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IMPACT OF AGE AND VITAMIN A SUPPLEMENTATION ON THERMOGENIC FACTORS IN A RAT MODEL OF CHILDHOOD OBESITY RISK

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ABSTRACT

Childhood obesity is major global health issue, affecting both developed and developing countries throughout the world. Despite the efforts of many health professionals, the percentage of children suffering from obesity has not been reduced over the last 15 years. Vitamin A (VA) deficiency is another troubling global health problem that affects many people, causing disorders like xerophthalmia or even early death. Recent studies have found that early postnatal supplementation of VA can increase expression of uncoupling protein 1 (UCP1), found in the brown adipose tissue of small mammals. Additional studies have found that a mixture of VA and retinoic acid (VARA) can 'carry over' some modifications in gene expression into later adolescence when compared to VA alone. This study aimed to determine if early postnatal VA supplementation will increase the expression of UCP1, and will therefore increase energy expenditure and promote weight loss in rats that are at a marginal VA status and on a high fat diet. Moreover, the study aimed to test if VARA could maintain a level of increased UCP1 expression in later adolescence compared to VA supplementation. Three treatment groups were created to test this hypothesis - VA, VARA, and placebo - and all groups were dosed and euthanized at a set schedule in order to mimic life stages of the human neonate and adolescent. Retinol content was measured using UPLC and genetic expression of UCP1 was determined by (q)RT-PCR. UPLC results indicated that VA and VARA supplementation increased tissue concentration of VA by more than 2 fold immediately after dosing, but returned to normal after several days. Furthermore, no significant change was found between the three groups with regard to UCP1 expression. In conclusion, while retinol was able to enter the BAT, neither VA nor VARA supplementation increased UCP1 mRNA expression, thus refuting our hypothesis.

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Chapter 1

Introduction and Literature Review

Obesity

Obesity is a medical condition that is described as having excess body fat. The terms 'overweight' and 'obese' in reference to adults are often classified by body mass index (BMI), a weight-to-height ratio that is a proxy for body fat percentage. BMI is calculated by dividing weight in kilograms by height in meters, squared (BMI= kg/m^2) (1). While body fat percentage is typically represented by BMI, there are several ways to accurately measure fat mass including multisegmental and multifrequency bioelectrical impedance analysis or quantitative magnetic resonance (2). The quantity of body fat deemed as 'excess' varies between age and sex. Peak body fat percentage in humans occurs between 3 to 6 months of age, approximately 32% in females and 29% in males (3). As a child leaves infancy, he experiences a decline in BMI in early childhood and then BMI rebounds at approximately age 5 to 6 years. After this adiposity rebound, BMI increases throughout the remainder of childhood and into adolescence and adulthood (4). While sexual dimorphisms in body fat are always present, they become more pronounced during adolescence. Women gain more fat mass relative to lean mass and normally procure the fat in their hips and thighs (5). In Western cultures, BMI typically increases during adulthood; the changes that occur are less dramatic than in ages of rapid growth (infancy, childhood, and adolescence). The increase in BMI during adulthood is largely attributed to increases in fat mass (6).

During childhood, body fat percentage changes dramatically as part of normal growth, therefore raw BMI is not used in children. Instead, weight status in children is classified by the comparison of BMI to published growth charts according to age and sex, to calculate BMI-for-age percentiles. The growth standards from the World Health Organization (WHO) and the growth references from the Centers for Disease Control and Prevention (CDC) are recommended for infants and children younger than 2 years old and children between 2 and 20 years of age, respectively (7). Children who have a BMI that falls between the 85th and 95th percentiles for their age and sex are defined as 'overweight' and children who have a BMI greater than the 95th percentile for age and sex are defined as 'obese'. The term 'severe obesity' is classified as greater than 120% of the 95th percentile or having a BMI greater than 35 kg/m^2 (7).

In 2003, 17.3% of children in the United States were classified as obese and the prevalence of obese children remained relatively stable between 2003 and 2011, despite the efforts of health professionals and other measures to increase public awareness of the problem (8). Unfortunately, while the prevalence of obese children has remained the same, the prevalence of 'severely obese' children increased from 5.1% in 2003 to 5.9% in 2012 (9). Obesity appears to be strongly linked to heredity; obesity in one parent increases a child's risk of obesity by 200-300% and obesity in both parents increases the child's risk by up to 1500%. Parental obesity has also shown to increase the risk of adult obesity in children younger than 10 years of age (10). This link between parents and offspring may also be attributed to lifestyle factors; parents unconsciously teach their children their food and activity habits. The prevalence of childhood obesity also appears to depend on racial factors - African American, American Indian, and Mexican American children have an increased risk as compared to non-Hispanic white children (11). Diet and physical activity habits are two major factors correlated with obesity. These two

factors can be influenced by genes that affect energy expenditure, fuel metabolism, appetite, and other factors (12).

Obesity is not a problem that solely affects children in the United States - it is a worldwide epidemic. Between 1990 and 2010, the prevalence of childhood overweight and obesity throughout the entire world increased from 4.2% to 6.7% (13). In addition, 43 million children were estimated to be overweight or obese in 2010, and approximately 81% of those children were from developing countries (13). The prevalence of childhood obesity in Africa is expected to rise from 8.5% to 12.7% between 2010 and 2020 (13). Obesity in general is very harmful to health because of the increased risk of comorbidities. The risk of developing dyslipidemia, insulin resistance, and cardiovascular dysfunction greatly increases with the development of obesity and these health issues can arise in childhood and persist into adulthood (14). Another study found that obesity in adolescence is a predictor of adverse health effects independent of adult weight (15). Studies in animal models have shown that a high-fat (HF) diet, a diet comprised of 40% animal fat, imitates the pathophysiology of human obesity and metabolic syndrome and increases body fat percentage (16).

Vitamin A: Background and Metabolism

Vitamin A (VA) is a term used to encompass retinol and all related compounds with its biologic activities. VA is a crucial part of diet because while all vertebrates require VA, none can synthesize it de novo (17). Dietary VA can be consumed as preformed VA (retinol) or provitamin A carotenoids such as β-carotene. Preformed VA is present in the tissues of animals as retinol and retinyl esters (RE). RE is the product of the esterification of all-*trans*-retinol with

long-chain fatty acids. The highest concentrations of preformed vitamin A are found in liver and other organ meats, but foods that are fortified with RE or β-carotene are also significant sources (17). In the developing world, the majority of VA consumption consists mainly as provitamin A carotenoids (17-19). β-carotene is naturally found in fruits and vegetables, such as carrots, tomatoes, and sweet potatoes (20).

In normal circumstances, approximately 70% of dietary vitamin A is absorbed; however, there are special conditions that can decrease absorption efficiency, such as a diet with less than 5% dietary fat (21). Before intestinal absorption, dietary vitamin A must be mixed with the digestive enzymes in the stomach in order to free the REs, then must be emulsified with fatty acids and bile salts, and incorporated into lipid micelles (22). Free retinol can then be absorbed by the enterocytes of the duodenum and jejunum by protein-mediated facilitated diffusion and passive diffusion, and finally exported to the lymphatic system or portal blood (22, 23). Figure 1 shows different metabolites of VA and the way they are formed; for example, VA is consumed as carotenoids and retinol but is stored in the form of retinyl esters. Retinal can also be transformed into retinoic acid (RA) and its polar metabolites. RA is thought to be the most biologically active retinoid in cells because the polar metabolites have equal or lesser biologically activity than retinoic acid. Studies suggest that RA is catabolized into polar metabolites as a protective measure to prevent the cells from constant exposure to RA's strong activity (24).

