RELATIONSHIP AMONG DIETARY FATS, FATTY ACID PROFILE AND EXPRESSION OF GENES INVOLVED IN TESTES FUNCTION IN ZUCKER (*fa/fa)* RATS

By

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Abstract

Six week old male obese *fa/fa* Zucker rats (n=10/group) were fed four different diets enriched in linoleic acid (LA), α-linolenic acid (ALA), eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) (safflower oil, flaxseed oil, EPA, and DHA oils, respectively) for 8 weeks. Fatty acids were analyzed in major lipid classes in the testes. Global gene expression was analyzed using the Affymetrix Rat Gene 2.0 ST Array. Annotated gene sets from the normal and underdeveloped testes were analyzed using Ingenuity Pathway Analysis (IPA). In lipid analysis, n-3 diet supplementation decreased n-6: n-3 polyunsaturated fatty acid and n-6: n-3 very long chain fatty acids (VLCFA) in most lipid classes in comparison to the LA diet. ALA increased the level of DHA, but not to the same level as DHA diet. Compared to the normal sized testis, the underdeveloped testis showed a marked decrease in n-6 pentaenoic PUFA and VLCFA while increasing n-6 tetraenoic fatty acids. Out of the 3192 genes detected, 1121 and 309 were differentially expressed in the underdeveloped and normal testes, respectively. The IPA indicated that transcripts that are upregulated in the normal testes relative to underdeveloped testes are involved in triacylglycerol biosynthesis, sphingomyelin metabolism and phosphatidylglycerol biosynthesis. Transcripts upregulated in underdeveloped testes relative to normal testes are involved in production of nitric oxide and reactive oxygen species. Downstream effect analysis showed an increased trend towards reproductive system diseases and endocrine system disorders in the underdeveloped testes compared to the normal testes. In conclusion, these results indicate that testicular lipids and their metabolism are closely related with normal testis development and function.

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AA	Arachidonic acid
ABP	Androgen-binding protein
AGPAT1-3	Acylglycerol-3-phosphate O-acyltransferase 1-3
AKT1	V-akt murine thymoma viral oncogene homolog 1
ALA	α-linolenic acid
ANOVA	Analysis of variance
A2M	Alpha-2-macroglobulin
APOE	Apolipoprotein E
BF3	Boron trifluoride
BMI	Body mass index
CDH2	Cadherin 2, type 1, N-cadherin (neuronal)
CE	Cholesterol ester
CGT	Ceramide galactosyltransferase
C7	Complement component 7
C1S	Complement component 1, s subcomponent
CP	Carbonyl protein
CST	Cerebroside sulfotransferase
Cx43	Connexin 43
D6D	Delta 6 desaturase
DHA	Docosahexaenoic acid
Dgat2	Diacylglycerol acyltransferase
DNA	Deoxyribonucleic acid
DPA	Docosapentaenoic acid

DnHP	Di-n-hexyl phthalate
E2	Estradiol
ELOVL-2	Elongation of very long chain fatty acids protein 2
EPA	Eicosapentaenoic acid
FABP	Fatty acid binding proteins
FADS-2	Fatty acid desaturase 2
FFA	Free fatty acids
FSH	Follicle stimulating hormone
GC	Gas Chromatography
GC-FID	Gas Chromatography-Flame Ionization Detector
GnRH	Gonadotropin-releasing hormone
GPR54	Kisspeptin/G protein coupled receptor
GSN	Gelsolin
INSL3	Insulin like factor 3
IL33	Interleukin33
iOAT	Idiopathic oligoasthenoteratozoospermic
IPA	Ingenuity Pathway Analysis
LA	Linoleic acid
LCFA	Long chain fatty acids
LH	Luteinizing hormone
LPCAT4	Lysophosphatidylcholine acyltransferase 4
MAP2K4	Mitogen-activated protein kinase kinase 4
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non alcoholic fatty liver disease

NCBI	National Centre for Biotechnology Information
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NRF2	Nuclear factor (erythroid-derived 2)-like 2
OMP	Olfactory marker protein
OSMR	Oncostatin M receptor
PAP	Prostatic acid phosphatase
PC	Phosphatidylcholine
PE	Phosphotidylethanolamine
Plin2	Perilipin
PL	Phospholipid
PLPLA2	Patatin-like phospholipase domain containing 2
PSA	Prostate specific antigen
PUFA	Polyunsaturated fatty acids
qRT-PCR	Quantitative real time polymerase chain reaction
RBL2	Retinoblastoma-like 2
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SGMS1	Sphingomyelin synthase 1
SHBG	Sex hormone binding globulin
SMPD2	Sphingomyelin phosphodiesterase 2
StAR	Steroidogenic acute regulatory protein
TAF7L	TAF7-like RNA polymerase II
TBX2	T-box 2
T4	Thyroxine

TG	Triglyceride
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A
VLCFA	Very long chain fatty acids
WC	Waist circumference
WHO	World Health Organization

CHAPTER I: LITERATURE REVIEW

Introduction

Male infertility is a commonly occurring yet not as extensively studied phenomenon. World Health Organization (WHO) defines male infertility as the failure to conceive following twelve months or more of unprotected sexual intercourse (WHO, 2009). The major organs responsible for male fertility are the testes. The main function of testes is steroidogenesis and spermatogenesis.

Normal function in testes is affected by a number of factors such as environmental toxins (Taxvig et al., 2013; Wang et al., 2015), disease conditions (Hadziselimovic & Herzog, 2001; Tvrda et al., 2015), and dietary influences (Merrells et al., 2009; Suh et al., 2011). More recently, obesity has become another contributing factor to abnormal testes function. Statistics Canada has reported an alarming 59.9% of males in Canada as obese in the year 2012 (Health Canada, 2012). A body mass index (BMI) in the range of 30-34.9, 35.0-39.9, and above 40 has been denoted as obese class I, II and III respectively, associated with an increasing risk of health problems (Health Canada, 2012). Studies show an inverse correlation between BMI, waist circumference (WC) and corresponding semen volume (Eisenberg et al., 2014). A similar trend was observed between increase in BMI and WC and reduced sperm count and motility (Hammiche et al., 2012; Jensen et al., 2004). Considering the increase of obesity worldwide, more attention to male reproductive health is warranted.

Recent evidence suggests that poor semen quality may be a linked to altered lipid metabolism. Abnormal lipid metabolism can result in an unstable sperm membrane (Bjorkgren et al., 2015). For example, decreased levels of polyunsaturated fatty acids (PUFA) are seen in the phospholipids of cryptorchid testes, with a specific decrease of

docosapentanoic acid (DPA, C22:5n-6) in rat testes (Furland et al., 2007a). Similarly, reduced levels of docosahexanoic acid (DHA, C22:6n-3) were observed in sperm samples of patients suffering from oligozoospermia (Zalata et al., 1998). However, our understanding of lipid metabolism in this organ is limited in relation to its function. A previous animal study conducted in our laboratory showed that obese *fa/fa* Zucker rats often had an unbalanced paired testes, leaving one of the testes underdeveloped. The underdeveloped testes showed a specific decrease in DPA, while maintaining its precursor arachidonic acid (AA, C20:4n-6) (Suh et al., 2011). Studying this obese animal model with the normal and underdeveloped testes will reveal the metabolic changes taking place, and the condition of lipid metabolism in the underdeveloped testes compared to the normal testes. This study also aims at studying the effect of dietary n3 and n6 oil supplementation on testes lipid composition. This chapter will review the male reproductive system, the effect of obesity on male reproduction, and how diet and gene expression can modify testes function.

Male reproductive system: Structure and function

General reproductive structure and function

The testes have two main functions: steroidogenesis and spermatogenesis. Sperm production takes place in the seminiferous tubules, from where the sperms are transported to the epididymis, where sperm maturation takes place and the sperms achieve motility (Shalet, 2009). The sperms are then either stored in the epididymis or transferred to the vas deferens prior to ejaculation. Seminal vesicles produce seminal fluid, which provides a medium and optimum pH for sperms, in addition to providing energy (Tortora & Derrickson, 2014). The prostate is responsible for producing certain enzymes that have a

proteolytic activity, such as prostate specific antigen (PSA), and prostatic acid phosphatase (PAP), which help in sperm activation (Drake et al., 2009).

Testes structure and function

The testes are contained in the scrotum, where regulation of temperature is facilitated by contraction and relaxation of the dartos muscles (Tortora & Derrickson, 2014). There are two coverings of the testes: tunica vaginalis and tunica albuginea. The tunica vaginalis forms a cavity that covers the testes during descent and stays as a protective layer. The tunica albuginea is a thick fibrous connective tissue that extends inwards to form the septa, which divide the testes into lobules. These lobes contain the seminiferous tubules that are the sites for spermatogenesis (deKretser & O'Donnel, 2000). The seminiferous tubules contain the germ cells for spermatogenesis, in addition to peritubular cells and Sertoli cells. The peritubular cells form a part of the covering of seminiferous tubules in human testicles in the form of concentric circles. They are responsible for contraction of the tubules and transport of sperm (Romano et al., 2005). They secrete proteins to aid in this function such as actin, myosin, panactin, desmin, and gelsolin (Holstein et al., 1996). Sertoli cells form a network from the basal membrane to the lumen of the seminiferous tubule, known as the blood testes barrier. This barrier functions as a protective sheath for germ cells and separates them from systemic circulation (Wong & Cheng, 2005). Sertoli cells themselves regulate spermatogenesis by producing cytokines, growth factors, opioids, steroids, and prostaglandins. The interstitial compartment contains Leydig cells, which are responsible for production of testosterone and insulin like factor 3 (INSL3) (Weinbauer et al., 2010). INSL3 was found to be responsible for activating the gene LGR8, and both these factors were associated with testicular descent (Kumagai et al.,

2002). The cellular structure of testes, the spermatozoa as well as the interstitial compartments are illustrated in Figure 1-1.

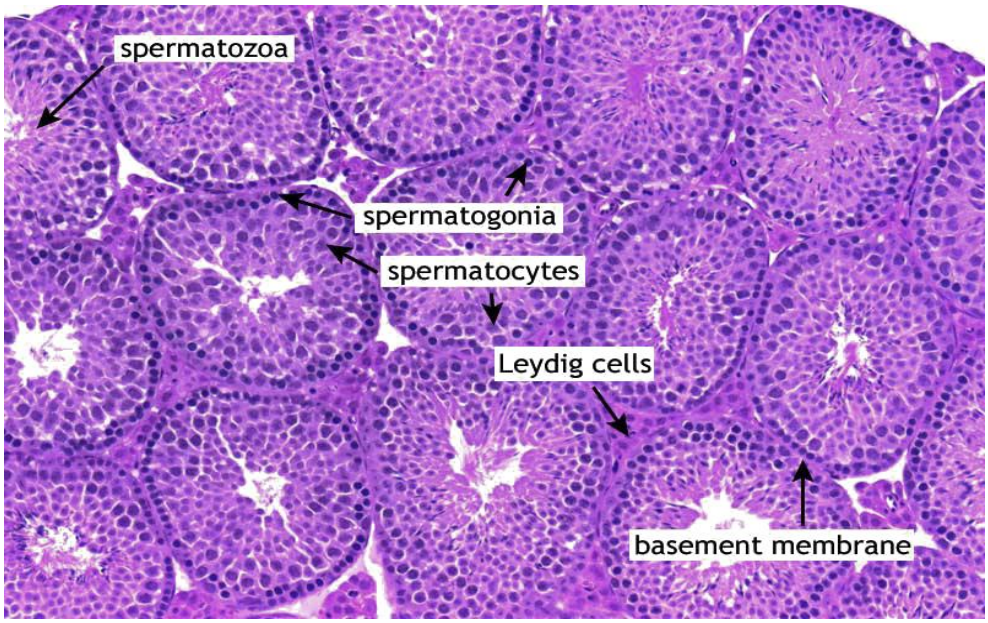

Figure 1-1: Histology of testes
Figure obtained from Deltagen, Inc. (San Mateo, CA, USA). Permission granted 27 July 2015 by Robert Driscoll, 1900 S. Norfolk St., San Mateo, CA, USA. (Deltagen, 2015)

Endocrine regulation of testes

Testicular function is controlled by hormonal regulation. Even the descent of testes is controlled by hormonal regulation, by the Leydig cells and secretion of INSL3 and testosterone (Bay et al., 2011). Testicular hormones are controlled by the hypothalamus. The release of gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary gland (Tortora & Derrickson, 2014). The LH stimulates the Leydig cells to produce testosterone and encourage spermatogenesis. The androgen binding protein (ABP) is responsible for binding to the testosterone and maintaining its

concentration in interstitial compartment (Tortora & Derrickson, 2014). FSH also acts on the Sertoli cells to initiate spermatogenesis (Tortora & Derrickson, 2014). Both LH and FHS are controlled by negative feedback mechanisms. Testosterone suppresses the secretion of GnRH, thereby controlling release of LH. Inhibin B is released by sustentacular cells located in the germinal epithelium, after the required degree of spermatogenesis has been achieved, which in turn inhibits FSH release (Weinbauer et al., 2010). This basic endocrine regulation is represented by Figure 1-2.

The GnRH release is regulated by the kisspeptin/ G protein coupled receptor (GPR54) system. This system is indicated to play a key role in activation of the hypothalamic-pituitary-gonadal axis at puberty (Rhie, 2013). Kisspeptin is a ligand that acts as a receptor for GPR54, and plays a key role in inducing GnRH secretion at puberty (Weinbauer et al., 2010). An increase of kisspeptin 54 in plasma is associated with an increase in levels of LH, FSH and testosterone in human males (Dhillo et al., 2005). In addition to this, impaired function of GPR54 has been indicated in hypogonadotropic hypogonadism (de Roux et al., 2003).

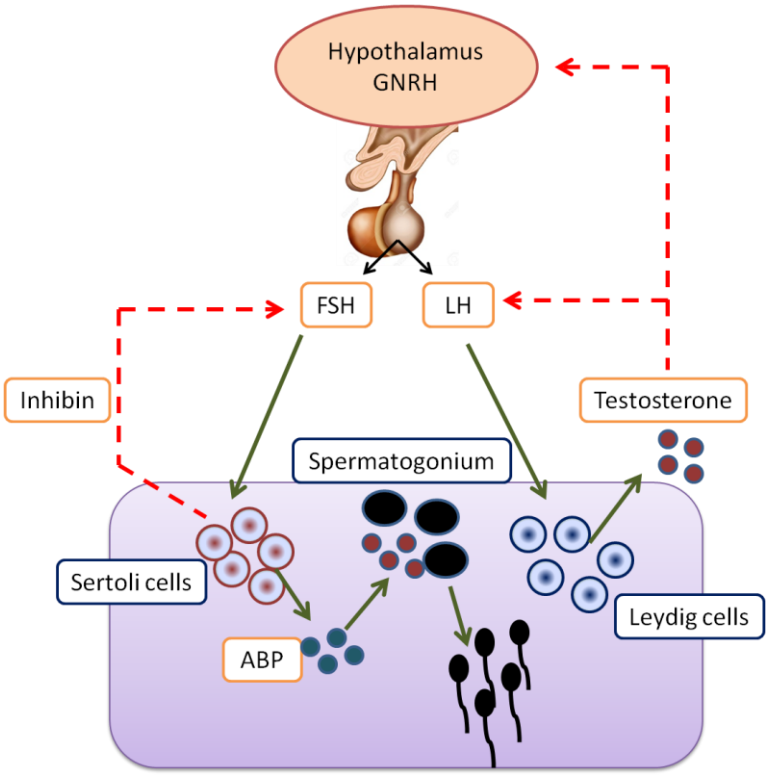

Figure 1-2: Endocrine regulation of testes
GNRH, Gonadotropin-releasing hormone; FSH, Follicle stimulating hormone; LH, leutinizing hormone; ABP, Androgen-binding protein

Spermatogenesis

Spermatogenesis begins with the germ cells present in the seminiferous tubules. It can be divided into four stages: 1. Mitosis and proliferation of germ cells; 2. Meiosis, where haploid germ cells (spermatids) are formed from tetraploid germ cells; 3. Development of spermatids into sperms (spermiogenesis); and 4. Entry of sperms into the tubular lumen (Weinbauer et al., 2010). Germ cells, or spermatogonia are of two types, A and B (De Rooij & Russell, 2000). The A type of spermatogonia are further divided into A pale and A dark spermatogonia. A dark are the stem cells and divide rarely. A pale divide more often to produce B spermatogonium. In rodents, the starting A stem cells are the ones that divide more often (Ehmcke & Schlatt, 2006). The B spermatogonium give rise to primary

spermatocytes by mitosis. The resulting haploid germ cells undergo meiosis to form secondary spermatocytes, and a second meiosis to form spermatids (Weinbauer et al., 2010). The final transition involves development of the round spermatids to sperm, by formation of the acrosome, flagellum, and multiplication of the mitochondria. This process is known as spermiogenesis (Tortora & Derrickson, 2014). In the last stage i.e. spermiation, the spermatids are released into the seminiferous tubule lumen (Weinbauer et al., 2010). The total duration of spermatogenesis including spermatogonia renewal is approximately 74 days in humans, 37-43 days in monkey species and 50 days in the rat (Amann, 2008; Weinbauer et al., 2010).

