAE levels at different time points across all dietary groups. All data are expressed as mean ± SEM (n=64).

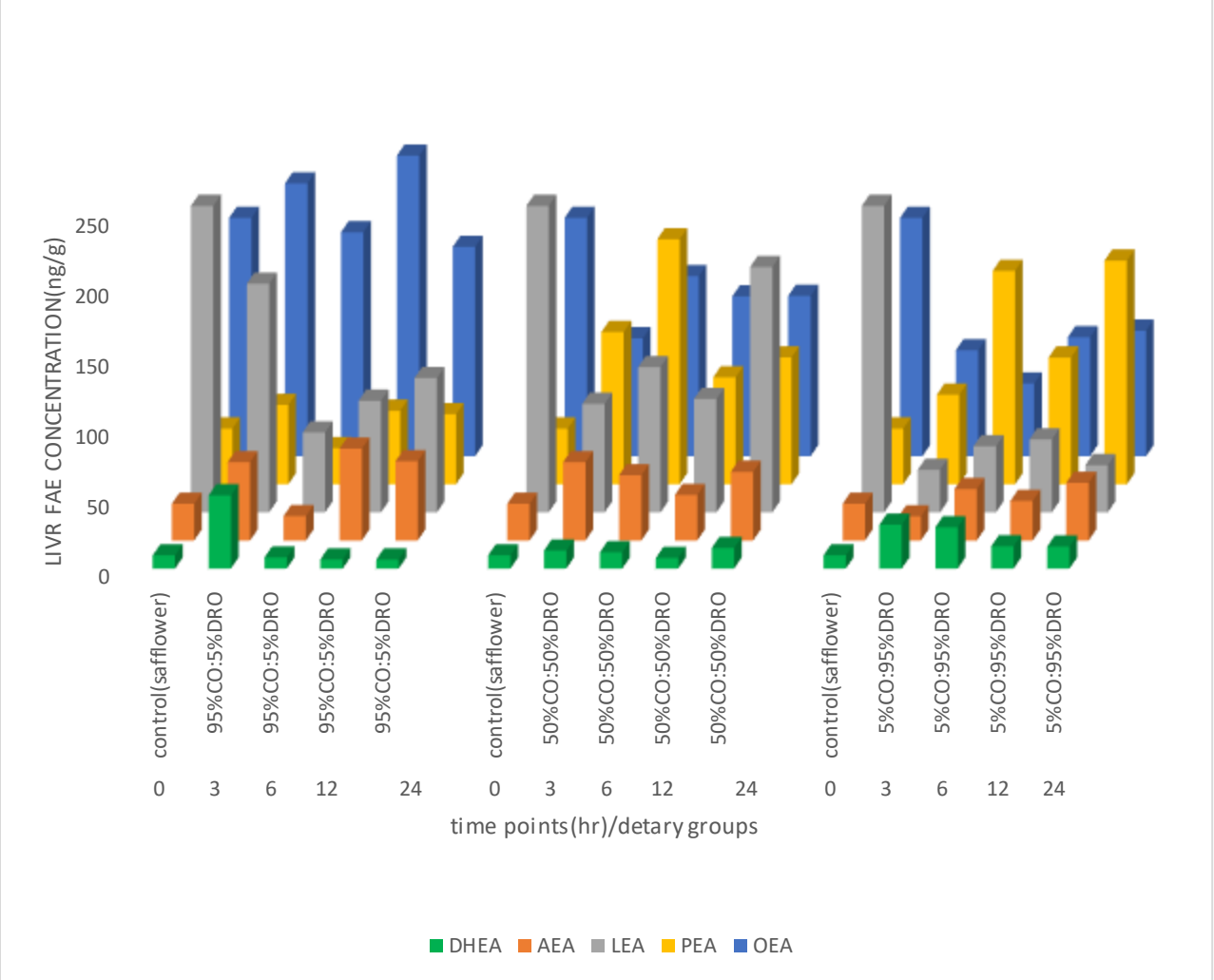

Figure 3-3: Liver FAE levels at different time points across all dietary groups. All data are expressed as mean ± SEM (n=64).