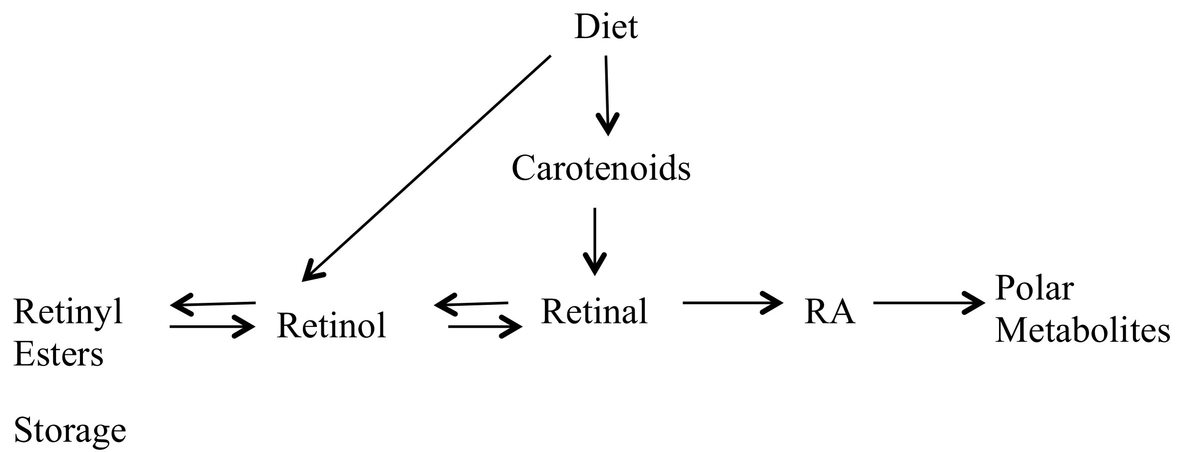

Figure 1. Metabolites of vitamin A. Many forms of VA are present in the body and VA is converted to the different forms to meet bodily needs. (VA = vitamin A; RA= retinoic acid)

Vitamin A Deficiency

While many specific studies have been performed on childhood obesity, few have focused on neonatal nutrition and its effects on later life, specifically early VA supplementation. Vitamin A deficiency (VAD) is a global health crisis and affects more than half of all countries in the world, especially in the developing countries of Africa and South-East Asia (25). The populations most directly affected by VAD are young children and pregnant women. The WHO estimates about 250 million preschool children worldwide to be deficient in VA (25). Researchers believe that the nutrient profile of the mother's breast milk is the major reason for children to become VA deficient (26). The diet of a lactating mother has a significant impact on the VA status of her exclusively breastfed infant. A study in Senegal found that VA supplementation of the mother increased the VA liver stores in her infant (27). The concentration of VA in breast milk increased drastically with 24 hours of maternal supplementation, resulting in infants having higher liver VA storage levels compared to infants of mothers who did not receive VA supplementation (27). Women in developing countries may have lower VA stores because women in developing countries spend a larger percentage of their life breastfeeding than women in industrialized societies (26). According to the Institute of Medicine, the recommended dietary allowance (RDA) for VA in pregnant women (19-50 years of age) is 770 mcg RAE and the RDA for lactating women of the same age group is 1,300 mcg RAE (23). Both recommendations are increased from the 700 mcg RAE recommendation for women who are not lactating or pregnant, but notably, the VA requirement for a lactating woman is almost double that of a pregnant woman (23). Some researchers also believe that VAD in children may be due to the micronutrient-poor food they receive during and after weaning from breast milk (26).

Deficiency in VA can cause many serious health problems, such as xerophthalmia and severe infection, and can increase risk of mortality; VAD accounts for 1 to 3 million of child deaths annually (28,29). VAD is the most common source of childhood blindness, causing approximately 500,000 children to lose their sight each year (29). While cases of VAD and xerophthalmia are more commonly found in developing countries, a recent study in Australia found several populations in developed countries where mild vitamin A deficiency is not a rare occurrence, like in those with cystic fibrosis, celiac disease or autism (30).

The WHO promotes VA supplementation for children between the ages of 6 months and 5 years old, for the purpose of reducing child mortality, but there is no recommendation of VA supplementation for children under the age of 6 months (31-34).

Brown Adipose Tissue

Fat composition in mammals consists of several metabolically different types of adipose tissue, including white adipose tissue (WAT) and brown adipose tissue (BAT). Both forms of fat are metabolically active and the mass of both tissues can be affected by similar factors, such as nutrition, drug therapy, and environment, so their characteristics and actions vary quite drastically (35). WAT is known to be a storage tissue due to its ability to store extra energy as triglycerides and release the fatty acids when the body requires additional fuel. The tissue can be found virtually everywhere in the body; for example, visceral WAT surrounds organs and is known to increase morbidity risk and subcutaneous WAT is found under the skin - both act as a form of insulation. WAT is also considered to be a metabolically active endocrine and secretory organ due to its role in glucose homeostasis and inflammatory processes (36). WAT secretes

many different proteins including leptin, adiponectin, adipsin, and resistin, and these proteins are responsible for energy metabolism and heavily contribute to health problems, like diabetes and obesity (36).

BAT is less prevalent compared to WAT in terms of percentage of body weight and is found in different regions of the body than WAT. BAT normally surrounds the anterior neck and supraclavicular fossa in humans (37), while in rats and other small mammals, a large deposit of BAT can be found between the two scapulae (38). BAT has recently been found to serve as a source of non-shivering thermogenesis in small mammals due to the presence of the highly specialized protein, uncoupling protein-1 (UCP1) (39). UCP1 is responsible for both cold- and diet-induced thermogenesis and its activity is protective against the production of excess body fat (40). This protein produces heat by separating oxidative phosphorylation from ATP synthesis and diminishing the proton gradient between the inner and outer membranes of the mitochondria (39). Because BAT produces heat as a byproduct, increased BAT can be helpful in regulating body temperature. Small mammals, such as rats and infant humans, cannot regulate body temperature through other mechanisms as well as human adults; therefore, quantity of BAT is higher (41). BAT appears to decrease with age in humans.

Many studies have been dedicated to researching ways to increase the energy expenditure of BAT, either by increasing the quantity of BAT through white-to-brown adipose tissue remodeling or through increasing the metabolic activity of BAT by increasing the expression of UCP1 (42,43). Researchers have found that quantity and UCP1 expression of BAT increases in small mammals when the mammals are exposed to cold temperatures (44). A study found that a HF diet increases thermogenic capability of BAT (44). Researchers have also tested numerous supplemental dietary thermogenic agents to accomplish this goal: selective thermochemicals,

like capsaicin, resveratrol, curcumin, green tea epigallocatechin gallate (EGCG), and berberine, dietary FAs, and all-*trans* retinoic acid (ATRA) (45. A study found that RA supplementation can stimulate expression of UCP1 in adipocytes (46). VARA, the combination of 90 mol-% VA and 10 mol-% ATRA, has been shown to increase the uptake of retinol and the storage of retinyl esters in lung tissues of neonatal rats, by approximately 5-fold (47,48). These studies concentrated on the effects of VARA on lung tissues, and very little is known about the effects of VARA on BAT.