Sperm structure and sperm abnormalities

The sperm structure can be divided into the head, neck and tail. The nucleus is located in the head and contains the chromosomes (Tortora & Derrickson, 2014). The head is covered by the acrosome which helps the sperm to penetrate the zona pellucida (Liu et al., 2003). Sperm motility is determined by the development of the tail, which is divided into the middle, principal, and end piece. The middle piece contains mitochondria which provide energy for sperm motility (Tortora & Derrickson, 2014). WHO defines normal semen quality as a volume of 1.5 ml, sperm concentration of 15 million per ml, progressive motility of 32%, and normal sperm form of 4% (WHO, 2010). The rat sperm has a similar structure to that of human sperm, but with the head in a hook shape (Industrial Reproductive Toxicology Discussion Group & Computer Assisted Sperm Analysis (CASA) Group, 2000). Changes in the sperm morphology affect function of the sperm. Defects such as flattened sperm head, pin head, bent neck, and bent tail are signs of an abnormal sperm (Industrial Reproductive Toxicology Discussion Group & Computer Assisted Sperm Analysis (CASA) Group, 2000).

Infertility has been linked to several sperm characteristics. A low sperm count coupled with higher sperm abnormalities is associated with low zona pellucida induced acrosome reaction, a step crucial in fertilization (Liu et al., 2003). Damaged mitochondria may provide insufficient energy for sperm motility (Carra et al., 2004). Holyoake et al. studied single nucleotide polymorphisms in the coding regions of mitochondrial genome and reported two common polymorphisms at nucleotide positions (nt) 9055 and 11719 that occur at higher frequencies showing associations with decreased sperm motility in men with such genotypes (Holyoake et al., 2001). A higher degree of sperm deoxyribonucleic acid (DNA) fragmentation is associated with low sperm count, sperm concentration, as well as sperm motility and morphology (Das et al., 2013; Evgeni et al., 2015). Sperm DNA damage in normozoospermic men shows positive correlation with age (Belloc et al., 2014). In addition to sperm DNA damage, abnormal sperms also show a change in their lipid composition. Fatty acid compositions of sperm phospholipids of infertile patients revealed a marked decrease in DHA, and an increase in n-6/n-3 ratio in oligozoospermic samples (Zalata et al., 1998). Infertility has also been associated with reduced levels of phosphatidylcholine (PC) and n-3 fatty acids, with an increase in phosphatidylserine and n-6 fatty acids in sperms (Gulaya et al., 2001). Presence of seminolipid, a sulfated glyceroglycolipid on the sperm head is also known to be partially responsible for affinity of the sperm head to the zona pellucida (White et al., 2000). Specific disease conditions such as varicocele are also responsible for overall poor sperm quality (Zedan et al., 2009). Thus, sperm structure as well as its protein and lipid composition determines its viability, ultimately impacting fertility.

Underdevelopment in testes

Along with providing a support structure for spermatogenesis, Sertoli cells to a certain degree are responsible for testicular volume (Weinbauer et al., 2010). It has also been

suggested that the size of the testes may be linked to production of germ cells (Luetjens et al., 2005). In addition to this, multiplication of Sertoli cells is largely governed by gonadotropins, and release of gonadotropins during infancy can cause a fourfold increase in Sertoli cells (Plant & Witchel, 2006). Extension of the division phase of Sertoli cells in rats via reduced levels of thyroid hormone can increase testes weight and sperm production by about 80% (Weinbauer et al., 2010). Thus testicular development depends largely on gonadotropic activity. Mice with FSH receptor knockout have underdeveloped testes, with 50% reduction in Sertoli cells (Sairam & Krishnamurthy, 2001). The absence of connexin 43 (Cx43), a gap junction protein in knockout mice impedes spermatogenesis (Brehm et al., 2007; Sridharan et al., 2007). A study performed on the cell culture of Sertoli cells indicated proteins such as occludin and β1-integrin are crucial to the formation of the blood testes barrier, as well as spermiation (Yan et al., 2008). In addition to this, proteins such as claudin-11, ZO-1, N-cadherin, and Cx43 which are present in the seminiferous epithelium show a difference in distribution when equine normal and cryptorchid testes are compared (Rode et al., 2015).

Along with proteins, testes are also sensitive to changes in lipid composition. In rat testes, a specific decrease of DPA has been associated with smaller testes and testicular underdevelopment (Furland et al., 2007a; Suh et al., 2011; Zanetti et al., 2007). In addition to this, seminolipids are crucial for formation of seminiferous tubules and spermatogenesis (Honke & Taniguchi, 2002).

More recent studies point to the role of gene expression in testes development. Mice, which were 10 weeks old with germ cell mutation of the Apc gene, Apc(Min/+) showed higher degree of underdeveloped seminiferous tubules (You et al., 2006). As previously mentioned, with regards to seminolipid, CGT deficient mice (*Cgt-/-*) showed

underdevelopment of the seminiferous tubules with lack of spermatids and spermatozoa (Fujimoto et al., 2000).

Specific disease conditions such as varicocele (Zini et al., 2005) and cryptorchidism (Zvizdic et al., 2014) are associated with decreased testicular volume. Environmental factors are also responsible for testicular underdevelopment. Saillenfait et al studied in-utero exposure to di-n-hexyl phthalate (DnHP) and found it to be teratogenic with the resultant male offspring exhibiting hypospadias, testes underdevelopment and undescended testes (Saillenfait et al., 2009).

Obesity: Effect on testes and infertility

Obesity is a condition characterized by excess body fat, or white adipose tissue which accumulates in the body to the extent where it interferes with normal body functions (Du Plessis et al., 2010). A strong association has been shown between obesity and poor semen quality, coupled with an inverse relationship between normal semen quality and BMI (Teerds et al., 2011). An increased occurrence of oligozoospermia, or low sperm concentration ($<20x10^6$/ml) was observed with an increase in BMI at a fertility clinic (Hammoud et al., 2008). Another study also correlated infertility with an increase in BMI. This correlation was found consistently in young and old men, indicating that obesity might play a bigger role than age in terms of male fertility (Sallmen et al., 2006). These changes can be attributed to the physiological changes induced by obesity.

Hormonal changes

As shown previously, hormones are the main regulators of testes function. Changes in the hormonal balance can cause physiological alterations in the testes. A case study of morbid male obesity found a high ratio of estrogen: testosterone followed by

hypogonadism and obesity. Symptoms were mitigated by administration of aromatase inhibitors, which caused a decrease in estrogen (Roth et al., 2008). Aromatase enzyme cytochrome P450, which is involved in estrogen synthesis, has high expression levels in white adipose tissue, and is hence responsible for the increased estrogen: testosterone ratio in obesity (Du Plessis et al., 2010). Obese men suffering from oligozoospermia had increased serum FSH, LH and estradiol (E2), prolactin and leptin (Hofny et al., 2010). On the other hand, elevated levels of serum E2 and thyroxine (T4) have been associated with decrease in sperm DNA damage in a study conducted at an infertility clinic, indicating that estradiol may play a protective role for sperms in the presence of increased levels of T4 (Meeker et al., 2008). This can be supported by the fact that estrogen receptor gene knockout mice exhibit low sperm count and impaired spermatogenesis (Eddy et al., 1996).Thus, presence of both estrogen and testosterone is essential for spermatogenesis, but a low estrogen: testosterone ratio is favorable. In addition to estrogen and testosterone levels, obesity has its main effect on leptin levels. Obese condition is accompanied with an increase in white adipose tissue, which is responsible for the production of leptin in the fed state that acts as a satiety factor (Du Plessis et al., 2010). In case of leptin deficiency, an upregulation of apoptosis regulating genes and impaired spermatogenesis have been found in *ob/ob* mice (Bhat et al., 2006), and significant sperm DNA damage from puberty, with abnormal organ weights and seminiferous epithelium formation in obese Zucker rats (Vendramini et al., 2014). Leptin supplementation in male *ob/ob* mice increased in serum FSH, elevated weights of testes and seminal vesicles, and improved sperm counts (Barash et al., 1996). Presence of leptin receptors on seminal fluid and spermatozoa further emphasizes the role of leptin even post spermatogenesis (Jope et al., 2003). However, high leptin levels are seen in the obese condition compared with lean, due to larger number of preadipocytes (Wang et al., 2008; Wozniak et al., 2009) which

leads to leptin resistance (Considine et al., 1996; Gonzaga et al., 2014). A buildup of leptin in obese individuals is associated with low levels of androgen, specifically free testosterone in the Leydig cells (Isidori et al., 1999). The increase in leptin was directly proportional to age, abnormal sperm morphology and BMI, FSH and LH, but inversely proportional to testosterone levels (Hofny et al., 2010). These studies indicate that although leptin is essential for normal testes function, its increased levels in an obese state can be detrimental to reproductive function.

In addition to leptin, the adipose tissue also secretes other hormones such as visfatin, adiponectin, resistin, as well as inflammatory cytokines (Wozniak et al., 2009). Resistin in particular is a contributing factor to insulin resistance, and further explains the connection between obesity and type 2 diabetes mellitus (Steppan et al., 2001). A study conducted by Bener et al in 857 diabetic Qatari men found 35.1% of diabetic men were infertile, with 50.6% of infertile men overweight and 29.1% obese (Bener et al., 2009). Comparative semen analysis of diabetic and non diabetic men has also revealed lower semen volume in diabetic men accompanied with higher nuclear DNA fragmentation and a greater number of mitochondrial DNA deletions (Agbaje et al., 2007).

Another impact of insulin resistance and an overall high level of insulin is the inhibition of sex hormone binding globulin (SHBG) and its synthesis (Lima et al., 2000). Jensen et al also found a decrease in serum testosterone inhibin and SHBG and they were inversely correlated with BMI, but an increase in BMI was correlated to increase in free androgen levels and estradiol (Jensen et al., 2004). These studies overall indicate that in an obese state there is a general imbalance of androgens, which is consistently associated with poor semen quality and infertility. Overall hormone imbalance in an obese condition is summarized in Figure 1-3.

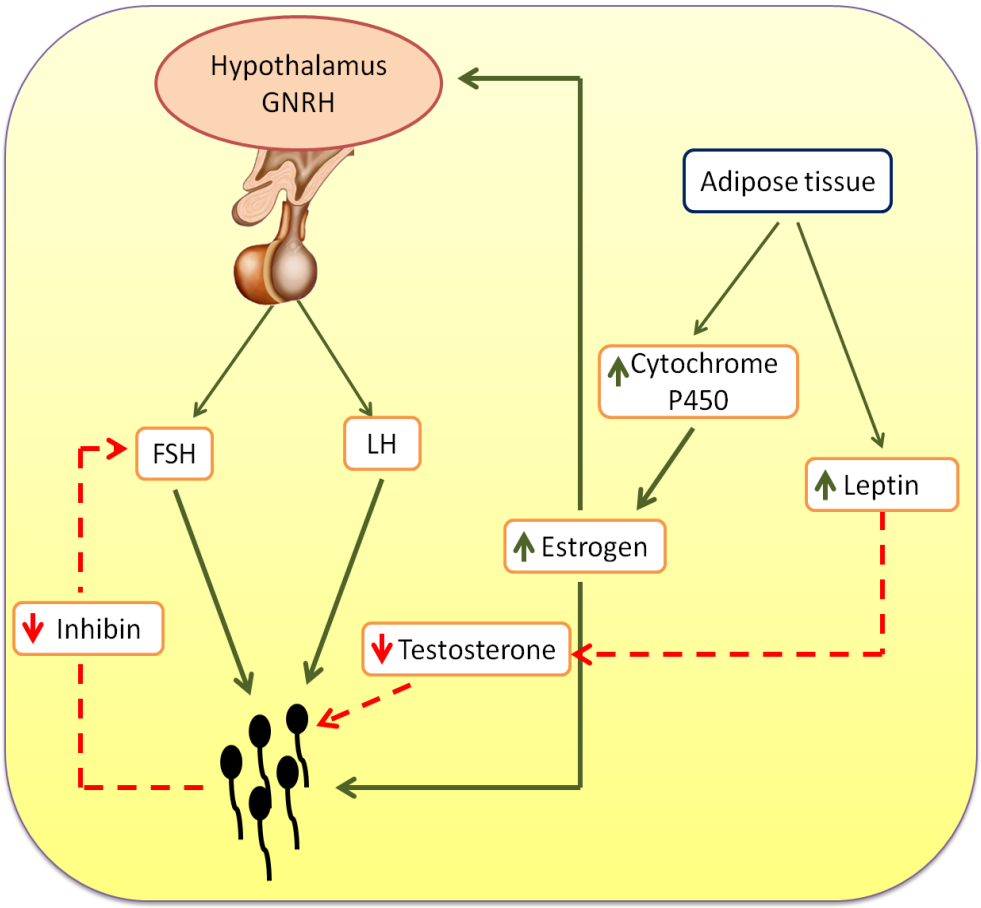

Figure 1-3: Hormonal imbalance in obesity
Green arrows indicate activating effects, red dotted arrows indicate inhibited activity. FSH- Follicle stimulating hormone, LH- luteinizing hormone, GNRH- Gonadotropin releasing hormone.

Oxidative stress

Oxidative stress is damage to tissue cells caused by the excess production of reactive oxygen species (ROS). These are unstable molecules which are highly reactive and can cause tissue damage (Du Plessis et al., 2010). Obesity, corresponding metabolic syndrome and diabetes have been associated with increased oxidative stress (Dandona et al., 2004). The testes and spermatozoa are enriched in lipids, specifically PUFA, which are the primary targets of lipid peroxidation (Alvarez & Storey, 1995). Measuring

carbonyl protein (CP) as a marker for ROS in patients with idiopathic oligoasthenoteratozoospermia (iOAT) showed a significant rise of CP in iOAT patients compared to normal fertile patients (El-Taieb et al., 2009). iOAT is a condition characterized by a low sperm count and defective sperm, the etiology of which is unknown (Cavallini, 2006). ROS production in human spermatozoa has also been linked with increase in seminal leukocytes, leading to poor sperm quality (Saleh et al., 2002). A study conducted at an infertility clinic found the DNA fragmentation index and oxidative stress were positively correlated in infertile patients (Saleh et al., 2003). ROS were also surmised to prompt germ cell apoptosis leading to a reduced sperm count (Agarwal & Said, 2003). Wang et al found similar results in patients with idiopathic infertility where sperm damage was positively correlated with ROS, cytochrome c, and caspases 9 and 3, which are proteins that mediate apoptosis (Wang et al., 2003). Said et al. also found similar results in the sperm of infertile men, with elevated levels of superoxide anions ($O_2^{\cdot -}$) and H_2O_2 and in the presence of exogenous beta nicotinamide adenine dinucleotide phosphate (NADPH) (Said et al., 2004). These studies show that oxidative stress is closely related with sperm quality. Considering that oxidative stress commonly occurs in an obese condition, these individuals are at a higher risk of developing infertility.

Physical changes in testes and accessory organs

There is a marked difference in the morphology of testes and accessory organs in obese conditions. The effect of obesity on reproductive organs has been studied in *fa/fa* Zucker rats, a genetic model for obesity with a dysfunctional leptin receptor that have been used studies for obesity and hypertension (Takaya et al., 1996). Obese *fa/fa* Zucker rats have significantly lower testes and epididymis weights in comparison with lean animals in the pubertal stage, along with loss of germ cells and disrupted seminiferous tubules

(Vendramini et al., 2014). Similar results were obtained in adult obese *fa/fa* Zucker rats compared with lean Zucker rats. The obese Zucker rats often have imbalanced paired testes with an overall significantly lower relative testes weight, smaller epididymis and seminal vesicles, but a larger prostate compared with lean (Suh et al., 2011). The physical changes in the testes and accessory organs between lean and obese animal are shown in Figure 1-4. Similar differences are seen in humans as well. Case studies show men with morbid obesity also suffer from hypogonadotropic hypogonadism (Roth et al., 2008). One of the main causes for this condition is the drop in testosterone which can lead to hypogonadism (Feeley & Traish, 2009; Traish et al., 2009).

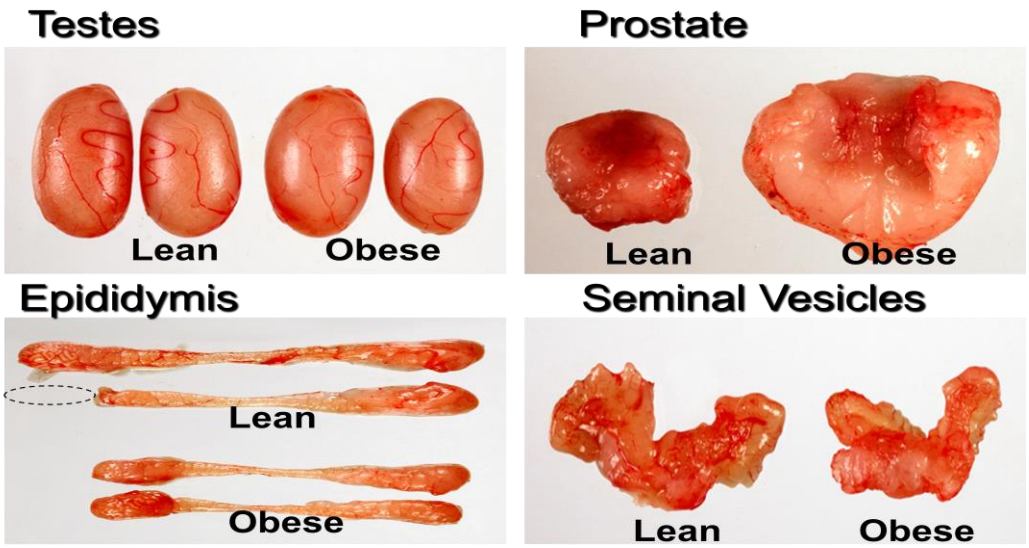

Figure 1-4: Phenotypic changes in testes and accessory organs
Dotted line represents cauda epididymis used for sperm morphology (Suh et al., 2011). Permission granted on 27 July 2015 by Cambridge University Press, Copyright Clearance Centre, 222 Rosewood Drive Danvers, MA 01923 USA.