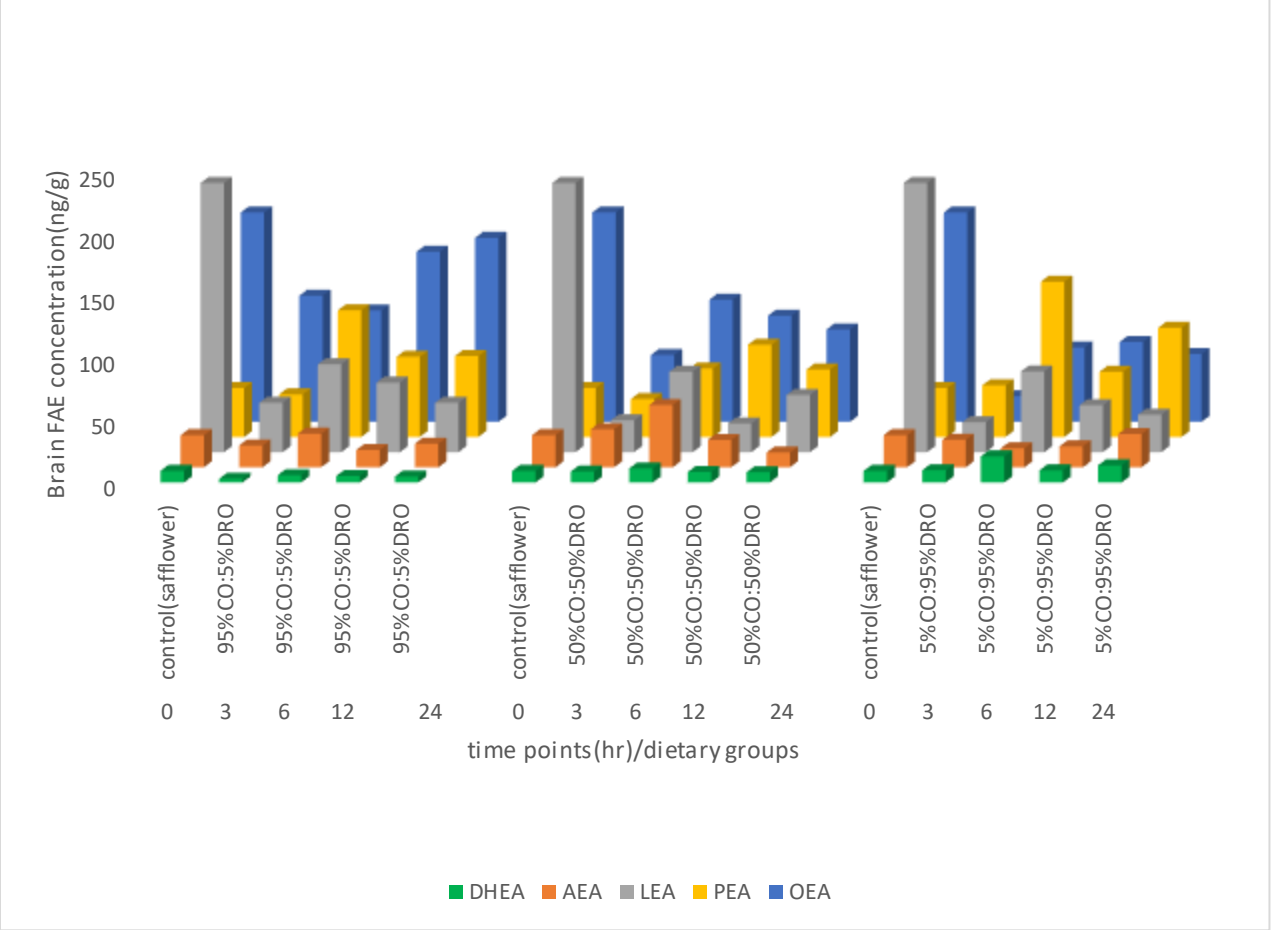

Figure 3-4: Brain FAE levels at different time points across all dietary groups. All data are expressed as mean ± SEM (n=64).

CHAPTER 4

Discussion and Conclusion

This study provided a time course-based examination of the effect of dietary fat consumption on plasma, liver, small intestine and brain in terms of fatty acid and fatty acid ethanolamide levels. To achieve this, we used the Sprague Dawley rat model, which has been utilized in different studies as an ideal animal model to mimic the physiological human system (138). The major finding from this present study was the rapid uptake of fatty acids after consumption of a single meal. Although many studies have shown that levels of fatty acids in plasma and tissue reflect the dietary fatty acid consumption, only very limited data are available looking at the time course of fatty acid change immediately after ingestion of a single meal. The present study provides more evidence that FAE levels are influenced by the levels of their precursor fatty acids derived from consuming diets rich in different concentrations of fatty acids during the 2hr window. A dose dependent OEA increase seen across all groups is in line with previous findings as stated earlier. Our inability to see increase in DHEA levels especially in the brain could imply that longer term feeding is needed to elevate DHEA levels. The primary finding of the present study shows that feeding a single meal containing n-9 and n-3 fatty acids to animals with very low concentrations of those fatty acid in their background diet caused significant effects on blood, liver, small intestine and brain fatty acid levels after a short period of time. This finding implies a rapid uptake and interconversion of ingested fat. Since most recommendations for development of CVD risk factors such as obesity place their focus on long term dietary fat consumption, the rapid uptake of dietary fat into plasma, small intestine and liver as seen in our study implies that the development of obesity and its related diseases commences within a time frame as short as a few hours. The present data also show the conversion of fatty acids to their ethanolamide derivatives. Only few studies have been able to show a positive effect of short

term feeding on these lipid mediators. It would be important to examine further whether the concentration derived within this short time frame is enough to induce a physiological response such as OEA's appetite regulatory role or AEA's hunger inducing role.

In conclusion, acute feeding of male rats with different concentrations of n-3 and n-9 fatty acid rich oils influenced the levels of OEA, PEA, LEA, AEA and DHEA in plasma, small intestine, liver and brain after only 3hr of feeding, reflecting the levels of their precursor fatty acids.

Future Directions

One major objective of this study was to investigate if the addition of DHA to an oleic acid rich diet would affect the production of OEA. Although, we saw a decrease in OEA levels after incorporating DHA into the diet, the gradual increase in concentration added to our oleic acid rich diet makes it difficult to determine if the decrease in OEA production to favor DHEA is as a result of the DHA added. Future studies investigating the relationship between oleic acid and DHA should be conducted and this should not alter the oleic acid concentration to which DHA will be added. Such studies will provide us with a stronger conclusion on whether the addition of DHA to an oleic acid rich diet suppresses the production of OEA to favor the production of DHEA.

Limitations and Strengths

The limitations of this study are that the background diet which served as control and time point zero had no subdivided dietary groups, making it difficult to compare other time points with 0hr of the same diet. There have been failed attempts to restrict the feeding window of several other types of animals, most especially mice and hamsters. However, our

ability to restrict and train our rats to eat within a 2hr time frame is a major strength of this study.

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