Rat Model for Experimental Use

Sprague-Dawley rats are widely accepted as a standard rodent model in laboratory research and are commonly used in studies involving nutrition and the investigation of common diseases, such as diabetes and obesity. Previous studies found that the pathophysiology of obesity in the rat model is very similar to obesity in humans (34). Unlike research in humans, the rat model more readily allows for BAT tissue collection and analysis, which is virtually impossible to perform on human subjects. After tissue collection, retinol levels in tissues are most often measured through high performance liquid chromatography (HPLC) analysis. The use of a rat model also eliminates certain influences, like race and socioeconomic factors, from biasing results. Human BAT is molecularly similar to the interscapular brown adipose tissue (iBAT) of rodents in constitutive UCP1 expression, myogenic origin, and homogenous multilocular morphology (42,43), additional support for using this model.

Importance of Sex Differences in Experiments

In 2014, the NIH began instating policies to require a balance of male and female animals in preclinical studies. These policies were instated due to the different reactions to treatments from sexes. Studies also found that the evaluation of sex differences may bridge the gap between animal studies and human work (49). There is a significant dimorphism with fat gain in humans. Women need a significantly higher percentage of total body fat than men. In addition, women and men gain weight in different places of the body. White adipose tissue is stored between two major fat depots in people of a normal weight, subcutaneous and visceral adipose tissue (50). Subcutaneous adipose tissue is stored under the skin and its presence is not associated with adverse health effects. Visceral adipose tissue, however, is located in the trunk of the body and surrounds organs. Its presence is considered to be a health risk because it increases risk of disease, like metabolic syndrome (51). Men typically suffer from android obesity, a condition in which adipose tissue is distributed mainly at the abdomen and chest (sometimes called "apple" shape). Women, however, usually gain fat primarily in the hips and thighs, a condition classified as have gynoid obesity (sometimes called "pear" shape) (52). Android obesity is heavily correlated with increased health risk compared to gynoid obesity (53). Studies have found that a larger waist circumference is associated with higher prevalence of undiagnosed diabetes and undiagnosed dyslipidemia (54). This sexual dimorphism was also observed in a study involving rats; a study observed that the body weight of adult male rats increased faster than female rats (55).

Chapter 2

Hypothesis and Aims

Previous studies have found VA supplementation to affect metabolic regulation, such as glucose regulation, insulin sensitivity, and thermogenesis in well-nourished animal models (35,39). Other studies have indicated retinoic acid (RA), a metabolite of VA, to be a regulator of the expression of mitochondrial uncoupling proteins, proteins that promote thermogenesis (39). Therefore, there is reason to hypothesize that VA supplementation may increase metabolic health by promoting thermogenesis, specifically through uncoupling proteins (UCP1 in BAT, and UCP3 in muscle). We hypothesized that treatment with VA in the neonatal period would have both 1) short-term effects during the early neonatal period, and 2) a possible carry-over, or priming, effect in young adolescent age using the rat model. Therefore, our aims were to evaluate body weight (by age and sex) and VA concentration in BAT, and the expression of a thermogenesis-related gene, UCP1, also in BAT, at neonatal age (postnatal day 12; P12) and young adolescence (5 weeks old).

Chapter 3

Materials and Methods

Animal Experiment

Diet and animal model

At the beginning of the experiment, adult Sprague-Dawley (Charles River Laboratories) males and females were mated on a high-fat vitamin A-marginal (HF VAM) diet. Table 1 shows the composition of the purified diet used in this experiment. The newborn rat pups were randomly placed into the three treatment groups: placebo, VA, or VARA. Additionally, the rat pups were randomly selected to be euthanized at either P12 or 5 weeks of age. Table 2 shows how the total population of rats was divided into different treatment groups.

Dose administration

Pups were weighed prior to each dose administration and the dose was calculated in proportion to body weight (0.4 µl/g of body weight). All doses were administered orally. The VA dose consisted of retinyl palmitate (6 mg/kg) delivered in canola oil. The VARA dose was the combination of retinyl palmitate (6 mg/kg) and 10% RA (0.6 mg/kg of body weight) suspended in canola oil. The placebo contained only canola oil.

Sample collection and tissue analysis

Once the pups reached P12 or 5 weeks of age, they were euthanized with carbon dioxide. The body weight and blood glucose were measured. The blood was drawn from the abdominal vena cava for the purpose of serum preparation. The body composition was measured at age 5

weeks by using a DEXA. Multiple types of tissues, including BAT, were dissected into tubes and immediately frozen in liquid nitrogen. Figure 2 demonstrates the location of BAT in Sprague Dawley rats. The BAT was differentiated from muscle through tissue density at the time of tissue collection.

Table 1. The composition of the diet utilized in this experiment. The high-fat VA-marginal diet was a purified diet designed for the study. The diet contains 45% fat by kcals and a marginal level of VA (0.35 μg retinol as retinyl palmitate per g diet).

Diet name	**High-Fat VA-Marginal**	
	gm%	**kcal%**
Protein	24.2	20.3
Carbohydrate	41.4	34.7
Fat	23.9	45.0
Total		100
kcal/gm	4.78	
Retinyl Palmitate, 500,000 USP/g	0.00275	0

Table 2. Sample size and treatment group breakdown. Pups were evenly separated into the 4 groups: three treatment groups and one control (normal chow diet). The groups were selected independently and randomly and comprised of pups from each litter. All treatments were calculated to be 0.4 µl/g of body weight and were administered orally.

Group #	**Total *n***	**Treatment and Period**			**Analysis times**	
		P0/1	**P4**	**P10**	**Neonatal (P12)**	**Adolescent (5 weeks)**
1	n=31	Placebo	Placebo	Placebo	n=18 (8M, 10F)	n=12 (7M, 6F)
2	n=31	VA	VA	VA	n=19 (9M, 10F)	n=12 (6M, 6F)
3	n=32	VARA	VARA	VARA	n=20 (9M, 11F)	n=12 (5M, 7F)
4 (control)	n=12	n/a	n/a	n/a	n/a	n=12 (5M, 7F)