Characteristic lipids in testes:

Testes are highly enriched in lipids, the main types of lipids being phospholipids, triacylglycerols, gangliosides, sulfolipids and polyunsaturated fatty acids (Coniglio, 1994).

Phospholipids

The phospholipids are the membrane lipids which are the predominant lipids found in the testes, being about 60-70% of the testes lipid (Bieri & Prival, 1965). The phospholipids have a glycerol backbone with two fatty acids at C1 and C2 and a phosphate group at C3. This structure and the closely packed phospholipid molecules form the lipid bilayer which is externally hydrophobic and internally hydrophilic (Saether, 2003). In the testes the main fractions of phospholipids are phosphotidylcholine (PC) and phosphotidylethanolamine (PE) (Merrells et al., 2009). A fluctuation in the levels of these phospholipid classes has been observed in infertile men, with a reduction in the levels of (PE) and a rise in the levels of (PS), suggesting that phospholipid composition plays an important role in male fertility (Gulaya et al., 2001). DHA, the predominant fatty acid found in human testes, is drastically reduced in oligozoospermic and asthenozoospermic (low sperm motility) semen samples, indicating a critical role of DHA in spermatogenesis, and sperm integrity (Zalata et al., 1998). In a study of the effect of fish oil supplementation on adult pig testes, pigs fed a menhaden oil containing diet had higher levels of PE and testosterone compared with the testes from tuna oil. These differences could be attributed to the levels of DHA and EPA, with menhaden oil having approximately equal proportions of EPA and DHA, but tuna oil having higher proportion of DHA. This indicates that phospholipid concentrations and compositions differ greatly with changing the lipid profile of the diet (Castellano et al., 2011). Phospholipid changes

have been observed in ram epididymal plasma membrane, where DHA was the predominant fatty acid in epididymal maturation of sperms (Parks & Hammerstedt, 1985). The phospholipid compositions of spermatozoa of mice in different sections of epididymis, and the degree of unsaturation and presence of lysophopholipid, are important for normal sperm maturation and development of sperm motility (Pyttel et al., 2014). Thus it can be concluded that phospholipid compositions change with diet as well as requirements during sperm growth and development.

Cholesterol

Normal cholesterol metabolism is essential for proper functioning of the male reproductive system. Cholesterol is involved in supporting the lipid rafts of sphingolipids in the phospholipid membranes (Parton & Hancock, 2004; Simons & Ikonen, 2000). Cholesterol has been found in varying quantities in the testes, the highest being in humans (3.4 mg/g) to the least being found in rat (2.0 mg/g) (Bieri & Prival, 1965). Changes in the lipid composition of sperms travelling in the epididymis show a loss of up to 50% cholesterol, and a reduction in the cholesterol: phospholipid ratio, which indicates that the membrane fluidity of sperm cells increases during maturation, to aid their mobility and membrane fusion (Whitfield et al., 2015). Despite this, cholesterol is still essential in lipid rafts in sperm membranes. Cholesterol trafficking is controlled by the activity of proteins such as calveolin 1, which is important in spermatozoa and their stability as well as cell signaling (Travis et al., 2001).

Apart from being a component of lipid rafts, the main function of cholesterol is steroid and testosterone synthesis. It is the precursor of testosterone, which is essential for spermatogenesis, muscle and bone mass and other male physiological parameters (Matsumoto, 2002). Uptake and use of cholesterol is regulated by several factors such as

steroidogenic acute regulatory protein (StAR), which regulates cholesterol uptake through the mitochondrial membrane. Here, the cholesterol side chain is cleaved with the help of cytochrome P-450, producing pregnenolone, which after further enzymatic actions produces testosterone (Anderson & Mendelson, 1988).

Seminolipids

Seminolipids (1-O-alkyl-2-O-acyl-3-O-D-(3-sulfo)-galactopyranosyl-sn-glycerol) are sulfogalactosylglycerolipids which are exclusively found in the testes (Goto-Inoue et al., 2009). Seminolipids are sulfoglycolipids, with a glycerol backbone, a carbohydrate moiety and two fatty acids differing from sulfatide only due to the presence of the glycerol moiety (Honke, 2013). Seminolipids are produced in the spermatocytes and are seen in consistent levels throughout the process of spermatogenesis (Fujimoto et al., 2000). Although their exact function is not known, their levels indicate that there is a definite role that seminolipids play in the cell stability and structure. White et al observed that sulfogalactosylglycerolipid liposomes on the sperm head showed affinity for the zona pellucida, but this affinity was not shown by galactosylglycerolipid, its precursor (White et al., 2000). Similar results were observed with regards to the sperm and egg affinity and the role of seminolipids (Feng et al., 2003; Weerachatyanukul et al., 2001). Recent data also shows seminolipid is involved in the membrane integrity and stability of the sperm, as it is one of the components in the lipid rafts (Attar et al., 2000; Bou Khalil et al., 2006; Rodemer et al., 2003). Mouse sperm that has been Percoll gradient centrifuged shows increased fertilizing capacity and higher concentration of seminolipids and DHA in the PC compared to washed capacitated mouse sperm (Furimsky et al., 2005).

The synthesis of sulfatide can be compared to that of the seminolipid, since they share the enzymes involved in their synthesis. Two major enzymes have been identified in the synthesis of seminolipids, namely ceramide galactosyltransferase (*cgt*), and cerebroside sulfotransferase (*cst*) (Honke & Taniguchi, 2002). The basic pathway of synthesis of seminolipid is illustrated in Figure 1-5. Mice with deficiencies of these enzymes have exhibited failure to synthesize seminolipids, and have also been seen to suffer from infertility (Fujimoto et al., 2000; Goto-Inoue et al., 2009). Thus, seminolipid has functional importance in spermatogenesis.

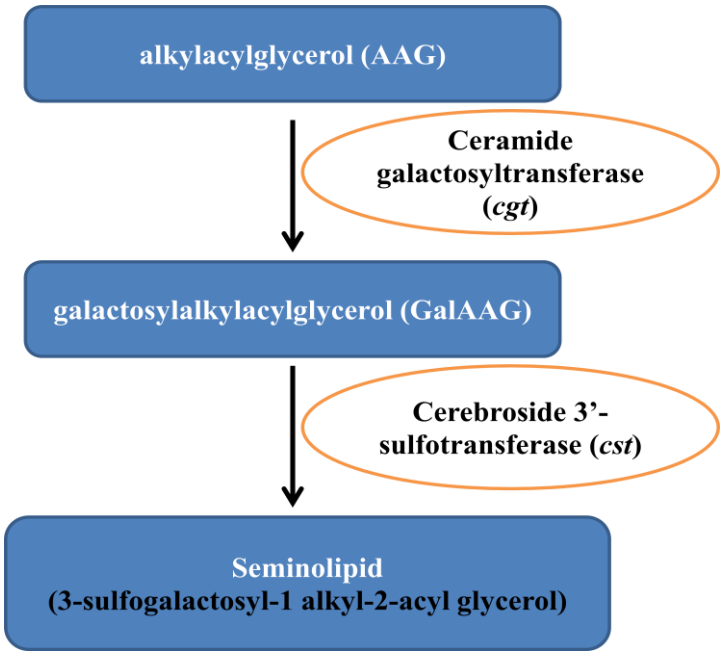

Figure 1-5: Pathway for synthesis of seminolipid

Fatty acids

The testes are composed of 65% of (PUFA), out of which AA (20:4n-6), DPA (22:5n-6) and DHA (22:6n-3) are the predominant fatty acids (Bieri & Prival, 1965). The biosynthesis of these fatty acids from their precursors was first clarified by Voss et al.,

and is known as Sprecher's pathway (Voss et al., 1991). The fatty acid production in the body is the result of a series of reactions of fatty acid elongation and desaturation steps which are responsible for the formation of DPA and DHA (Saether, 2003). The substrates for the metabolic process are linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3), which are essential fatty acids required in the diet (Skiba et al., 2015). The conversion of ALA to 18:4n-3 and LA to 18:3n-6 is carried out by Δ6 desaturase enzyme, which is the rate limiting step in the metabolism of n-3 fatty acids (Roqueta-Rivera et al., 2010). Further elongation and desaturation steps form EPA and AA respectively. In the last step of LC PUFA metabolism these intermediates are then converted to DHA and DPA by the activity of Δ6 desaturase enzyme (Roqueta-Rivera et al., 2010), followed by peroxisomal oxidation. A flowchart of the LCFA metabolism is shown in Figure 1-6.

Fatty acids, especially PUFA are of special importance in testes. Cell culture studies looking at the conversion of AA to DPA and from EPA to DHA in rat germ cells showed a very low activity of Δ 6 desaturation, but this activity was much higher in Sertoli cells, indicating that perhaps these fatty acids may be transported to germ cells after synthesis (K. Retterstol et al., 2001). In vivo germ cells show a higher content of DPA and DHA in comparison to Sertoli cells (K. Retterstol et al., 2001). Although these fatty acids are present in all species, the proportion of n-6 and n-3 fatty acids is different in every species. For example, in rat testes 22:5n-6, DPA is the predominant fatty acid, but in human testes 22:6n-3, DHA is the predominant fatty acid (K. Retterstol et al., 2001). A decrease in the level of 22:5n6 in rat testes can affect testes development (Suh et al., 2011). Similarly, low levels of DHA in human semen samples have been found in oligozoospermic males (Zalata et al., 1998), and infertile males (Gulaya et al., 2001). Thus we can see that testes are sensitive to even small changes in the fatty acid composition.

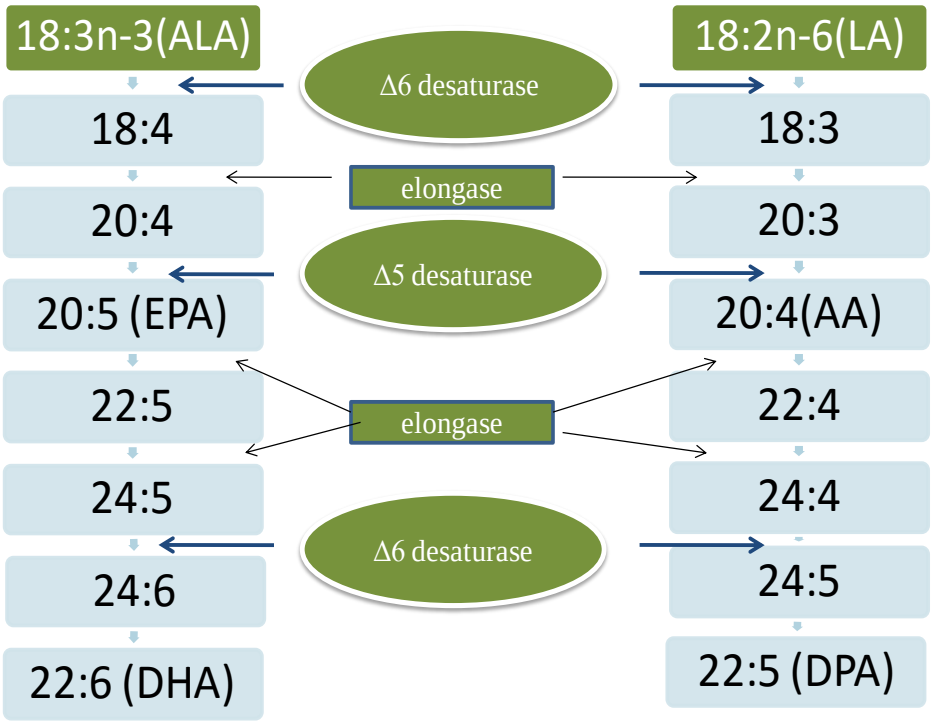

Figure 1-6: Pathway of n-3 and n-6 PUFA metabolism

Testes function affected by dietary lipids

Due to the high lipid content of testes, the membrane composition of testes is susceptible to changes in dietary lipid composition. Commonly used dietary oils such as coconut, soybean, olive and grapeseed oil have an impact on the lipid composition as well as hormone levels in rat testes (Hurtado de Catalfo et al., 2008). The main n-3 fatty acids that available through the diet are ALA, EPA and DHA (Riediger et al., 2009). Recent evidence shows that testes function may be affected by changes in the level of these fatty acids in the diet. Supplementation with n-3 fatty acids increases DHA in plasma membrane, and is accompanied with low levels of AA and DPA (Sebokova et al., 1990). The ratio of n-6: n-3 fatty acids in the diet can affect male reproductive function, with improved function associated with an increased ratio (Yan et al., 2013).

Several studies have looked at the beneficial effects of n-3 fatty acid supplementation. Fish oil supplementation in boars affected lipid composition in testes as well as testosterone production (Castellano et al., 2011). Improved reproductive function was seen in rats having non alcoholic fatty liver disease (NAFLD) when supplemented with n-3 fatty acids (Li et al., 2013).The role of n-3 fatty acids is further seen in the production of series 3 eicosanoids, prostaglandin I3, thromboxane A3, leukotriene B5 via cyclooxygenase and epoxygenase pathways. These molecules are responsible for vasoconstriction, platelet aggregation and regulation of inflammation. Supression of the cycloxygenase 2 pathway, for example, has shown to reduce sperm motility and fertility (Balaji et al., 2007). Supplementation with dietary n3 fatty acids could play a crucial role in alteration of these factors (Riediger et al., 2009). However there is still limited knowledge of the effect of n-3 fatty acid supplementation on testes in an obese condition, and the response of underdeveloped testes to such dietary supplementation. Table 1-1 summarizes some of the studies looking at the effect of diet supplementation on testes.

Table 1-1: Effect of dietary interventions on testes

Reference	Animal model and diet	Measurement	Findings	Conclusion
(Suh et al., 2011)	*Ln* and *fa/fa* Zucker rats (n=12/group) 6 weeks old. Fed 15% (w/w) total fat diet, containing 0 or 5% (w/w) n-3 LCFA from fish oil for 8 weeks	Organ weights, sperm morphology. Fatty acid composition of PC and PE	Obese rats had smaller epididymis, seminal vesicles but larger prostate compared to lean. Underdeveloped testes had low DPA. n-3 diets increased DHA in PC and PE	Testes sensitive to dietary lipids; obese rats respond more to dietary LCFA compared to lean. DPA is selectively reduced in underdeveloped testes, indicating its importance in male reproduction
(Merrells et al., 2009)	Weanling Sprague Dawley rats 3 weeks old (4-5/group). Zn control (ZC; 30 mg Zn/kg), Zn marginally deficient (ZMD; 9mg Zn/kg), Zn deficient (ZD; 1mg Zn/kg), pair fed to the ZD group (PF; 30 mg Zn/kg) for 3 weeks	Epididymal sperm morphology. Lipid profiles of testis phospholipids	ZD rats had lower testes, seminal vesicles and prostate weight, 34–35% more abnormalities in spermatozoa and 24% shorter tail length, and higher cholesterol concentration compared to ZC. Testes phospholipids enriched in n-6, but DPA low in ZD rat testes	Zinc deficient diet is associated with abnormal testes function. Impaired testes function could be due to changes in membrane fatty acids, and decline of DPA in phospholipids
(Yan et al., 2013)	Sprague Dawley rats (n=8/group), 90 days old. Fed diets containing soybean and flaxseed oil, with varying PUFA n-3:n-6 ratio (0.13, 0.40, 0.85, 1.52, 2.85) for 60 days	Hormone: GnRH, LH, FSH and T. Testes and sperm microscopic examination. Reproductive capacity observed through mating	The 1.52 ratio group among others: higher sperm density and motility, higher birth weights and litter sizes. Increase in GnRH, FSH, LH and T with increasing n-3:n-6 ratios	n-3:n-6 ratio found to affect male reproductive function; increase of n-3:n-6 ratio improves seminal parameters