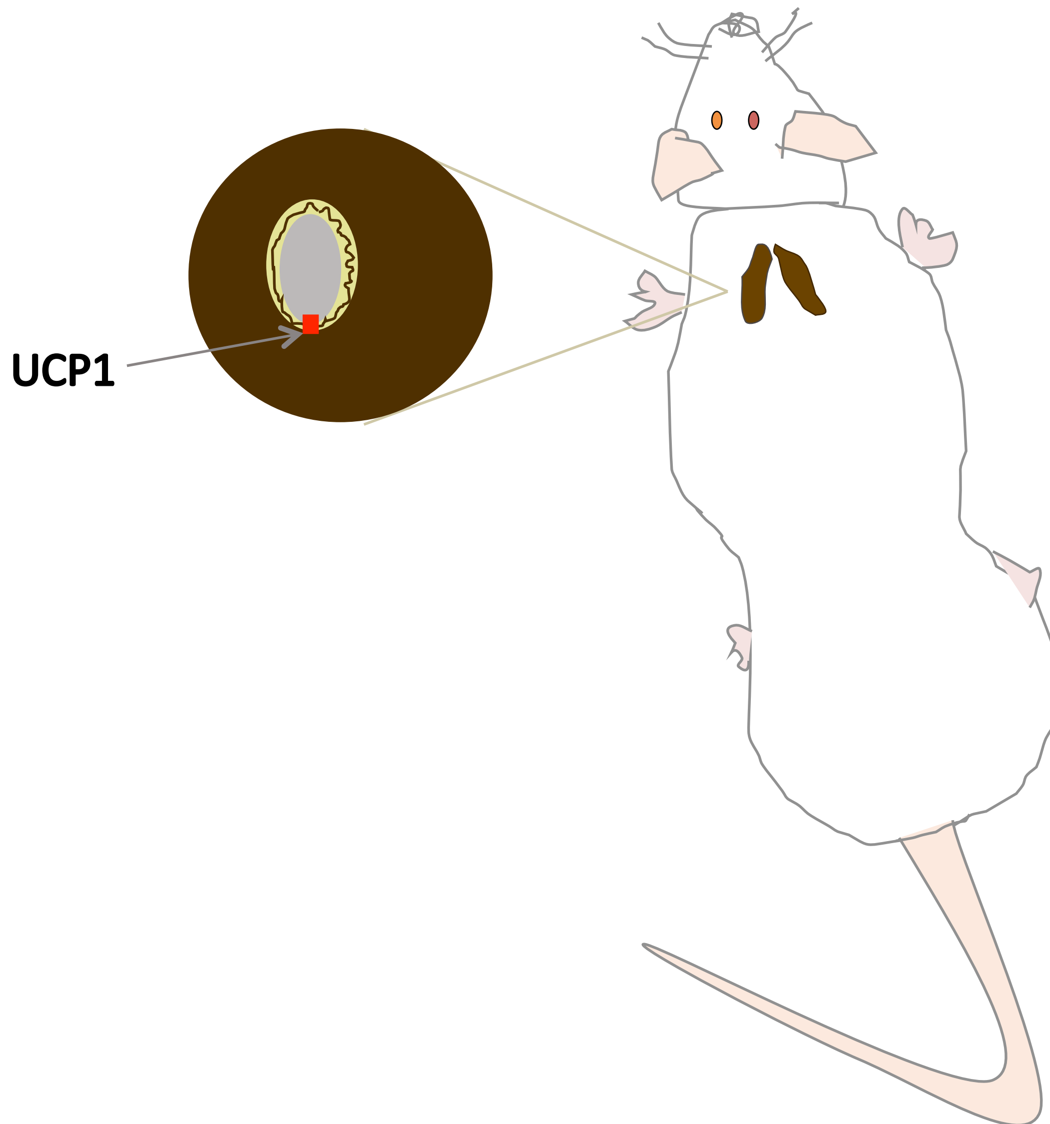

Figure 2. The location of BAT. A large deposit of BAT is found in the interscapular region of rodents, represented by the brown color on the rat figure. The color of BAT is similar to skeletal muscle, but is quite different in texture and can be easily distinguished from muscle. through the texture differences.

Polymerase Chain Reaction

The total RNA was extracted from the BAT samples using the TRIzol® (Life Technologies) method. The cDNA was prepared through reverse transcription and the mRNA expression was measured using (q)RT-PCR.

RNA Isolation

The TRIzol method was followed in order to isolate the total RNA from the BAT samples. Approximately 100 mg of each sample was added to 1 ml of TRIzol reagent before the tissue was thawed to prevent degradation. The tissue samples were homogenized for 1 minute and were incubated at room temperature for 5 minutes to permit complete dissociation of nucleoprotein complexes. The volume of tissue used did not exceed 10% of the TRIzol volume used for homogenization. Then, 200 µL of chloroform was added per 1 mL of TRIzol reagent used in homogenization, and the tubes were shaken vigorously for 20 seconds and then left at room temperature for 2 to 3 minutes. Next, the sample tubes were placed in a 4˚C centrifuge at 12,000xg for 15 minutes to separate the mixture into 3 phases: a phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains solely in the upper aqueous phase; therefore, the upper phases was isolated and transferred to new centrifugation tubes with great care to prevent the transfer of other phases. The remaining phases were discarded.

Next, 500 µl of 100% isopropyl alcohol were then added per 1 ml of TRIzol reagent in order to precipitate the RNA from the aqueous phase. The isopropyl alcohol and aqueous phase were combined by gently inverting the tubes for 20 seconds and left at room temperature for an additional 10 minutes. The samples were centrifuged again at a 4°C centrifuge at 12,000xg for 10 minutes to precipitate the RNA and form a pellet on the bottom of the tube. The supernatant

was removed and the pellet was rinsed with 75% ethanol and air-dried for 10 minutes. The RNA pellet was then reconstituted with 100µl DEPC-treated water and stored in an -80°C freezer.

RNA purification

RNA was purified through the use of the RNeasy® Mini Kit (QIAGEN Group). First, 350 µl of Buffer RLT, provided by the RNeasy kit, were added to the reconstituted RNA pellet (100 µl) and mixed well. Next, 250 µl of ethanol was then added to the diluted RNA and mixed together through pipetting. The sample was immediately transferred to the RNeasy Mini spin column and 2 ml collection tube to be centrifuged for 15 seconds at 8000xg. The flow-through was immediately discarded.

Next, the RNeasy spin column was washed and rinsed with 500 µl of Buffer RPE twice and the flow-through was discarded. Finally, 50 µl of DEPC-treated water were added to the spin column membrane to elute the RNA. The purified RNA was then stored in a -80°C freezer. The total RNA sample purity and concentration was tested with a NanoDrop 1000 Spectrophotometer. The ratios of 260/280 ranged from 2.10 to 2.18 for all samples.

cDNA synthesis and PCR analysis

The purified total RNA samples were removed from the freezer and thawed on ice. The samples were then mixed well and spun down twice and placed on ice while master mix 1 solution was prepared (Table 3). Next, 5 µl of master mix 1 solution and 10 µl of purified total RNA were added to each tube. The tubes were mixed well, spun down, and incubated at 70°C for 5 minutes. Next, the tubes were incubated on ice for at least 2 minutes while master mix 2 solution was prepared (Table 4). Then,10 µl of master mix 2 solution was added to each tube while the tubes were sitting on ice. The tubes, which contained the total of 25 µl solution, were mixed well and spun down. The tubes were incubated according to a preset program in order to

synthesize the cDNA (Table 5). The cDNA samples were then diluted and placed in a -20°C freezer.

The cDNA samples were then combined with master mix 3 solution (Table 6) in individualized wells of a PCR plate (see Figure 2 for arrangement). The master mix 3 contained the appropriate primer for the gene being sequenced. See Table 6 for the composition of the master mix 3 and see Table 7 for the gene sequence of the primers used. The plate was centrifuged at 1600xg for 3 minutes at 4°C. The plate was then placed in the StepOnePlus PCR machine for replication and analysis.

Table 3. Master mix 1 solution for cDNA synthesis. 1X indicates the composition of the total mixture in each tube used to make the cDNA.

	1X
dH_2O (DEPC-treated)	3.55 µl
Total RNA	10 µl
Oligo dT (0.5 µg/µl; PROMEGA)	0.2 µl
dNTP (10 mM each; PROMEGA)	1.25 µl
Total amount	15 µl

Table 4. Master mix 2 solution for cDNA synthesis. 1X indicates the composition of the total mixture in each tube used to make the cDNA.

	1X
dH_2O (DEPC-treated)	3.5 µl
5x Buffer (cDNA synthesis buffer; PROMEGA)	5 µl
RNasin (40 U/µl; PROMEGA)	0.5 µl
Reverse Transcriptase (200 U/µl; PROMEGA)	1 µl
Total amount	10 µl

Table 5. Preset program for cDNA synthesis. The machine used preprogrammed settings to synthesize the cDNA.

Step	Temperature	Time
1	42°C	90 min
2	95°C	5 min
3	4°C	Unlimited (30 min)

	1	2	3	4	5	6	7	8	9	10	11	12
A	1a	8	15	26	34	42	50	62	70	81	91	99
B	1b	9	16	27	35	43	51	63	71	82	92	100
C	2	10	20	29	36	44	52	64	72	83	93	101
D	3	11a	21	30	37	45	53	65	73	84	94	102
E	4	11b	22	31a	38	46	54	66	74	85	95	103
F	5	12	23	31b	39	48	57	67	75	86	96	104
G	6	13	24	32	40	49a	60	68	78	88	97	105
H	7	14	25	33	41	49b	61	69	80	90	98	106

Figure 3. PCR 96-well plate setup. The figure shows the arrangement of samples on the StepOnePlus PCR plate. Samples 1, 11, 31, and 49 were duplicated to ensure continuity between the two PCR runs.

Table 6. The composition of master mix 3. 15 µl of this mix was used with 5 µl of sample in the StepOnePlus PCR machine.

	1X
SYBR Green (PROMEGA)	10 µl
dH_2O (DEPC-treated)	4 µl
Primer (UCP1 or β-actin)	1 µl
Total Volume	15 µl

Table 7. The gene sequence of the primers designed for PCR amplification. The forward and reverse gene sequences of the primers for UCP1 and the housekeeping gene, β-actin, are listed as followed.

Gene		Gene sequence of the primers
UCP1	FORWARD	5'-GGC CAA GAC AGA AGG ATT GC-3'
	REVERSE	5'-GAC AAG CTT TCT GTG GTG GC-3'
β-actin	FORWARD	5'-GCT TCG CGG GCG ACG ATG-3'
	REVERSE	5'-TCT CTT GCT CTG GGC CTC G-3'

Ultra Performance Liquid Chromatography

All lights were turned off and window shades were drawn while working with BAT in order to prevent VA degradation that naturally occurs with light. The BAT samples were weighed and cut into 0.1 g pieces and placed in tubes that contained 1 mL ethanol and 6 beads for the preparation of homogenization (Bead Ruptor 12; OMNI International). Samples were homogenized and the tubes were rinsed with 1 mL ethanol, followed by one-hour extraction in room temperature. Then, 100% KOH and 20% pyrogallol were added to each tube and mixed well. Samples were then incubated in a 55°C water bath for 30 minutes and were then taken out and cooled to room temperature. Once cooled, 4 mL of hexanes and 2 mL of distilled water were added to each sample and mixed well. Next, the samples were centrifuged at 1600 rpm at 20°C for 15 minutes. Centrifugation separated the samples into two phases and the upper hexanes phase (about 3.5 mL) was transferred into a 7 mL scintillation vial. A known concentration of internal standard, trimethylmethoxyphenyl-retinol (TMMP-retinol), was added to each sample. The mixture of hexanes and TMMP-retinol was dried using nitrogen gas in a 37°C. It was important to ensure that the hexanes had completely evaporated in the drying process in order to ensure an accurate retinol content. Finally, 100 µL of methanol was added for reconstitution of the total retinol (ROH) content. The samples were loaded into the AcquityTM UPLC instrument and processed.

The ROH concentration (pmol/g) in each BAT sample was determined through a series of calculations. Using the known TMMP-retinol concentration (pmol) and area under the curve (AUC) of both TMMP-retinol and ROH determined by UPLC, the ROH concentration (pmol) can be computed using the following ratio:

TMMP-retinol concentration/TMMP-retinol AUC = ROH concentration/ROH AUC

ROH (pmol/g) of tissue was calculated using the computed ROH concentration (pmol) and the weights of tissue of BAT sample used.

Chapter 4

Results

Changes in Body Composition

No differences in growth rate were found between the diets and treatment groups; on average, all rats grew continuously at an exponential rate. The similarities in growth rate between each group signify success in randomization of the subjects into each group. Figure 4 shows the similar increase in body weight throughout all treatment groups. Body composition was performed on the 5 weeks age group, using a DEXA machine. The average body fat percentage of each rat had increased in all rats on the HF VAM diet (regardless of treatment) compared to the normal chow diet. No differences were noted between the three treatment groups. In addition, no differences were noted between the two sexes. This result was expected due to fat's higher energy density in relation to carbohydrates. Figure 5 illustrates the relationship between each treatment group and sex and the percent body fat of the rats at 5 weeks of age.

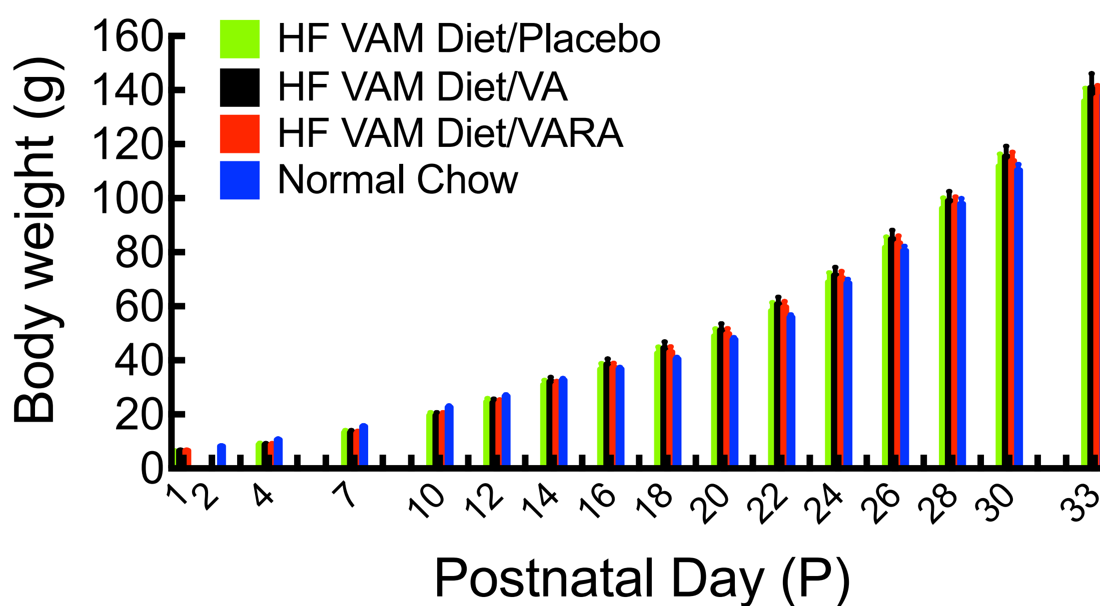

Figure 4. Change in body weight with respect to age and treatment group. The graph disregards sex and age of euthanasia and shows the change in weight of the subjects overall. Bars (mean ± SEM). *n*=106.