(Castellano et al., 2011)	Duroc boars (n=8/group), 204±9 days, fed diets containing hydrogenated fat (AF) menhaden oil (MO) (EPA 16%, DHA 18%) or tuna oil (TO) (EPA 7%, DHA 33%) for 7 months	Phospholipid distributions, T, dihydrotestosterone, estradiol in testes	PE content of testes-TO< MO<AF. Sphingomyelin in testes- TO>MO>AF. T and estradiol- TO<MO	Fish oil supplementation affected lipid composition of boar testes, as well as T levels
(Hurtado de Catalfo et al., 2008)	Wistar rats, (n=10/group) fed diets containing soybean oil (S), olive oil (O), coconut oil (C), or grapeseed oil (G) for 60 days	Lipid sub classes, fatty acids, PC and PE, hormone levels in interstitial cells	Testosterone higher in O and C compared with S and G. Diet O showed higher levels of PC, PE and PI	Dietary oils modify lipid composition, and influence steroidogenic function in interstitial cells
(Li et al., 2013)	Sprague Dawley rats 4 weeks old (n=20/group) fed diets containing high fat diet (HFD, 45% kcal fat) to induce NAFLD. Fed n-3 fatty acids for 4 weeks	Hormones, sperm count, morphology and motility, mating reproductive capacity	Improvement in reproductive function	n-3 fatty acid supplementation can improve reproductive function in rats having NAFLD
(Sebokova et al., 1990)	Weanling Sprague Dawley rats (n=6/group). Fed 20% (w/w) fat diet, containing beef tallow (BT) (18% beef tallow, 2% safflower oil), linseed oil (LO) (16% linseed oil, 4% beef tallow) or fish oil (FO) (20% fish oil)	Fatty acid analysis of testes plasma membrane. testosterone synthesis assay	Decrease in AA and DPA of testes membrane in animals fed FO. High cholesterol and FO diet increased testosterone synthesis	Fat composition of diet influences phospholipid composition of testicular plasma membrane

NAFLD,Non alcoholic fatty liver disease; PC,Phosphatidylcholine; PE,Phosphatidylethanolamine; PI,Phosphatidylethanolamine; PUFA, polyunsaturated fatty acids; AA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; GnRH, gonadotropin releasing hormone; LH, leutinizing hormone; FSH, follicle stimulating hormone; T, testosterone

Testes function as affected by enzymes and gene expression

Fatty acid metabolism in mammals is controlled by several different enzymes, the main enzymes being the elongases and desaturases (Guillou et al., 2010). Deficiency of these enzymes have shown a disruption of fatty acid metabolism, and consequently impaired spermatogenesis. Mice deficient in the Δ6 desaturase enzyme showed impaired spermatogenesis and infertility (Roqueta-Rivera et al., 2010). Similarly, mice deficient in ELOVL2, an elongase enzyme, showed impaired reproductive function (Zadravec et al., 2011). Transgenic animal models with knock out genes have exhibited impaired reproductive function, which could be the cause of modifications in the lipid composition through gene expression (Stoffel et al., 2008). Deletion of genes such as Wt1 involved in the steroidogenic pathway can result in disruption of levels of several hormones and impair spermatogenesis in mice (Chen et al., 2014). Similarly, *cgt*$^{-/-}$ mice showed deficiency of seminolipid and a disrupted spermatogenesis (Fujimoto et al., 2000). This indicates that the expression of genes regulating lipid metabolism determines normal reproductive function, however, the expression of these genes in an obese condition and their subsequent effect on male reproduction needs to be studied further. Table 1-2 lists some of the recent studies that have been conducted with regards to the gene expression and the resulting effect on enzymes involved in lipid metabolism.

Table 1-2: Study of genes involved in reproductive function through transgenic animal models

Reference	Animal model and diet	Measurements	Findings	Conclusion
(Roqueta-Rivera et al., 2010)	Wild type $^{+/+}$ (n=9/group), heterozygote $^{+/-}$, D6D null $^{-/-}$ mice fed AIN93G, with 0.2% w/w AA, or 0.2% w/w DHA	Fertility evaluated through breeding, fatty acid analysis, sperm count and motility, and gene expression	Fertility, sperm counts and motility restored by DHA. DHA required for sperm head and flagellum formation. No change in gene expression	Fertility and spermatogenesis restored by DHA supplementation in D6D null mice
(Zadravec et al., 2011)	ELOVL-2 $^{-/-}$ (n=2/group) mice fed DHA enriched diet (19.5% of total fatty acid) for 3 months	Lipid profiling histological examination of testes, and reproductive function tested	Impaired reproductive function in ELOVL $2^{-/-}$ mice, increase in 22:4 n-6- CoA and low levels of 22:5-n-6-CoA, absence of VLCFAs	ELOVL2 is essential in the metabolism of n-6 fatty acids, and for spermatogenesis
(Stoffel et al., 2008)	FADS 2 $^{-/-}$ (n=10-12/group) mice generated by targeted FADS 2 gene expression in mice	Fatty acyl compositions mating test	LC PUFAs absent, mice were sterile with hypogonadism	FADS 2 expression is essential for PUFA metabolism and spermatogenesis
(Fujimoto et al., 2000)	UDP-galactose *cgt*$^{-/-}$ (n=6-8/group) mice generated by gene targeting	Histological analysis and lipid analysis of testicular germ cells was performed	Anatomical defects in reproductive organs of *cgt*$^{-/-}$ mice observed, deficient in seminolipid, disrupted spermatogenesis	UDP-galactose:ceramide galactosyltransferase is essential for synthesis of seminolipid, and spermatogenesis
(Roqueta-Rivera et al., 2011)	FADS2$^{+/+}$ and FADS2 $^{-/-}$ (n=3-5/group) mice fed diets supplemented with 0.2% (w/w) AA, or 0.2% (w/w) DHA	Periodic acid Schiff histology, and immunohistochemistry	DHA supplementation in mice restored intact acrosome	Deficiency of n-3 fatty acids results in failure of acrosome biogenesis in mice

D6D,Delta 6 desaturase; ELOVL-2, Elongation of very long chain fatty acids protein 2; FADS 2, Fatty acid desaturase 2; VLCFA, Very long chain fatty acids (C>24); UDP-galactose, Uridine diphosphate galactose

CHAPTER II: FATTY ACID PROFILES IN TESTICULAR LIPID CLASSES ARE DIFFERENTLY AFFECTED BY DIETARY n-3 ALA, EPA AND DHA IN OBESE *(fa/fa)* ZUCKER RATS

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Abstract

Testes are organs with a unique lipid environment, which can be disturbed in an obese condition leading to infertility. Polyunsaturated fatty acids (PUFA) are of prime importance in normal testes function, however, our understanding of PUFA metabolism in testis is still limited. This study examined the changes in fatty acid composition of testes in an obese condition, and the response of testes to dietary supplementation with n-3 fatty acids from different sources. Male *(fa/fa)* Zucker rats, six weeks old, were fed four different diets enriched in either linoleic acid, α-linolenic acid, eicosapentanoic acid (EPA), or docosahexanoic acid (DHA) oils for 8 weeks. Lean animals were fed LA diet only. Cauda epididymal sperm morphology was assessed. Fatty acid profiles were measured in phospholipid (PL), triacylglyceride (TG), cholesterol (CE) and free fatty acids (FFA) by focusing on polyunsaturated (PUFA) and very long chain fatty acids (VLCFA). Obese animals had higher sperm abnormalities compared to lean animals with an abundance of head abnormalities. In obese animals, the ratios of n-6/n-3 PUFA were signficantly ($p<0.05$) lower in all testis lipid classes except CE in comparison to the lean animals. Among obese animals, the ratios of n-6/n-3 PUFA were significantly lower regardless of the dietary n-3 fatty acid sources. ALA provided in the diet increased DHA in all lipid classes, but not to the same level as EPA and DHA. n-6 series of VLCFA were significantly abundant in all testis lipid classes and the ratios of n-6/n-3 VLCFA were

significanly lower with dietary n-3 fatty acid in TG and FFA. The results indicate that the testes are sensitive to changes in the fat types provided in the diet. While the majority of n-6 and n-3 fatty acids were altered by different dietary n-3 fatty acid sources, ALA was not effective for increasing DHA levels in testes compared to rats receiving diets with preformed EPA and DHA. Whether these changes were related with sperm maturation needs to be tested in the future.

Keywords: Obesity, Male infertility, Testes, Omega 3 fatty acids, Omega 6 fatty acids, Very long chain fatty acids

Introduction

Male infertility has recently been added to the list of health complications linked to obesity, which is a global health issue. An increase in body mass index (BMI) has been associated with poor semen quality, such as low semen volume, sperm output, and a decline in testosterone and other hormones crucial to spermatogenesis (Hart et al., 2015). Reduced progressive sperm concentration and total sperm count were observed in men with high BMI and waist circumference (Hammiche et al., 2012; Jensen et al., 2004). Saleh et al., (2003) stated that oxidative stress, a side effect of obesity contributes to DNA fragmentation in the sperm and has a strong positive correlation with infertility. Despite the implications of obesity associated male infertility, there is still limited information available in this field.

Testes are enriched in polyunsaturated fatty acids (PUFA), and studies suggest that infertility could stem from changes in the lipid environment of the testes. Although testes are rich in PUFAs, levels of these fatty acids are different with respect to species. Rat testes have DPA as the predominant fatty acid in the testes, whereas in humans higher concentrations of DHA are present (Retterstol et al., 2001). A variation in the level of these fatty acids has been observed in abnormal testes function. Low levels of DHA in human semen samples have been found in oligozoospermic males (Zalata et al., 1998), and infertile males (Gulaya et al., 2001). Similarly in rats, a specific decrease of DPA was identified in testes phospholipids (Suh et al., 2011), and DPA is involved in spermatid maturation (Davis et al., 1966).

Diet supplementation as a means of providing PUFAs in the testes has been studied. Dietary oils such as coconut, soybean, olive and grapeseed oil have an impact on the lipid composition as well as hormone levels in rat testes (Hurtado de Catalfo et al., 2008).

Supplementation with fish oil, rich in n-3 PUFA, affected the lipid composition of boar testes, as well as the testosterone levels (Castellano et al., 2011). Providing n-3 PUFA improved reproductive function in rats having non alcoholic fatty liver disease (NAFLD) (Li et al., 2013). More specifically, providing DHA restored spermatogenesis and fertility of Δ6 desaturase null mice (Roqueta-Rivera et al., 2010). These changes occurring as a result of dietary intervention have been attributed to a modified n-6/n-3 fatty acid ratio in the diet. Different dietary n-3/n-6 fatty acid ratios result in changes in male reproductive function in rats (Yan et al., 2013). An increase in the n-6/n-3 fatty acid ratio in testes was inversely related with sperm morphology and motility in boar testes (Am-in et al., 2011). A question then arises as to what optimal ratio of these fatty acids is needed for normal testis function.

Dietary EPA and DHA, known as conditionally essential PUFA for neural cell development, can be obtained from α-linolenic acid (ALA, 18:3n-3), which is then converted to EPA and DHA. Providing ALA in the diet increased the concentration of EPA in various tissues (Mantzioris et al., 1994). However, the efficiency of this conversion to DHA has been a point of controversy. Studies have observed that the conversion of ALA to EPA and DHA is not as efficient as providing preformed EPA and DHA (Talahalli et al., 2010). An n-6/n-3 fatty acid ratio of 4:1 in human diets (Ghafoorunissa, 1992) or a bioavailability of 2.3:1 in human peroxisomes (Masters, 1996) has been recommended for the required conversion of ALA to DHA. With respect to male reproduction, a ratio of 0.65 showed higher sperm density and motility in rats (Yan et al., 2013). However, the efficiency of this conversion and the incorporation into testes is still debatable in normal or disease states, such as obesity. It would be of interest to see how this conversion occurs in n-6 fatty acid enriched rodent testes.

Previous studies looking at effects of diet on lipid composition of testes have mainly focused on specific lipid classes, with membrane lipids or phospholipids as their main target. These studies have measured changes in levels of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) and its impact on fertility (Gulaya et al., 2001; Suh et al., 2011), and phospholipids in spermatozoa (Zalata et al., 1998). However, information about the fatty acid composition and changes of other major lipid classes in testes is limited. Thus a complete characterization of fatty acid distribution in the different lipid classes such as triacylglycerides (TG), cholesterol esters (CE) and free fatty acids (FFA) in addition to phospholipids will provide a clearer picture of changes in the fatty acid metabolism.

A complete characterization of all the lipid classes will also make it possible to observe the distribution of very long chain polyunsaturated fatty acids (VLCFA, C>24) in testis. VLCFA cannot be obtained from the diet and are synthesized *in situ* in the testes and spermatozoa from shorter chain fatty acids (Zadravec et al., 2011). Although the exact function of VLCFA is not known, it has been reported that VLCFA in testes are mainly located in the sphingomyelin, accounting for 40% (w/w) of total fatty acids found in the ceramides and 15% (w/w) of the fatty acids found in sphingomeylin (Furland et al., 2007b) and phospholipids (Robinson et al., 1992). Aveldano et al (1993) detected presence of VLCFA in triacylglycerols in isolated rat seminiferous tubules. This has spurred the need to look at all the lipid classes to better understand the distribution of VLCFA in tesicular lipids.

Therefore, the purpose of this study was to investigate changes in fatty acid composition of testes in an obese condition, and the response of testes to dietary supplementation with n-3 fatty acids. Our objective is to provide diets enriched in ALA, EPA or DHA, and compare the level of DHA in testes with ALA versus EPA and DHA supplementation.

Lastly, by studying the fatty acid profile of PL,TG, CE and FFA, we aim to obtain a complete picture of PUFA metabolism in the testes, as well as VLCFA distribution in testes lipid classes.

Materials and methods

Animals and diets

This study protocol was approved by the University of Manitoba Animal Care Committee. Five week old male lean and *fa/fa* Zucker rats were obtained from Charles River Laboratories (St-Constant, PQ) and randomly divided into diet groups after 1 week acclimatization. All animals were initially fed the basal adaptation diet based on AIN 93G diet (Reeves et al., 1993) containing soybean oil for 1 week. Formulations of these diets and their fatty acid compositions are listed in Tables 2-1 and 2-2 and reported in Hong (2015).The soybean oil provided ~63% of PUFAs, with an n-6/n-3 ratio of 6:1. The obese *(fa/fa)* Zucker rats were then fed the experimental diets containing PUFA (70-75% w/w total fatty acids) using different sources of oils as follows: i) Safflower oil group serving as control obese animals (Ob. Saff, n-6:n-3 fatty acids = 240:1); ii) Flaxseed oil (Ob. Flax, n-6:n-3 fatty acids = 1:3); iii) EPA oil (Ob. EPA, n-6: n-3 fatty acids = 1:1.1) iv) DHA oil (Ob. DHA, n-6: n-3 fatty acids = 1:1.1). The lean Zucker rats were fed diet containing safflower oil (Ln. Saff) to serve as a reference group. Animals were sacrificed by carbon dioxide asphyxiation followed by decapitation. Testes, epididymis, cauda and prostate were collected and weighed. All organs were immediately frozen in liquid nitrogen and stored at -80°C.

Table 2-1 Experimental diets

Ingredients (g/kg)	Soybean Oil Diet	Safflower Oil Diet	Flaxseed oil Diet	EPA Diet	DHA Diet
Cornstarch	348	348	348	348	348
Maltodextrin	132	132	132	132	132
Sucrose	100	100	100	100	100
Egg white	213	213	213	213	213
Cellulose	50	50	50	50	50
Mineral mix AIN-93G-MX	35	35	35	35	35
Vitamin mix AIN- 93- VX	10	10	10	10	10
Choline	3	3	3	3	3
Biotin mix	10	10	10	10	10
Soybean oil	100	0	0	67	67
Safflower oil	0	100	0	0	0
Flaxseed oil	0	0	87	0	0
Canola oil	0	0	10	0	0
Coconut oil	0	0	3	0	0
EPA oil	0	0	0	33	0
DHA oil	0	0	0	0	33

Diet ingredients were purchased from Dyets, Inc (Bethleham, PA). Safflower oil was obtained from Alnor Oil Company (Valley Stream. NY), flaxseed oil from Omega Nutrition (Vancouver, BC), canola oil from Smuckers Food Services (Markham, ON), coconut oil from Nutiva (Richmond, CA), and EPA and DHA oils from Larodan Fine Chemicals (Malmö, SE) (Hong, 2015).

Table 2-2: Fatty acid composition of experimental diets

Fatty Acids (g/100 g)	Soybean Oil Diet	Safflower Oil Diet	Flaxseed Oil Diet	EPA Diet	DHA Diet
18:2n-6 (LA)	54	72	18	36	36
18:3n-3 (ALA)	9	0.3	52	6	6
20:5n-3 (EPA)	0	0	0	32	0
22:6n-3 (DHA)	0	0	0	0	33
ΣSFA	15	10	11	10	10
ΣMUFA	21	17	19	15	15
ΣPUFA	63	72.3	70	75	75
Σn6:n3 ratio	6:1	240:1	1:3	1:1.1	1:1.1

ΣSFA, Total saturated fatty acids, ΣMUFA, Monounsaturated fatty acids, LA, Linoleic acid, ALA, linolenic acid, EPA, eicosapentaenoic acid, DHA, docosahexaenoic acid, PUFA, polyunsaturated fatty acid (Hong, 2015).

Sperm morphology

Spermatozoa were derived from the caudal epididymis, which was minced in 2 ml of 0.9% NaCl. Minced caudal epididymis was fixed with 200 ml formalin and stained with eosin Y. Head, tail and neck abnormalities were assessed using 400X conventional light microscopy (Olympus EH, Tokyo, Japan; Sony CCD model XC-711 camera, Tokyo, Japan). Sperm morphology was assessed in 200 sperms as established in our laboratory (Merrells et al., 2009) as well as others (Industrial Reproductive Toxicology Discussion Group & Computer Assisted Sperm Analysis (CASA) Group, 2000; Sprando et al., 1999).