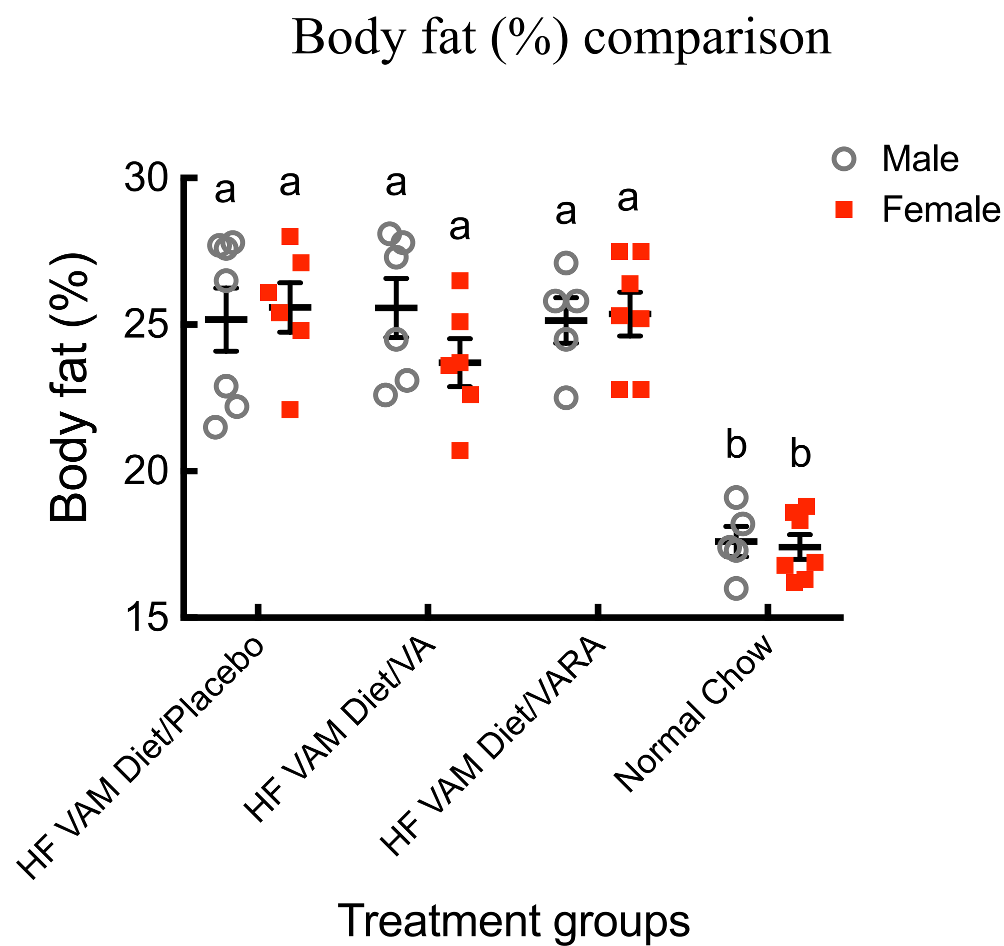

Figure 5. The percent body fat of the rats at 5 weeks of age. There was no significant difference between all three treatment groups (placebo, VA, and VARA) and sex. Bars (mean ± SEM). Different letters denote $p<0.05$ by two-way ANOVA and post-hoc test. *n*=49.

Blood Glucose Concentration

There were no statistically significant differences between the blood glucose concentrations of any treatment groups within the P12 group. In addition, no significant differences were found between any of the treatment groups within the 5 weeks group. The VARA treatment group within the 5 weeks group was significantly different from every P12 treatment group. Furthermore, the P12 placebo group was significantly different from the VA, VARA, and normal chow groups of the 5 weeks groups. Figure 6 shows the differences in blood glucose concentration in relation to the different treatments.

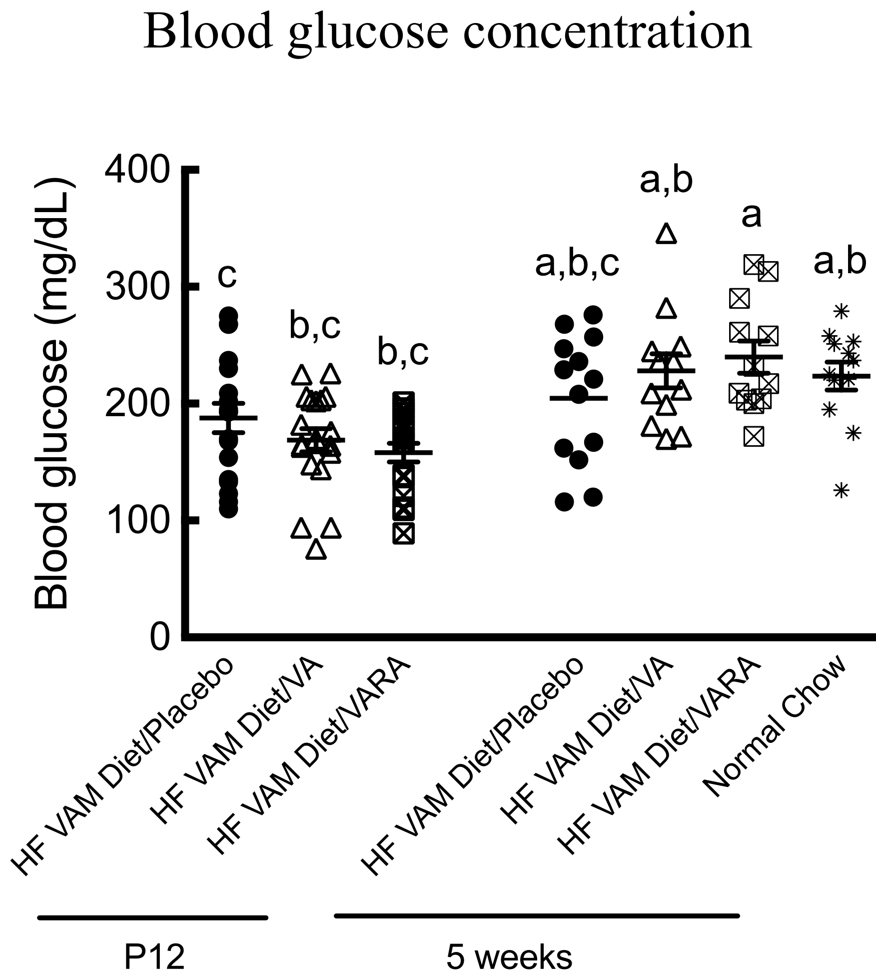

Figure 6. The blood glucose concentration at P12 and 5 weeks. This figure includes subjects from both P12 and 5-week. One-way ANOVA test was performed in the figure. n=106.

Tissue Total Retinoid Results

In P12 rats, both the VA and VARA treatment groups had significantly higher concentrations of retinol in the BAT than the placebo group. This finding is not surprising given that rats were dosed two days prior to euthanasia in the P12 group. In the 5 weeks, group, however, no differences were noted between any of the treatment groups. In addition, no differences were found between males and females in the P12 group or the 5 week group. Figure 7 and 8 show the differences in retinol concentrations between the three treatment groups at P12 and 5 weeks, respectively.

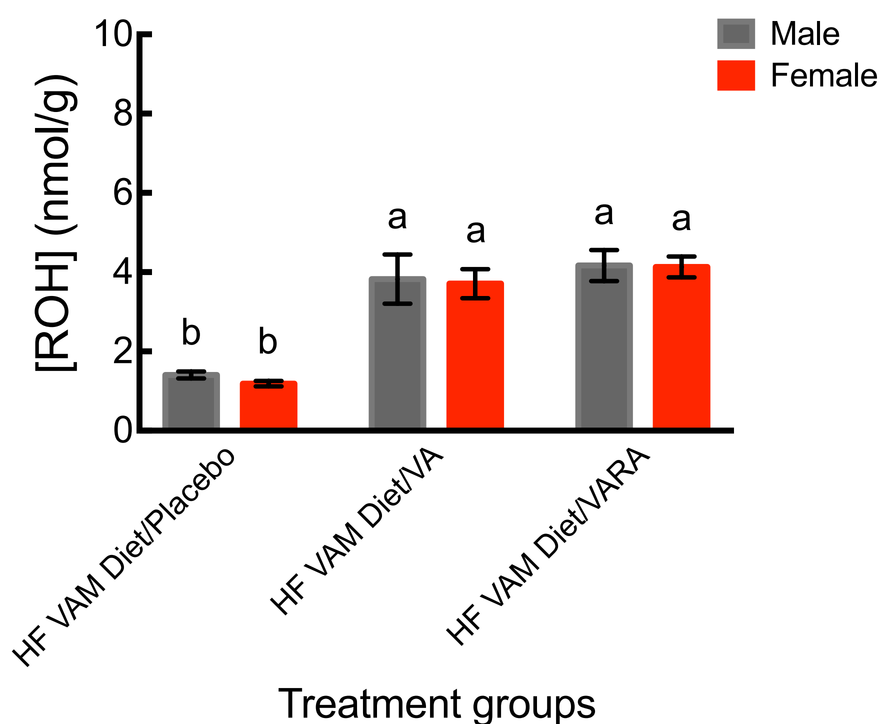

Figure 7. Comparison of BAT retinol concentration at P12. No significant difference was found between sexes but the placebo group had significantly lower levels of retinol compared to the other two treatment groups. Bars (mean ± SEM). Different letters denote $p<0.05$ by two-way ANOVA and post-hoc test. $n=56$.

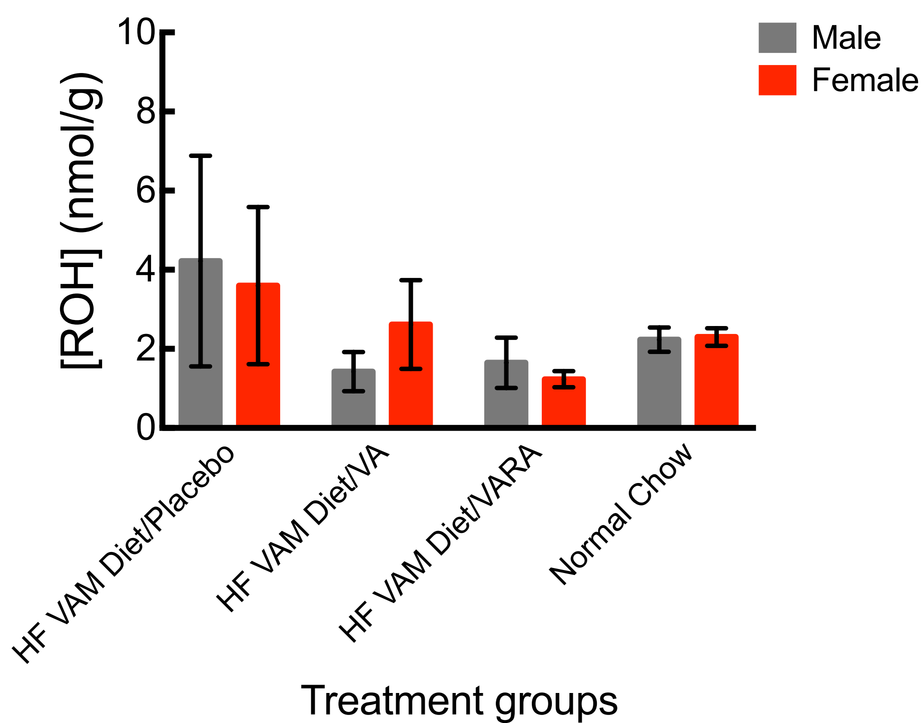

Figure 8. Comparison of BAT retinol concentration at 5 weeks. No significant differences were found between the sexes or treatment groups. Bars (mean ± SEM). n=49.

Gene Expression Results

The group containing rats on the HF VAM Diet and placebo treatment at 5 weeks of age was used as the standard of "1" in comparing the UCP1 expression in BAT. That group was selected as the standard as the UCP1 expression was expected to be the lowest in this group in comparison to the other groups. In the P12 group, expression of UCP1 in the VA treatment group was significantly lower than the placebo group. There was no significant difference between the placebo and VARA groups or between the VA and VARA groups. The results from the 5 weeks group were similar to the P12 group. There was a significant difference between the VA treatment group and the placebo at 5 weeks, while there was no difference between the placebo and VARA groups or between the VA and VARA groups. Figure 9 and 10 show the gene expression results of the PCR and the changes in UCP1 expression between the three treatment groups at P12 and 5 weeks of age, respectively. No differences were noted between sexes.

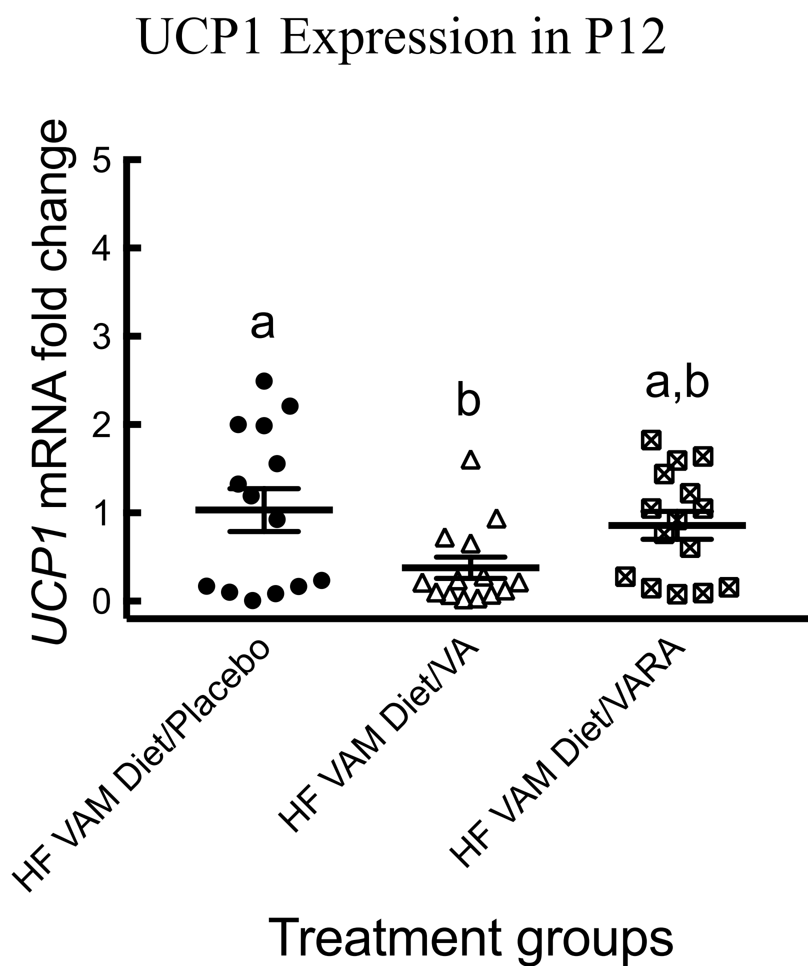

Figure 9. BAT UCP1 expression in P12. The VA treatment is significantly different from the placebo group. Different letters denote $p<0.05$ by ordinary one-way ANOVA and post-hoc test. Bars (mean ± SEM). $n=56$.