Lipid analysis

Total lipids were extracted from testes using the Folch method (Folch et al., 1957). Decapsulated testes were homogenized in 0.025% calcium chloride to form a 20% (w/w) solution, 1 ml of which was used for extraction of total lipids. Ten ml of chloroform and methanol were used for extraction in the ratio 2:1 (v/v). Separations into lipid classes, including phospholipid (PL), triacylglycerides (TG), cholesterol ester (CE) and free fatty acid (FFA), were carried out using silica-gel G-plates (Analtech silica-gel G; MandelScientific Co., Guelph, Ontario, Canada) prewashed in hexane. The separation mobile phase was composed of petroleum ether: diethyl ether: acetic acid in the ratio 80:20:1 (v/v/v). Lipid classes were visualized with 0.1% (w/v) aniline naphthalene sulfonic acid under UV light. CE and TG bands were saponified to cleave fatty acids using 0.5 moles of methanolic KOH with heating in a sand bath at 110°C for 1.5 hours. Fatty acid methyl esters were prepared for all the bands using boron trifluoride (BF3) in methanol by incubating in a sand bath at 110°C for 1 hour.

Fatty acid analysis

Separation of fatty acids was carried out on a SGE BPX-70 column (10 m X 0.10 mm ID and 0.2 μm film thickness) using a Varian 450 gas chromatography instrument coupled to a flame ionization detector (Vista 6010 GLC and Vista 402 data system; Varian Instruments, Mississauga, Ontario, Canada). The temperature program was 120°C for 0 min, then raised to 175°C at 10°C/min, held for 0.3 min, raised to 250°C at 15°C/min, held for 0 min, and finally raised to 280°C at 6°C/min and held for 4 min. Samples were run with a 50:1 split ratio and a 0.4 ml/min column flow. Hydrogen was the carrier gas and the injector/detector temperatures were 280°C and 290°C, respectively. The fatty

acid peaks were identified using a standard (NuChek Prep 461) as well as standards developed in our lab (Castellano et al., 2011; Suh et al., 2009).

Statistical analysis

The effect of diets on testes fatty acid composition and sperm morphology were tested with one way analysis of variance (ANOVA) using statistical analysis software (SAS SAS version 9.2, SAS Institute, Inc., Cary, NC, USA). Duncan's multiple range test was used for the mean comparisons between groups considering probability of $P \leq 0.05$ be significant. Data are expressed as mean values and standard deviations (SD).

Results

Diet and genotype effects on body and organ weights

Body weights and weights of testes and prostate are shown in Table 2-3. As expected body weights reflected genotypic differences between the lean and obese animals, with the obese animals having a significantly ($P<0.001$) higher body weight. Within obese animals, the body weight of Ob-Saff group was highest, which was sigfinicantly higher than the EPA and DHA groups. Regarding testis weights, although no significant differences were observed in absolute weights, relative weights of paired testes (% of body weight, w/w) were significantly lower in the Ob. Saff group compared to the Ln. Saff group ($P<0.0001$). Among obese animals, relative weight of testes in EPA group was significantly higher than the Ob. Saff group ($P<0.0001$). Obese animals had significantly larger prostate compared to lean animals in both absolute and relative weights ($P<0.0001$). Within the obese animal diet groups, animals fed the safflower diet had significantly larger prostate (absolute + relative weight) compared to EPA diet fed group ($P<0.0001$).

Table 2-3: Body weights and weights of accessory organs

	Ln. Saff n=6		Ob. Saff n=5		Ob. Flax n=7		Ob. EPA n=7		Ob. DHA n=8		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Significant effects (P)
Body weight (g)	428.81^{d}	21.81	675.06^{a}	34.76	651.69^{ab}	46.13	616.48^{bc}	44.26	578.90^{d}	50.33	<0.001
Testes*(g)	3.52	0.64	3.20	0.72	3.31	0.41	3.59	0.84	3.27	0.43	NS
Prostate gland (g)	0.68^{c}	0.19	1.87^{a}	0.34	1.56^{b}	0.31	1.41^{b}	0.19	1.46^{b}	0.49	<0.0001
Relative to body weight (%w/w)											
Testes*	0.82^{a}	0.16	0.47^{c}	0.10	0.51^{bc}	0.09	0.58^{b}	0.14	0.56^{bc}	0.06	<0.0001
Prostate gland	0.16^{c}	0.04	0.28^{a}	0.05	0.24^{ab}	0.04	0.23^{b}	0.03	0.25^{ab}	0.07	<0.0001

Values are Mean±SD. Significant differences were identified using one way analysis of variance (ANOVA). Values within a row having different superscipt letters are different by Duncan's multiple range test ($P<0.05$).
* Paired weights. NS, not significant

Diet and genotype influences on sperm morphology

Sperm morphology was studied to see the effect of diets (Figure 2-1). Abnormalities in sperm were classified on the basis of abnormal head, abnormal tail, abnormal head and tail, bent neck, and total abnormalities.When comparing the genotype effects in animals fed safflower diets, the obese animals had significantly fewer normal sperms, and higher sperm abnormalities compared to the lean animals ($P<0.0176$). Head abnormality were more abundant compared to the tail abnormaily in both lean and obese animals. Overall sperm abnormalities were not significantly different in the obese animal groups.

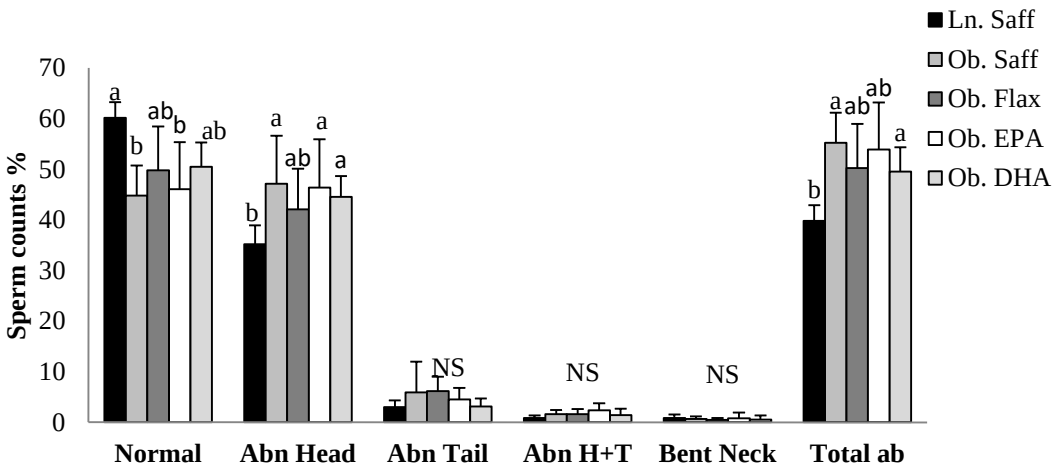

Figure 2-1: Effect of diet on sperm morphology
Mean±SD (n=5-6). Morphology was classified on the basis of Normal, normal sperm; Abn Head, abnormal head; Abn Tail, abnormal tail; Abn H+T, abnormal head+tail; bent neck; and Total ab, total abnormalities. Y axis shows sperm counts in %. Significant effects of diet were identified using one way analysis of variance (ANOVA). Bars within a sperm category having different letters are different by Duncan's multiple comparison ($P<0.05$). Ln. Saff, Lean animal fed safflower oil; Ob. Saff, Ob. Flax, Ob. EPA, Ob. DHA, Obese animals fed safflower, flax, EPA and DHA oils respectively.

Diet and genotype influences on fatty acid profiles in testes phospholipids

Effects of different n-3 dietary oil sources on fatty acid profiles of testis phospholipids were tested in lean and obese rats (Table 2-4). Specific changes were observed with changes in diet. With respect to the genotypes within the same diet (safflower), no major fatty acid composition chages were identified. However, the ratio of n-6/n-3 PUFAwas significantly lower in obese animal groups compared to lean animals ($P<0.0001$) (Figure 2-2). Within the obese groups, n-6/n-3 PUFA ratio was significantly lower in the Ob. Flax, Ob. EPA and Ob. DHA compared to the Ob. Saff group ($P<0.0001$). 18:3n-3 in the Ob. Flax diet was elongated and desaturated to provide 20:5n-3, however this level was lower compared to 20:5n-3 in the profile of Ob. EPA group ($P<0.0001$). Subsequent elongation and desaturation from EPA to DHA was observed in Ob.Flax group, but the level of DHA was significantly lower compared to Ob. EPA and Ob. DHA groups ($P<0.0001$). Ob. Saff had low levels of 20:5n-3 and 22:5n-6 compared to the n-3 supplemented groups ($P<0.0001$). In comparison, n-3 supplemented diets significantly decreased n-6 PUFAs compared to the n-6 enriched control diet. 22:5n-6 was significantly lower in n-3 diet groups compared with Ob. Saff diet ($P=0.0014$). Its precursor 20:4n-6 was also lower in Ob. EPA and Ob. DHA group compared with Ob. Saff ($P=0.0358$). Total VLCFA level in PL was about 3% with n-6 VLCFAs being abundant. n-6 total tetraenoic VLCFA was significanly decreased in animals fed EPA and DHA diets whereas n-3 pentaenic VLCFA were increased. VLCFAs 26:5n-3, 26:6n-3, 28:5n-3, and 30:5n-3 were negligible in testicular PL.

Table 2-4: Effect of diet and genotype on fatty acids in phospholipids

	Ln. Saff n=6		Ob. Saff n=5		Ob. Flax n=7		Ob. EPA n=7		Ob.DHA n=8		Significant
Fatty acid % w/w	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	effects (P)
14:0	0.23	0.08	0.17	0.04	0.17	0.03	0.18	0.04	0.16	0.04	NS
16:0	36.32	3.72	34.66	1.93	34.29	1.80	35.43	1.48	34.17	1.65	NS
18:0	7.51^{ab}	0.75	8.08^{a}	0.39	7.58^{ab}	0.62	7.29^{b}	0.50	6.90^{b}	0.71	0.0335
18:1n-9	9.03^{c}	1.24	9.39^{bc}	1.04	10.56^{a}	0.69	10.66^{a}	0.65	10.20^{ab}	0.60	0.0057
18:1n-7	1.51^{b}	0.19	1.52^{b}	0.12	1.70^{a}	0.09	1.72^{a}	0.08	1.69^{a}	0.10	0.0058
18:2n-6	3.20^{c}	0.47	2.85^{c}	0.65	3.85^{b}	0.14	2.92^{c}	0.41	4.42^{a}	0.62	<0.0001
18:3n-6	0.10	0.05	0.10	0.03	0.10	0.03	0.08	0.02	0.11	0.04	NS
18:3n-3	0.00^{b}	0.00	0.00^{b}	0.00	0.02^{a}	0.01	0.00^{b}	0.00	0.00^{b}	0.00	<0.0001
20:0	0.08^{b}	0.02	0.14^{a}	0.08	0.10^{b}	0.02	0.11^{ab}	0.02	0.09^{b}	0.02	0.0685
20:3n-6	1.20^{c}	0.28	1.20^{c}	0.09	1.48^{b}	0.07	1.49^{b}	0.24	2.02^{a}	0.16	<0.0001
20:4n-6	13.01^{ab}	2.22	13.94^{a}	1.61	13.64^{a}	0.41	11.15^{b}	2.33	12.35a^{b}	0.99	0.0358
20:5n-3	0.00^{c}	0.01	0.01^{c}	0.02	0.31^{b}	0.05	0.62^{a}	0.09	0.26^{b}	0.09	<0.0001
22:2n-6	0.02^{b}	0.05	0.07^{a}	0.07	0.02^{b}	0.02	0.01^{b}	0.01	0.01^{b}	0.01	0.0790
22:4n-6	1.57^{a}	0.27	1.67^{a}	0.20	1.37^{b}	0.10	1.25^{b}	0.07	1.21^{b}	0.11	<0.0001
22:5n-6	16.85^{a}	2.51	17.13^{a}	2.70	14.23^{b}	1.20	13.72^{b}	1.58	13.58^{b}	0.72	0.0014
22:5n-3	0.06^{c}	0.08	0.07^{c}	0.05	0.21^{b}	0.05	0.50^{a}	0.12	0.18^{b}	0.06	<0.0001
22:6n-3	0.51^{c}	0.12	0.69^{c}	0.06	2.22^{b}	0.39	3.42^{a}	0.60	3.95^{a}	1.24	<0.0001
VLCFA											
24:4n-6	0.93^{a}	0.12	0.95^{a}	0.05	0.89^{ab}	0.04	0.84b^{c}	0.07	0.78^{c}	0.06	0.0010
24:5n-6	0.91	0.16	1.00	0.19	0.90	0.05	0.93	0.09	0.87	0.08	NS
24:5n-3	0.00^{c}	0.00	0.00^{c}	0.00	0.05^{b}	0.04	0.13^{a}	0.05	0.06^{b}	0.04	<0.0001
24:6n-3	0.00^{c}	0.00	0.03^{c}	0.04	0.11^{b}	0.05	0.24^{a}	0.08	0.20^{a}	0.08	<0.0001
26:4n-6	0.07	0.07	0.08	0.07	0.07	0.02	0.04	0.04	0.05	0.04	NS
26:5n-6	0.06	0.03	0.09	0.02	0.09	0.02	0.09	0.03	0.07	0.04	NS

26:5n-3	nd		nd		nd		nd		nd		ND
26:6n-3	nd		nd		nd		nd		nd		ND
28:4n-6	0.11	0.08	0.10	0.04	0.07	0.02	0.09	0.04	0.09	0.03	NS
28:5n-6	0.07	0.07	0.04	0.02	0.07	0.03	0.04	0.05	0.05	0.02	NS
28:5n-3	nd		nd		nd		nd		nd		ND
28:6n-3	0.02	0.05	0.01	0.02	0.01	0.01	0.03	0.04	0.02	0.03	NS
30:5n-6	0.08	0.04	0.07	0.04	0.07	0.02	0.08	0.03	0.08	0.02	NS
30:5n-3	nd		nd		nd		nd		nd		ND
30:6n-3	0.14	0.19	0.11	0.09	0.08	0.05	0.16	0.08	0.10	0.10	NS
32:5n-6	0.21	0.11	0.25	0.26	0.10	0.13	0.18	0.09	0.08	0.06	NS
SFA	47.56	2.94	46.38	0.68	45.92	1.23	46.75	1.78	45.42	1.76	NS
MUFA	11.34^{b}	1.32	11.74^{b}	1.02	13.15^{a}	0.72	13.43^{a}	0.52	12.95^{a}	0.58	0.0004
n-6 PUFA	36.02^{ab}	2.51	37.01^{a}	1.94	34.77^{b}	1.30	30.75^{c}	1.84	33.82^{d}	1.72	<0.0001
n-3 PUFA	0.58^{c}	0.20	0.77^{c}	0.10	2.76^{b}	0.46	4.54^{a}	0.62	4.38^{a}	1.32	<0.0001
n-6 VLCFA	2.44^{a}	0.52	2.59^{a}	0.36	2.27^{ab}	0.12	2.28^{ab}	0.10	2.07^{b}	0.24	0.0438
n-3 VLCFA	0.25	0.33	0.31	0.41	0.32	0.20	0.67	0.21	0.41	0.27	NS
n-6/n-3 PUFA	66.58^{a}	17.72	49.15^{b}	8.73	12.85^{c}	1.89	6.91^{c}	1.29	8.42^{c}	2.68	<0.0001
n-6/n-3 VLCFA	14.89	15.23	12.32	12.17	12.38	13.28	3.60	0.80	7.72	5.76	NS
n-6TetraVLCFA	1.11^{ab}	0.24	1.14^{a}	0.09	1.04^{abc}	0.06	0.97^{bc}	0.08	0.91^{c}	0.09	0.0171
n-6 Penta VLCFA	1.33	0.28	1.45	0.27	1.23	0.10	1.31	0.10	1.15	0.16	NS
n-3 Penta VLCFA	0.00^{c}	0.00	0.00^{c}	0.00	0.05^{b}	0.04	0.13^{a}	0.05	0.06^{b}	0.04	<0.0001
n-3 Hexa VLCFA	0.25	0.33	0.31	0.41	0.27	0.17	0.54	0.18	0.35	0.24	NS

Values are Mean±SD. Significant differences were identified using one way analysis of variance (ANOVA). Values within a row having different superscipt letters are different by Duncan's multiple range test ($P<0.05$). Ln. Saff, Lean group supplemented with safflower. Ob. Saff, Ob. Flax, Ob. EPA, Ob. DHA, Obese animals supplemented with safflower, flax, EPA and DHA oils respectively. NS, not significant; ND, not detected; SFA, total saturated fatty acids; MUFA, total monounsaturated fatty acids; PUFA, total polyunsaturated fatty acids; VLCFA, total very long chain fatty acids; n-3, omega 3 fatty acids; n-6, omega 6 fatty acids.

Table 2-5: Effect of diet and genotype on fatty acids in triacylglycerides

	Ln. Saff n=4		Ob. Saff n=3		Ob.Flax n=5		Ob. EPA n=5		Ob. DHA n=6		Significant
Fatty acid % w/w	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	effects (P)
14:0	0.67	0.18	0.74	0.30	0.72	0.15	0.78	0.17	0.68	0.08	NS
16:0	27.96^{c}	0.28	29.54b^{c}	2.51	30.62^{b}	1.13	33.06^{a}	2.04	30.31^{b}	1.08	0.0015
18:0	3.15^{b}	0.64	4.40^{a}	0.98	3.84a^{b}	0.27	3.90^{ab}	0.42	3.49^{b}	0.25	0.0355
18:1n-9	8.08^{b}	1.15	9.98^{a}	1.99	10.14^{a}	0.75	9.66^{a}	0.84	10.09^{a}	0.75	0.0535
18:1n-7	2.54	0.16	2.22	0.12	2.54	0.36	2.70	0.25	2.43	0.31	NS
18:2n-6	5.43b^{c}	1.66	7.05^{a}	0.75	5.39b^{c}	0.72	4.63^{c}	0.63	6.60a^{b}	0.78	0.0089
18:3n-6	0.23	0.21	0.23	0.13	0.18	0.03	0.12	0.02	0.16	0.02	NS
18:3n-3	0.09^{b}	0.07	0.10^{b}	0.04	0.90^{a}	0.21	0.14^{b}	0.03	0.18^{b}	0.02	<0.0001
20:0	0.14	0.03	0.13	0.05	0.12	0.07	0.14	0.05	0.11	0.04	NS
20:3n-6	1.55^{bc}	0.12	1.31^{c}	0.24	1.71^{b}	0.19	1.57^{bc}	0.20	2.20^{a}	0.20	<0.0001
20:4n-6	5.00^{ab}	1.07	5.69^{a}	1.24	4.31^{bc}	0.58	3.82^{dc}	0.41	3.08^{d}	0.36	0.0005
20:5n-3	0.03^{c}	0.02	0.06^{c}	0.03	0.41^{b}	0.14	0.96^{a}	0.31	0.26^{b}	0.08	<0.0001
22:2n-6	0.03	0.06	0.06	0.07	0.05	0.03	0.05	0.04	0.06	0.08	NS
22:4n-6	5.11^{a}	0.69	5.07^{a}	0.56	4.08^{b}	0.23	3.39^{c}	0.37	3.37^{c}	0.33	<0.0001
22:5n-6	24.43^{a}	4.25	18.70^{b}	3.13	17.86^{b}	0.84	15.83^{b}	0.93	17.18^{b}	1.20	0.0002
22:5n-3	0.08^{c}	0.02	0.16^{c}	0.02	1.09^{b}	0.25	2.06^{a}	0.50	0.73^{b}	0.19	<0.0001
22:6n-3	0.57^{c}	0.02	1.22^{c}	0.34	3.17^{b}	0.71	3.28^{b}	0.32	7.64^{a}	1.55	<0.0001
VLCFA											
24:4n-6	2.78^{a}	0.14	2.30^{b}	0.20	2.02^{c}	0.13	1.65^{d}	0.03	1.60^{d}	0.21	<0.0001
24:5n-6	5.22^{a}	0.44	4.29^{b}	0.36	4.04b^{c}	0.35	3.74b^{c}	0.07	3.63^{c}	0.49	<0.0001
24:5n-3	nd		nd		nd		nd		nd		ND
24:6n-3	0.14^{d}	0.05	0.19^{d}	0.04	0.46^{c}	0.06	0.97^{a}	0.10	0.65^{b}	0.15	<0.0001
26:4n-6	0.36^{a}	0.06	0.22^{b}	0.09	0.22^{b}	0.04	0.20^{b}	0.06	0.21^{b}	0.04	0.0020
26:5n-6	0.77^{a}	0.04	0.75 ^{ab}	0.16	0.60^{bc}	0.07	0.57^{c}	0.17	0.44^{c}	0.09	0.0017

26:5n-3	nd		nd		nd		nd		nd		ND
26:6n-3	0.01^{c}	0.01	0.00^{c}	0.00	0.08^{a}	0.01	0.19^{b}	0.06	0.12^{b}	0.03	<0.0001
28:4n-6	0.25	0.02	0.18	0.06	0.19	0.04	0.20	0.04	0.18	0.03	NS
28:5n-6	1.08^{a}	0.07	0.82^{b}	0.07	0.71^{b}	0.10	0.74^{b}	0.30	0.65^{b}	0.12	0.0100
28:5n-3	nd		nd		nd		nd		nd		ND
28:6n-3	0.00^{b}	0.00	0.00^{b}	0.00	0.05^{b}	0.04	0.22^{a}	0.22	0.06^{b}	0.03	0.0309
30:5n-6	0.58^{a}	0.03	0.38^{b}	0.11	0.42^{b}	0.10	0.41^{b}	0.10	0.44^{b}	0.07	0.0423
30:5n-3	nd		nd		nd		nd		nd		ND
30:6n-3	0.00	0.00	0.04	0.07	0.11	0.16	0.08	0.06	0.05	0.04	NS
32:5n-6	0.09	0.01	0.06	0.03	0.08	0.04	0.08	0.05	0.07	0.02	NS
SFA	32.88^{b}	1.69	35.89^{b}	4.24	36.03^{b}	1.25	39.75^{a}	2.87	35.22^{b}	1.14	0.004
MUFA	12.19^{b}	1.27	14.25^{a}	2.44	14.57^{a}	1.07	14.27^{a}	0.67	14.45^{a}	0.72	0.0527
n-6 PUFA	42.06^{a}	2.37	38.24^{b}	3.28	33.76^{c}	0.90	29.62^{d}	1.63	32.85^{c}	1.18	<0.0001
n-3 PUFA	0.76^{c}	0.12	1.55^{c}	0.27	5.56^{b}	1.19	6.44^{b}	1.10	8.82^{a}	1.78	<0.0001
n-6 VLCFA	11.13^{a}	0.63	8.98^{b}	0.70	$8.29b^{c}$	0.68	7.59^{c}	0.57	7.22^{c}	1.00	<0.0001
n-3 VLCFA	0.15^{c}	0.05	0.23^{c}	0.10	0.71^{b}	0.14	1.46^{a}	0.25	0.89^{b}	0.20	<0.0001
n-6/n-3 PUFA	56.51^{a}	10.59	25.07^{b}	3.43	6.27^{c}	1.14	4.69^{c}	0.66	3.93^{c}	1.23	<0.0001
n-6/n-3 VLCFA	81.42^{a}	28.03	45.88^{b}	22.99	11.99^{c}	2.80	5.25^{c}	0.49	8.62^{c}	2.84	<0.0001
n-6TetraVLCFA	3.39^{a}	0.11	2.69^{b}	0.35	2.44^{b}	0.16	2.05^{c}	0.06	2.00^{c}	0.27	<0.0001
n-6 Penta VLCFA	7.74^{a}	0.52	6.29^{b}	0.43	$5.85b^{c}$	0.52	$5.54b^{c}$	0.55	5.22^{c}	0.74	<0.0001
n-3 Penta VLCFA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	NS
n-3 Hexa VLCFA	0.15^{c}	0.05	0.23^{c}	0.10	0.71^{b}	0.14	1.46^{a}	0.25	0.89^{b}	0.20	<0.0001

Values are Mean±SD. Significant differences were identified using one way analysis of variance (ANOVA). Values within a row having different superscipt letters are different by Duncan's multiple range test ($P<0.05$). Ln. Saff, Lean group supplemented with safflower; Ob. Saff, Ob. Flax, Ob. EPA, Ob. DHA, Obese animals supplemented with safflower, flax, EPA and DHA oils respectively. NS, not significant; ND, not detected; SFA, total saturated fatty acids; MUFA, total monounsaturated fatty acids; PUFA, total polyunsaturated fatty acids; VLCFA, total very long chain fatty acids; n-3, omega 3 fatty acids; n-6, omega 6 fatty acids.

Table 2-6: Effect of diet and genotype on fatty acids in cholesterol esters

	Ln. Saff n=4		Ob. Saff n=3		Ob. Flax n=5		Ob. EPA n=5		Ob. DHA n=6		Significant
Fatty acid % w/w	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	effects (P)
14:0	0.57	0.21	0.41	0.18	0.70	0.25	0.66	0.21	0.58	0.39	NS
16:0	20.32	3.53	19.65	3.07	19.77	3.39	23.48	2.07	20.64	2.85	NS
18:0	6.18	1.85	6.49	0.64	6.85	1.66	7.15	0.63	6.92	1.85	NS
18:1n-9	6.69	2.07	7.83	1.35	6.87	1.07	8.80	1.11	7.00	0.80	NS
18:1n-7	1.43^{c}	0.20	1.49b^{c}	0.11	1.56b^{c}	0.14	1.98^{a}	0.35	1.79^{ab}	0.20	0.0073
18:2n-6	3.82	1.67	4.76	1.08	3.49	0.83	4.21	0.59	4.32	0.91	NS
18:3n-6	0.11	0.08	0.20	0.04	0.19	0.08	0.15	0.03	0.12	0.08	NS
18:3n-3	0.04^{b}	0.07	0.01^{b}	0.01	0.16^{a}	0.05	0.03^{b}	0.00	0.05^{b}	0.04	0.0003
20:0	0.00	0.00	0.23	0.30	0.00	0.00	0.00	0.00	0.51	1.34	NS
20:3n-6	1.19^{b}	0.24	1.32^{b}	0.16	1.39^{b}	0.11	1.40^{b}	0.13	1.84^{a}	0.27	0.0004
20:4n-6	10.93^{ab}	5.50	14.93^{a}	3.97	8.06^{b}	2.69	11.16^{ab}	1.99	7.93^{b}	2.14	0.0353
20:5n-3	0.02^{c}	0.04	0.07^{c}	0.04	0.54^{b}	0.09	0.98^{a}	0.15	0.41^{b}	0.24	<0.0001
22:2n-6	0.40	0.17	0.33	0.18	0.51	0.42	0.13	0.12	0.91	1.34	NS
22:4n-6	2.88^{a}	0.43	3.28^{a}	0.03	2.75^{ab}	0.56	2.14^{b}	0.32	2.20^{b}	0.43	0.0037
22:5n-6	14.23^{a}	0.77	14.56^{a}	0.34	11.36^{b}	1.39	10.50^{b}	1.13	11.14^{b}	1.10	<0.0001
22:5n-3	1.17	0.58	0.86	0.42	1.76	0.37	1.32	0.18	1.24	0.71	NS
22:6n-3	0.70^{c}	0.14	0.95^{c}	0.24	2.26^{b}	0.45	2.39^{b}	0.56	4.34^{a}	1.26	<0.0001
VLCFA											
24:4n-6	3.81^{a}	1.11	3.32^{ab}	0.95	3.89^{a}	0.69	2.40^{b}	0.40	3.00^{ab}	0.70	0.0385
24:5n-6	5.80	1.77	4.74	0.82	5.79	1.40	3.83	0.75	4.94	1.77	NS
24:5n-3	0.48	0.21	0.40	0.18	0.17	0.26	0.37	0.17	0.39	0.23	NS
24:6n-3	0.12^{b}	0.24	0.24^{b}	0.37	1.81^{a}	0.73	1.08^{a}	0.39	1.08^{a}	0.69	0.0019
26:4n-6	0.89	0.33	0.55	0.31	0.83	0.22	0.46	0.10	0.61	0.31	NS
26:5n-6	1.95^{ab}	0.69	1.58^{ab}	0.42	2.07^{a}	0.46	1.11^{b}	0.26	1.30^{ab}	0.70	0.0554

26:5n-3	nd		nd		nd		nd		nd		ND
26:6n-3	0.00^{b}	0.00	0.01^{b}	0.01	0.47^{a}	0.16	0.28^{a}	0.18	0.37^{a}	0.16	0.0002
28:4n-6	0.35	0.12	0.21	0.09	0.29	0.06	0.20	0.02	0.23	0.11	NS
28:5n-6	3.37	1.48	2.24	0.74	2.94	0.87	1.55	0.40	2.11	0.94	NS
28:5n-3	nd		nd		nd		nd		nd		ND
28:6n-3	0.01^{b}	0.01	0.00^{b}	0.01	0.26^{a}	0.06	0.33^{a}	0.04	0.42^{a}	0.19	0.0001
30:5n-6	0.98	0.35	0.46	0.44	0.93	0.30	0.53	0.14	0.67	0.37	NS
30:5n-3	0.25^{a}	0.11	0.10^{b}	0.09	0.25^{a}	0.11	0.09^{b}	0.06	0.16^{ab}	0.10	0.0504
30:6n-3	0.04	0.09	0.07	0.12	0.20	0.30	0.16	0.10	0.16	0.07	NS
32:5n-6	0.12	0.07	0.06	0.03	0.12	0.10	0.07	0.05	0.07	0.05	NS
SFA	29.01	5.30	28.31	3.45	29.53	4.09	36.69	7.42	31.38	3.99	NS
MUFA	9.34^{b}	2.15	10.61^{ab}	1.48	9.87^{b}	0.71	12.21^{a}	1.46	10.27^{ab}	1.09	0.0478
n-6 PUFA	33.64^{ab}	7.39	39.40^{a}	5.20	27.77^{b}	3.87	29.73^{b}	3.62	28.50^{b}	2.87	0.0122
n-3 PUFA	2.02^{b}	0.79	1.90^{b}	0.65	4.77^{a}	0.73	4.76^{a}	0.86	6.09^{a}	1.74	<0.0001
n-6 VLCFA	17.26	5.84	13.18	3.56	16.85	3.34	10.16	1.93	12.93	4.79	NS
n-3 VLCFA	0.89^{b}	0.14	0.83^{b}	0.63	3.17^{a}	0.70	2.31^{a}	0.60	2.58^{a}	0.77	<0.0001
n-6/n-3 PUFA	20.44^{a}	14.09	22.73^{a}	8.72	5.97^{b}	1.42	6.35^{b}	1.03	5.06^{b}	1.66	0.0008
n-6/n-3 VLCFA	19.12^{a}	5.00	23.45^{a}	14.87	5.36^{b}	0.35	4.48^{b}	0.61	5.25^{b}	2.25	<0.0001
n-6TetraVLCFA	5.04^{a}	1.55	4.09^{ab}	1.35	5.00^{a}	0.83	3.06^{b}	0.51	3.84^{ab}	1.07	0.0459
n-6 Penta VLCFA	12.21	4.34	9.09	2.27	11.85	2.61	7.10	1.48	9.09	3.75	NS
n-3 Penta VLCFA	0.72	0.32	0.50	0.27	0.42	0.20	0.46	0.16	0.55	0.22	NS
n-3 Hexa VLCFA	0.17^{c}	0.24	0.32^{c}	0.49	2.75^{a}	0.72	1.85^{b}	0.47	2.03^{ab}	0.75	<0.0001

Values are Mean±SD. Significant differences were identified using one way analysis of variance (ANOVA). Values within a row having different superscipt letters are different by Duncan's multiple range test ($P<0.05$). Ln. Saff, Lean group supplemented with safflower; Ob. Saff, Ob. Flax, Ob. EPA, Ob. DHA, Obese animals supplemented with safflower, flax, EPA and DHA oils respectively. NS, not significant; ND, not detected; SFA, total saturated fatty acids; MUFA, total monounsaturated fatty acids; PUFA, total polyunsaturated fatty acids; VLCFA, total very long chain fatty acids; n-3, omega 3 fatty acids; n-6, omega 6 fatty acids.