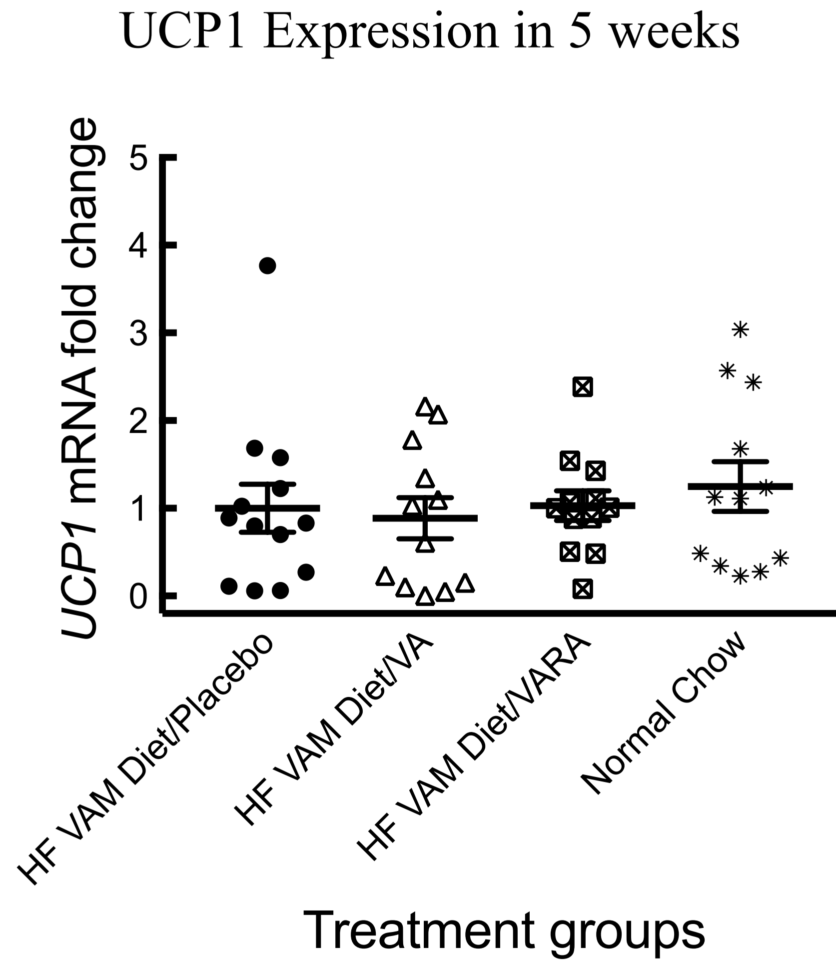

Figure 10. BAT UCP1 expression in 5 weeks. No significant differences were found between groups by ordinary one-way ANOVA. Bars (mean ± SEM). n=49.

Chapter 5

Discussion

Two different diets were used in this experiment: HF VAM and normal chow. In the HF VAM diet, 45 percent of total calories come from fat and the fat percentage in the chow diet is significantly lower than the HF diet. Unfortunately, the production of the normal chow diet is unregulated and there is much variation between batches. There is no way to know the specific macronutrient distributions in the normal chow diet or the source of macronutrients; some batches may be higher in fiber than other batches. While all rats gained weight at a steady rate, independent of diet, the rats eating a diet high in fat had a higher fat mass compared to rats eating a normal chow diet. The HF diet is more similar to a typical child's diet, where the recommendation is 30-40% of calories from fat. This finding also highlights the importance of macronutrient distribution in a child's diet. In order to maintain a healthy body composition and fat mass, calories and fat intake should be held in consideration - not just calories alone. The importance of macronutrient distribution could be tested by decreasing the fat composition of the rats' diet after inducing adiposity through the HF diet to see if body fat percentage decreases. Our study analyzed the body fat composition of the 5 weeks group, but not the P12 group. Future studies could benefit from analyzing the body fat in the P12 to see if the HF diet increases body fat percentage at an early age.

Blood glucose findings were very irregular and there was a lot of variation within groups. The results did not lead to impactful trends or data with regards to diet. The most significant finding in blood glucose was the difference in blood glucose concentration between ages. The 5

weeks groups had a significantly higher blood glucose concentration compared to the P12 groups. There were no significant correlations noted between blood glucose levels and the treatment groups. Concentration of blood glucose varies constantly throughout the day and depends on time of meal, composition of meal, and level of activity. The rats' activity level was approximately the same in all groups and meal composition was separated into the two diets. The major difference between all groups is the time of meal. Because the rats had constant access to food throughout the entire experiment, there was no way to know when the rats last ate. Despite the fact that all rats' blood glucose levels were tested at approximately the same time, the rats could be at different stages of a meal (postprandial, post absorptive, etc.) and could give different and inaccurate blood glucose readings on the portable glucometer. Glucose readings could be more accurate if food access was restricted so that the rats' stage of meal could be known and tested at the same time.

There was a significant difference between BAT retinol concentrations in both the VA and VARA treatment groups compared to the placebo group in P12 rats. The rats that were treated with either VA or VARA had significantly higher retinol levels than the rats treated with the placebo. This result tells us that VA is reaching the BAT and being absorbed shortly after supplementation. The 5 weeks group, however, displayed no significant differences between any of the treatment groups. Because euthanasia in this group occurs approximately 3 weeks after the last dose of VA, the VA had time to mobilize from the tissues and to be excreted from the body. This tells us that BAT is not a storage tissue for retinol, but because the tissue absorbed the VA shortly after supplementation, BAT may be a transient absorptive tissue for VA. In addition, no differences between sexes were noted in either age group.

We observed a treatment effect for VA in the BAT at P12, but there was no difference between the VA and VARA groups. This suggests that RA did not have an effect and the treatment effect was most likely from VA itself in storage. While there was a significant difference between the VA group and the placebo at P12, the UCP1 mRNA expression did not increase- it decreased. This finding refutes our hypothesis. In addition, VARA supplementation did not increase expression of the UCP1 and therefore, refuted our hypothesis.

There were no increase in UCP1 mRNA expression at 5 weeks, but this is expected as there was no increase in UCP1 expression in the P12 group. In addition, the treatment effect of UCP1 mRNA fold change in the VA group disappeared at 5 weeks. This result could be due to the mobilization of VA out of the BAT or the expansion of BAT mass. In this experiment, we did not weigh the total BAT collected from each rat, so we cannot deduce if the BAT mass expanded. The UCP1 expression results from the P12 group indicated that the treatment effect was the result of an increase of storage of VA after supplementation two days prior to euthanasia; therefore, one could deduce that the disappearance of the treatment effect in the 5 weeks group may be due to mobilization of VA stores because the last treatment occurred approximately 3 weeks prior to euthanasia. Future experiments could benefit from weighing the BAT after euthanasia in order to determine if the quantity of BAT increased after treatment.

This experiment determined that BAT can transiently absorb VA, but BAT is not a storage tissue for VA. We did There were no significant differences noted in the 5 weeks age group, indicating that the effects of treatment did not carry over into young adolescence.

Chapter 6

Conclusions

While this experiment was successful in finding that BAT is a tissue capable of VA absorption, we found that VA or VARA supplementation did not increase UCP1 mRNA expression in BAT. In addition, this experiment found that a HF diet can increase body fat percentage in the adolescent age group. The experiment refuted our hypothesis because we did not see an increase in UCP1 mRNA expression in BAT at age groups P12 and 5 weeks. This finding indicates that VA or VARA treatments did not increase expression of UCP1 in the neonatal period and did not have a priming effect in early adolescence. Through this experiment, we found that BAT is not a storage tissue for VA, but may transiently absorb VA. While the results our experiment did not support our hypothesis, the findings provided insight on the effects of the HF diet. We found that rats on the HF VAM diet had a higher body fat percentage in comparison to the rats eating a normal chow diet. This finding has implications for the effect of macronutrient distribution of a child's diet on body fat percentage and should be tested further. We also found that the 5 weeks group had a higher level of blood glucose than the P12 group, but there was significant variation within groups. Future studies could benefit from regulating feeding times and glucose readings to standardize the rats' stage of absorption. In addition, future studies could also find analysis of neonatal body composition to be informative.

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