Table 2-7: Effect of diet and genotype on fatty acids in free fatty acids

	Ln. Saff n=4		Ob.Saff n=3		Ob. Flax n=5		Ob.EPA n=5		Ob. DHA n=6		Significant
Fatty acid % w/w	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	effects
14:0	0.24	0.06	0.27	0.06	0.35	0.18	0.23	0.07	0.31	0.09	NS
16:0	30.66	0.88	30.06	0.87	30.08	2.71	30.78	2.47	30.65	2.33	NS
18:0	5.54	1.51	6.58	2.12	4.75	1.51	6.15	1.44	5.68	1.71	NS
18:1n-9	9.68	0.77	10.57	0.80	10.81	0.51	11.28	1.79	10.92	0.62	NS
18:1n-7	2.15	0.15	2.14	0.21	2.28	0.22	2.98	1.89	2.15	0.45	NS
18:2n-6	6.88^{b}	0.86	6.97^{b}	0.61	7.25^{b}	0.54	6.65^{b}	0.81	8.38^{a}	1.27	0.0071
18:3n-6	0.16a^{b}	0.04	0.17^{a}	0.02	0.16a^{b}	0.04	0.12^{b}	0.02	0.18^{a}	0.06	0.0744
18:3n-3	0.02^{b}	0.03	0.03^{b}	0.02	0.32^{a}	0.13	0.08^{b}	0.05	0.07^{b}	0.04	<0.0001
20:0	0.09	0.06	0.09	0.08	0.23	0.38	0.11	0.09	0.14	0.15	NS
20:3n-6	1.35^{c}	0.10	1.31^{c}	0.14	1.62^{b}	0.09	1.57^{b}	0.10	2.21^{a}	0.14	<0.0001
20:4n-6	18.64^{a}	1.28	19.16^{a}	0.80	16.70^{b}	1.92	16.32^{bc}	1.21	14.94^{c}	1.38	<0.0001
20:5n-3	0.05^{c}	0.02	0.07^{c}	0.02	0.82^{b}	0.17	1.85^{a}	0.56	0.68^{b}	0.20	<0.0001
22:2n-6	0.18	0.34	0.08	0.12	0.28	0.40	0.04	0.05	0.11	0.17	NS
22:4n-6	1.97^{a}	0.15	2.08^{a}	0.28	1.62^{b}	0.11	1.38^{c}	0.19	1.35^{c}	0.17	<0.0001
22:5n-6	13.07^{a}	1.11	11.76^{b}	0.60	10.45^{c}	0.61	9.36^{d}	0.68	9.93^{cd}	1.13	<0.0001
22:5n-3	0.05^{d}	0.01	0.08^{d}	0.02	0.39^{b}	0.13	0.83^{a}	0.11	0.25^{c}	0.07	<0.0001
22:6n-3	0.40^{d}	0.06	0.69^{d}	0.09	2.01^{c}	0.43	2.60^{b}	0.23	3.77^{a}	0.79	<0.0001
VLCFA											
24:4n-6	1.54^{a}	0.21	1.42^{ab}	0.20	1.32^{b}	0.15	1.02^{c}	0.13	1.12^{c}	0.15	<0.0001
24:5n-6	1.50^{a}	0.15	1.43^{a}	0.19	1.34^{ab}	0.18	1.15^{c}	0.19	1.24b^{c}	0.24	0.0247
24:5n-3	0.01^{bc}	0.02	0.00^{c}	0.00	0.04a^{bc}	0.03	0.09^{a}	0.07	0.06^{ab}	0.03	0.0057
24:6n-3	0.03^{d}	0.02	0.06^{d}	0.03	0.16^{c}	0.02	0.29^{a}	0.07	0.23^{b}	0.06	<0.0001
26:4n-6	0.26^{a}	0.04	0.23^{ab}	0.03	0.22^{ab}	0.07	0.16^{c}	0.03	0.18^{bc}	0.03	0.0031
26:5n-6	0.35^{a}	0.05	0.32^{a}	0.06	0.33^{a}	0.07	0.24^{b}	0.05	0.28^{ab}	0.05	0.0097

26:5n-3	nd		nd		nd		nd		nd		ND
26:6n-3	0.00^{b}	0.00	0.00^{b}	0.00	0.08^{a}	0.04	0.07^{a}	0.04	0.06^{a}	0.04	0.0001
28:4n-6	0.15^{a}	0.03	0.13^{ab}	0.03	0.13^{ab}	0.03	0.10^{b}	0.02	0.11^{b}	0.03	0.0441
28:5n-6	0.57^{a}	0.09	0.48^{ab}	0.11	0.47^{ab}	0.10	0.31^{c}	0.08	0.40^{bc}	0.10	0.0007
28:5n-3	0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.01	0.02	0.02	NS
28:6n-3	0.00	0.00	0.00	0.00	0.03	0.05	0.05	0.04	0.04	0.04	0.0536
30:5n-6	0.32	0.08	0.29	0.09	0.25	0.08	0.17	0.09	0.19	0.14	NS
30:5n-3	0.01	0.01	0.01	0.01	0.05	0.11	0.07	0.10	0.07	0.11	NS
30:6n-3	0.02	0.01	0.02	0.02	0.03	0.02	0.05	0.04	0.03	0.02	NS
32:5n-6	0.04	0.02	0.03	0.02	0.04	0.04	0.02	0.02	0.04	0.04	NS
SFA	38.84	1.57	38.53	1.94	38.69	2.48	38.99	2.29	39.03	2.01	NS
MUFA	13.12^{c}	0.68	14.21^{b}	0.97	14.62^{b}	0.41	15.92^{a}	0.76	14.57^{b}	0.48	<0.0001
n-6 PUFA	42.41^{a}	0.91	41.64^{a}	1.00	38.23^{b}	1.99	35.60^{c}	1.62	$37.27b^{c}$	1.88	<0.0001
n-3 PUFA	0.51^{c}	0.11	0.87^{c}	0.14	3.55^{b}	0.80	5.36^{a}	0.65	4.77^{a}	0.94	<0.0001
n-6 VLCFA	4.72^{a}	0.57	4.33^{a}	0.65	4.09^{ab}	0.56	3.17^{c}	0.47	3.57^{bc}	0.66	0.0004
n-3 VLCFA	0.07^{c}	0.02	0.09^{c}	0.03	0.40^{b}	0.12	0.63^{a}	0.14	0.49^{b}	0.12	<0.0001
n-6/n-3 PUFA	85.81^{a}	16.31	49.07^{b}	8.10	11.15^{c}	2.11	6.70^{c}	0.57	8.16^{c}	1.98	<0.0001
n-6/n-3 VLCFA	66.95^{a}	14.46	51.55^{b}	21.01	10.77^{c}	2.54	5.18^{c}	0.88	7.72^{c}	2.41	<0.0001
n-6TetraVLCFA	1.95^{a}	0.24	1.77^{ab}	0.24	$1.66b^{c}$	0.24	1.28^{d}	0.17	1.42^{cd}	0.20	<0.0001
n-6 Penta VLCFA	2.45^{a}	0.28	2.27^{ab}	0.36	2.18^{ab}	0.35	1.71^{c}	0.32	1.97^{bc}	0.41	0.0077
n-3 Penta VLCFA	0.04^{b}	0.02	0.03^{b}	0.03	0.11^{ab}	0.09	0.18^{a}	0.06	0.15^{a}	0.11	0.0048
n-3 Hexa VLCFA	0.05^{c}	0.03	0.08^{c}	0.02	0.31^{b}	0.06	0.47^{a}	0.09	0.36^{b}	0.14	<0.0001

Values are Mean±SD. Significant differences were identified using one way analysis of variance (ANOVA). Values within a row having different superscipt letters are different by Duncan's multiple range test ($P<0.05$). Ln. Saff, Lean group supplemented with safflower; Ob. Saff, Ob. Flax, Ob. EPA, Ob. DHA, Obese animals supplemented with safflower, flax, EPA and DHA oils respectively. NS, not significant; ND, not detected; SFA, total saturated fatty acids; MUFA, total monounsaturated fatty acids; PUFA, total polyunsaturated fatty acids; VLCFA, total very long chain fatty acids; n-3, omega 3 fatty acids; n-6, omega 6 fatty acids.

Diet and genotype influences on fatty acid profiles in testes triacylglycerides

Effects of different n-3 dietary oil sources on fatty acid profiles of testis triacylglyceride were tested in lean and obese rats (Table 2-5). With respect to genotype, obese testis had significantly lower n-6 DPA and higher DHA leading to significantly lower n-6/n-3 PUFA ratio compared to the lean counterpart ($P<0.0001$) (Figure 2-2). The level of 20:4n-6 was similar in both groups but LA was significantly higher in the obese group compared to the lean group ($P=0.0089$). Also 18:0 and 18:1 was significantly higher in obese testis ($P<0.05$).Within the obese diet groups, the n-6/n-3 PUFA ratio was significantly lower in all n-3 supplemented diets compared to Ob. Saff group ($P<0.0001$) and this was accompanied with a significantly higher level of DHA ($P<0.0001$). With respect to the n-3 supplemented diet groups, the Ob.Flax diet showed highest levels of 18:3n-3 compared to Ob. EPA and Ob. DHA ($P<0.0001$), but levels of 20:5n-3 were lower compared to Ob. EPA ($P<0.0001$), and levels of 22:6n-3 were lower compared to Ob. DHA. 22:6n-3 was also significantly lower in Ob. EPA compared to Ob. DHA ($P<0.0001$), which was not observed in phospholipid fatty acid composition. A look at n-6 LCFA metabolism in obese rats showed n-3 supplemented diets had lower levels of 18:2n-6 ($P=0.0089$) and 20:4n-6 ($P=0.0005$) in comparison to Ob. Saff, but no significant changes in the levels of 22:5n-6 were observed in any of the obese animals. The fatty acid profile of VLCFA showed genotype and diet influences. Total VLCFA level in TG was about 9-11% with n-6 VLCFAs being abundant. Total n-6 VLCFA were significantly lower in Ob. Saff compared to Ln ($P<0.0001$). The n-6/n-3 VLCFA ratio in Ob. Saff was also lower than Ln. Saff ($P<0.0001$). Within the obese animals, n-3 supplemented diets had lower total n-6 VLCFA compared to Ob. Saff. Total n-3 VLCFA were the highest in Ob. EPA, followed by Ob. Flax and Ob. DHA ($P<0.0001$). n-3 VLCFA 24:5n-3, 26:5n-3, 28:5n-3, 30:5n-3 were negligible in TG.

Diet and genotype influences on fatty acid profiles in testes cholesterol esters

Effects of different n-3 dietary oil sources on fatty acid profiles of testis cholesterol were tested in lean and obese rats (Table 2-6). The effect of genotype was minor in CE. Within the obese diet groups, the n-6/n-3 PUFA ratio was lower in all n-3 supplemented groups compared to Ob. Saff ($P=0.0008$) (Figure 2-2). Within the n-3 supplemented groups, the Flax oil fed group was lower in 20:5n-3 and 22:6n-3 compared to the Ob. EPA ($P<0.0001$), and Ob. DHA ($P<0.0001$). Due to a compensatory effect, Ob. Saff had lowest levels of 20:5n-3 and 22:5n-6 compared to the n-3 supplemented groups ($P<0.0001$). With respect to n-6 LCFA metabolism, total n-6 fatty acids were low in all the n-3 diets compared to Ob. Saff ($P=0.0122$). Among the obese diet groups, Ob. Flax and Ob. DHA had lower 20:4n-6 compared to Ob. Saff ($P=0.0353$), and all n-3 diets had lower 22:5n-6 compared to Ob. Saff ($P<0.0001$). Total VLCFA level in CE was about 12-18% with n-6 VLCFAs being abundant. The VLCFA profile showed n-3 supplemented diets had higher n-3 VLCFA compared to Ob. Saff ($P<0.0001$), but no significant difference was observed in levels of total n-6 VLCFA. The n-3 VLCFA 26:5n-3 and 28:5n-3 were negligible in CE.

Diet and genotype influences on fatty acid profiles in testes free fatty acids

Effects of different n-3 dietary oil sources on fatty acid profiles of testis free fatty acids were tested in lean and obese rats (Table 2-7). The genotype impact on free fatty acid profile showed a lower n-6/n-3 PUFA ratio in obese testis compared to the lean animals, with 22:5n-6 specifically lower in Ob. Saff compared to Ln. Saff ($P<0.0001$) (Figure 2-2). In the obese animal groups, the n-6/n-3 PUFA ratio was low in all the n-3 diet groups compared to Ob. Saff group ($P<0.0001$). n-3 LCFA metabolism showed Ob. Flax had higher levels of 18:3n-3 compared with Ob. EPA and Ob. DHA ($P<0.0001$), but 22:5n-3

and 22:6n-3 levels were not as high in the Ob. Saff diet compared to Ob. EPA and Ob. DHA diets (P<0.0001). 22:6n-3 levels were seen in the order of Ob. DHA>Ob. EPA> Ob. Flax> Ob. Saff, with Ob. DHA having highest level of 22:5n-6. A look at n-6 LCFA metabolism showed all n-3 diet groups had lower levels of 20:4n-6 and 22:5n-6 compared to Ob. Saff. Within the n-3 groups, 18:2n-6 was highest in Ob. DHA group, but 20:4n-6 was the lowest in this group. Total VLCFA level in FFA was about 4-5% with n-6 VLCFAs being abundant. VLCFA profile showed total n-6 VLCFAs were lowest in Ob. EPA, followed by Ob. DHA. n-3 VLCFA were highest in Ob. EPA group compared to Ob. Flax and Ob. DHA groups, with Ob. Saff having the lowest level of total n-3 VLCFAs. 26:5n-3 was the only VLCFA not detected in FFA.

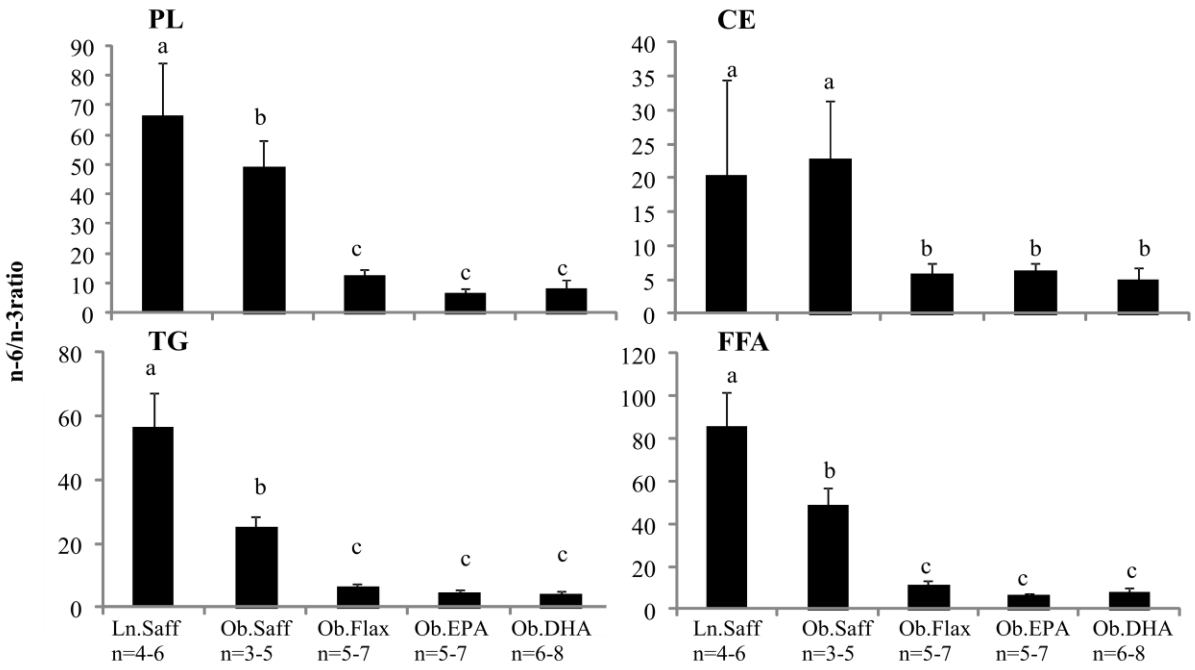

Figure 2-2: Effect of genotype and diet on n-6/n-3 polyunsaturated fatty acid ratio in each lipid classes in testis

Bars are Mean ± SD. Significant effects of diet were identified using one way analysis of variance (ANOVA). Bars within each lipid class having a different superscipt letters are

different by Duncan's multiple comparison ($P<0.05$). Ln. Saff, Lean group supplemented with safflower; Ob. Saff, Ob. Flax, Ob. EPA, Ob. DHA, Obese animals supplemented with safflower, flax, EPA and DHA oil, respectively; PL, phospholipids; TG, triglycerides; CE, cholesterol esters; FFA, free fatty acids.

Discussion

This study presents a complete fatty acid profile in all lipid classes in obese testis. While the majority of n-6 and n-3 fatty acids were altered by different dietary n-3 fatty acid sources, this study demonstrated that ALA was not as effective in increasing DHA levels in comparison to EPA and DHA diets. While the levels of PUFA and VLCFA were different in each lipid class, the n-6 series of PUFA and VLCFA were abundant in obese rat testis, and were sensitive to modulation by dietary treatment.

Effect of obesity on testes fatty acid compositions

There were genotypic differences in the PL, TG and FFA profiles in terms of n-6/n-3 PUFA ratios. In all lipid classes except CE, the ratio of n-6/n-3 PUFA was lower in the testes of obese animals compared to the lean animals, despite the same n-6 enriched diet being provided; this suggests a suboptimal n-6 PUFA metabolism in the obese animals. A look at the major fatty acids in n-6 PUFA metabolism showed LA was in fact higher in obese testis TG compared to lean testis. But DPA, known to be the predominant fatty acid in rat testes, was decreased in obese compared to lean testis in TG and FFA lipid classes. Our findings regarding PL n-6 fatty acids are consistent with previous studies that show the n-6 lipid environment is maintained in obese rat testes (Suh et al., 2011), but the TG and FFA classes in fact show decreased DPA. Since DPA is predominantly a membrane lipid, the variation could be much less between normal and obese conditions, or TG and FFA could be more affected by obesity induced hormone sensitive lipase; this needs further study.

Effect of n-3 fatty acid supplementation on testes fatty acid composition

Diet supplementation with n-3 fatty acids, regardless of its source reduced the n-6/n-3 PUFA ratio compared with the control safflower diet in all lipid classes. This shows testes are sensitive to changes in diet, and these changes are seen in all lipid classes. One of the objectives of this study was to test the conversion of dietary ALA to DHA and its efficiency against direct consumption of DHA. Flax oil was used to provide ALA, keeping the n-6/n-3 ratio 1:3. In all lipid classes, DHA was higher in DHA enriched diet compared to Flax diet. Similarly, EPA levels in all groups were higher in the EPA enriched group compared to Flax diet. This indicates that by providing ALA enriched Flax oil, although ALA is converted to EPA and DHA, higher levels of EPA and DHA in testes can be achieved by direct dietary supplementation with EPA and DHA oil. Providing EPA directly did result in its conversion to DHA in the phospholipids, however, EPA supplementation did not provide the same level of DHA as direct DHA oil supplementation in the TG, CE and FFA lipid classes. With respect to n-6 LCFA, 22:5n-6 levels were low in all n-3 diet groups except TG compared to Ob. Saff, indicating perhaps a preference for n-3 LCFA metabolism in n-3 supplemented diets. Whether these changes directly affect spermatid maturation needs to be tested.

Genotype and diet influences on VLCFA in different lipid classes in testes

Previous studies have found VLCFA in testes phospholipids (Robinson et al., 1992) and sphingomyelins and ceramides (Furland et al., 2007b). Other studies also detected the presence of VLCFA in lipid classes other than phospholipids (Aveldano et al., 1993). This study found presence of VLCFA in FFA of the testes as well, and this has not been commonly studied. No significant differences were found in the VLCFA in testes PL with respect to diet. While the majority of n-3 VLCFA were not detected, the PL and TG,

FFA presented clearly most of the n-6 VLCFA. This indicates that perhaps metabolism of n-6 VLCFA is preferred for testis function. In the TG and CE lipid classes, the EPA enriched diet had the highest total n-3 VLCFA. Previous studies have shown that EPA, but not DHA, gets converted to pentaenoic and hexaenoic VLCFA in the retinal phospholipid (Suh & Clandinin, 2005), indicating EPA is metabolically more active than DHA in terms of chain elongation and desaturation in both tissues.

In conclusion, there is limited research available on the impact of obesity on the lipid environment of testes and the response of testes to dietary treatment. Although no significant changes in sperm morphology were observed in this study, sperm concentration and sperm maturation in the obese animals needs to be tested with respect to changes in lipid environment of testes. In addition, a larger sample size looking at sperm morphology would help clarify the effects of dietary fat on sperm morphology. Our findings show that an obese condition affects the lipid metabolism in testes, more specifically n-6 PUFA metabolism. Dietary supplementation with n-3 fatty acids changed the fatty acid composition of PL, TG, CE and FFA. The ratio of n-6/n-3 PUFA was lowered in all n-3 fatty acid diet groups, indicating that testes are sensitive to changes in dietary fat. Flax oil inclusion in the diet increased EPA and DHA in the testes, indicating that through Sprechers pathway ALA is converted to its successive fatty acids, however it does not provide the same levels of EPA and DHA as direct supplementation with EPA and DHA oil. Previous studies have investigated VLCFA in testes membrane lipids, i.e. phospholipids. This study provides a complete profile of VLCFA in major lipid classes in the testes, and demonstrates their sensitivity to diet changes.

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CHAPTER III: PENTAENOIC LONG AND VERY LONG CHAIN FATTY ACIDS (C>24) ARE SELECTIVELY DECREASED IN THE LIPID CLASSES OF UNDERDEVELOPED TESTIS

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Abstract

A previous study has shown that a significant decrease in n-6 docosapentaenoic acids (n-6 DPA) in phosphatiddylcholine and phosphatidylethanolamine is associated with testis underdevelopment. This study further investigated whether the same phenomenon takes place in other lipid classes in underdeveloped testis. Adult male obese *(fa/fa)* Zucker rats presenting both under- and normal sized testis were used for this study. Testis with more than 30% weight differences in a paired testes was considered underdeveloped. n-6 long chain fatty acids and their elongated and desaturated products of very long chain fatty acids (C>24, VLCFA) were measured in phospholipid (PL), triacylglycerides (TG), cholesterol ester (CE) and free fatty acids (FFA) in testis. In comparison to normal size testis, underdeveloped testes showed a marked decrease in n-6 DPA in PL and FFA, and total n-6 pentaenoic VLCFA in PL, TG and FFA. Whereas, the precursor of DPA, 20:4n-6 was significantly increased in PL; C22:4n-6 and C28:4n-6 were increased in PL, CE and FFA. This study indicates that conversion of tetraenoic to pentaenoic long chain fatty acids and VLCFA in the underdeveloped testes was limited in underdeveloped testis PL, TG and FFA, indicating a crital role of fatty acids in testis development.

Keywords: Testes, Underdevelopment, Docosapentaenoic acids, Very long chain fatty acids, Phospholipids, Free fatty acids, Triacylglyceride, Cholesterol ester

Introduction

Recent evidence has highlighted the importance of lipid metabolism, specifically long chain polyunsaturated fatty acids (LCPUFA) in normal testes function. More interesting evidence shows LCPUFA is closely related with testis development including size. For example, in experimental cryptorchidism in rats, a 50% loss in testicular weight , due to loss in germ cells was accompanied with a reduction in n-6 docosapentaenoic acid (DPA,22:5n-6) and 28:4n-6 in phospholipids (Furland et al., 2007a). Similarly, testes exposed to X ray irradiation showed decrease in weight, followed by loss of DPA rich phospholipids and non-hydroxy very long chain fatty acids (C>24,VLCFA) (Oresti et al., 2010). This shows a definite link between testis development and fatty acid composition in testicular lipids. However, there is still limited information on lipid changes in relation to testis underdevelopment.

Previous studies conducted in our lab showed that about 60% of obese *(fa/fa)* Zucker rats, and 35% of lean Zucker rats had unbalanced testes, leaving one of the testes underdeveloped (Suh et al., 2011). Fatty acid profiles of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) showed a specific reduction in DPA in the underdeveloped testis compared to normal testis, whereas its precursor, arachidonic acid (AA, C20:4n-6), was significantly higher in PC (Suh et al., 2011). However, whether this phenomenon occurs in other lipid classes such as triacylglycerols (TG), cholesterol esters (CE) and free fatty acids (FFA) is not known. In addition to LCFA, information about VLCFA metabolism in an underdeveloped testis is limited.

Thus, this study will determine the changes in composition of major lipid classes in the underdeveloped testis in comparison to normal testis. Obtaining a full fatty acid profile of

major lipid classes will help us understand the normal development of the testis, thereby maintaining normal function of testis.

Materials and methods

Animals and diets

This study protocol was approved by the University of Manitoba Animal Care Committee. Adult male *fa/fa* Zucker rats (Charles River Laboratories St-Constant, PQ), fed AIN 93G diet (Reeves et al., 1993) containing safflower oil for 8 weeks were used in this study. A difference greater than 30% in testis size within the paired testes was counted, and the smaller testis of the pair was considered underdeveloped (Suh et al., 2011). The average weight of testes of the normal and underdevelped testis was 1.86 ± 0.31 and 0.93 ± 0.06 grams, respectively. Animals were sacrificed by carbon dioxide asphyxiation followed by decapitation. Testes were collected, weighed, and immediately frozen in liquid nitrogen and stored at -80°C.

Lipid analysis

Total lipids were extracted from testes using the Folch method (Folch et al., 1957). Decapsulated testes were homogenized in 0.025% calcium chloride to form a 20%w/w solution, 1 ml of which was used for extraction of total lipids. Ten ml of chloroform and methanol (2:1, v/v) were used for extraction. Separations into lipid classes phospholipid (PL), triglycerides (TG), cholesterol ester (CE) and free fatty acid (FFA) were carried out using silica-gel G-plates (Analtech silica-gel G; MandelScientific Co., Guelph, Ontario, Canada) prewashed in hexane. The separation mobile phase was petroleum ether, diethyl ether and acetic acid in the ratio 80:20:1 (v/v/v). Lipid classes were visualized with 0.1% (w/v) aniline naphthalene sulfonic acid under UV light. CE and TG bands were

saponified to cleave fatty acids using 0.5 moles of methanolic KOH and heating in a sand bath at 110°C for 1.5 hours. Fatty acid methyl esters were prepared for all the bands using boron trifluoride (BF3) in methanol by incubating in a sand bath at 110°C for 1 hour.

Fatty acid analysis

Separation of fatty acids was carried out on a SGE BPX-70 column (10 m X 0.10 mm diameter and 0.2 μm film thickness), using a Varian 450 gas chromatography instrument coupled to a flame ionization detector (Vista 6010 GLC and Vista 402 data system; Varian Instruments, Mississauga, Ontario, Canada). The temperature program was 120°C for 0 min, then was raised to 175°C at 10°C/min, held for 0.3 min, raised to 250°C at 15°C/min, held for 0 min, and finally raised to 280°C at 6°C/min and held for 4 min. Total run time was 19.8 min, and samples were run with a 50:1 split ratio and a 0.4 ml/min column flow. Hydrogen was used as the carrier gas for the method. The injector/detector temperatures were 280°C and 290°C, respectively.

Statistical analysis

The effect of testis size on fatty acids in testicular lipids was tested by student t-test using SAS (version 9.2; SAS Institute, Inc., Cary, NC, USA). Probability of $P \leq 0.05$ was considered significant. Data are expressed as mean values and standard deviations.

Results and Discussion

Separation of lipid classes in normal and underdeveloped testes

The total lipids were separated into phospholipids, monoacylglycerols, free fatty acids and triacylglycerols using diethyl ether and acetic acid in the ratio 80:20:1 (v/v/v).

Separations were visualised under UV using 0.1% aniline naphthalene sulfonic acid (w/v).

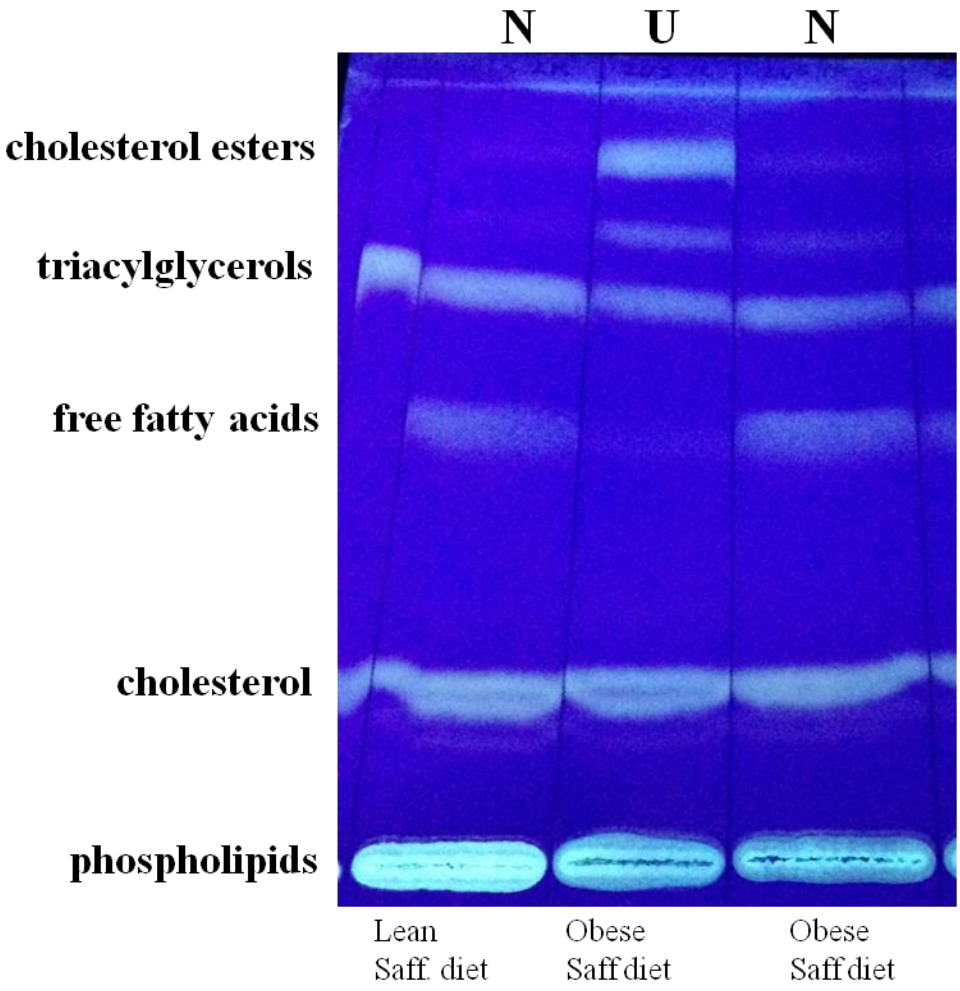

Figure 3-1: Typical lipid classes in normal and underdeveloped testis in obese Zucker rats

Pictures showing the band separation observed in normal and underdeveloped testes. N, normal sized testis; U, underdeveloped testis; Lean- testes from a lean animal supplemented with safflower; Obese, Obese group supplemented with safflower. Approximately 1mg of total lipid was loaded and separated by using a mobile phase of petroleum ether, diethyl ether and acetic acid in the ratio 80:20:1 (by volume), and bands were visualized under UV with 0.1% aniline naphthalene sulfonic acid (w/v).

Fatty acid profiles of normal and underdeveloped testes

A typical lipid class distribution is shown in Figure 3-1 between normal and underdeveloped testis. Although quantitative measurement of each lipid was not made, CE was accumulated while FFA was decreased in underdeveloped testis. A similar significant increase of CE was noted after X-ray irradiation of adult rat testes, where the accumulated DPA- rich CE were found to be Sertoli cell products (Oresti et al., 2010). Oresti et al., (2010) interpreted that Sertoli cells hydrolyze injury- associated accumulated fats to FFA, which are converted to CoA esters in the endoplasmic reticulum, and then coupled with free cholesterol to produce CE. In this study the FFA fraction would be expected to be higher, but our findings in Figure 3-1 indicate otherwise. Thus, the lipid distribution and processing mechanisms occurring in underdeveloped testes of obese rats may be different from that of testicular atrophy resulting from injury.

Fatty acid profile of normal and underdeveloped testes was obtained. Figure 3-2 shows a typical chromatogram of normal and underdeveloped testis in TG. There was a specific lowering of DPA in the phospholipid profile of underdeveloped testes, despite its precursor AA having the same levels in both testes. There was also a change in the concentration of VLCFA in underdeveloped testes compared to normal.

To better understand the changes in fatty acid composition taking place in underdeveloped testes, the fatty acid profiles of PL, TG, CE, and FFA were studied (Table 3-1).

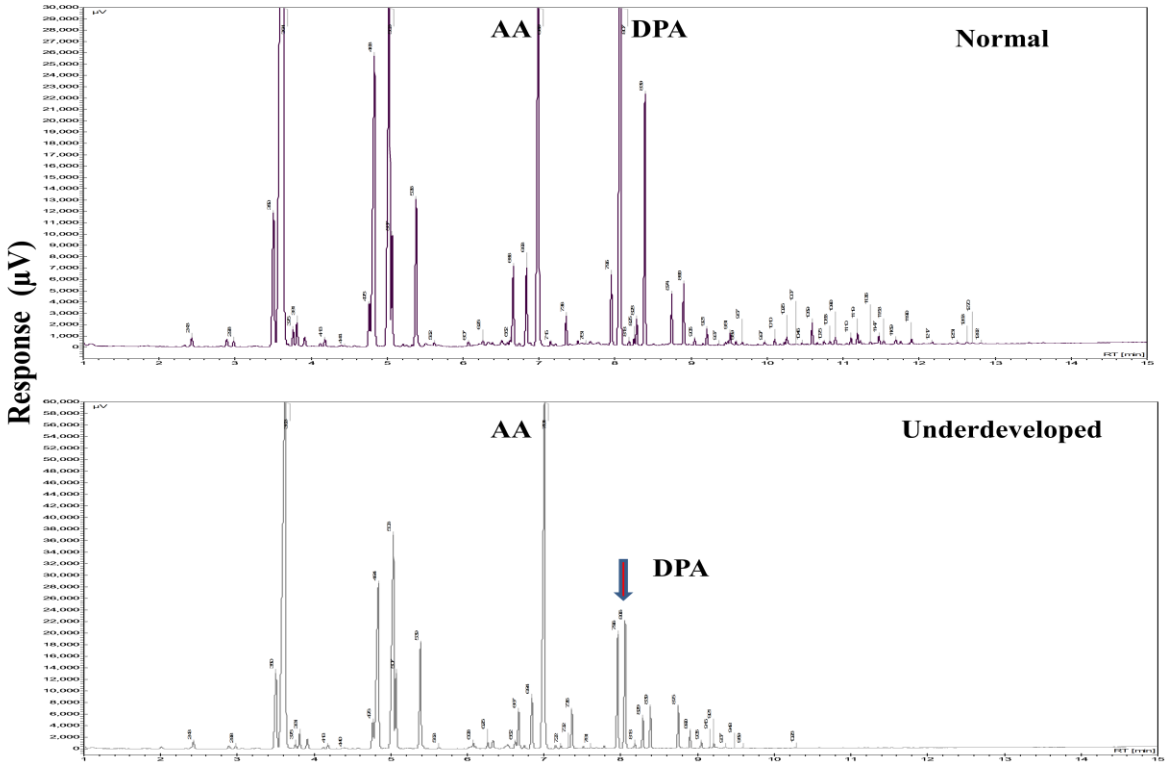

Figure 3-2: Chromatogram of normal and underdeveloped testes triacyglycerides
A chromatogram showing the content of arachidonic acid (20:4n-6) and DPA (22:5n-6) in phospholipids of normal and underdeveloped testes. AA, arachidonic acid; DPA, docosapentaenoic acid.

Table 3-1: Fatty acid profile of normal and underdeveloped testes

	Phospholipids				Triacylglycerols				Cholesterol esters				Free fatty acids			
	Normal		Under		Normal		Under		Normal		Under		Normal		Under	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Fatty acid % w/w	n=5		n=3		n=3		n=3		n=3		n=3		n=5		n=3	
14:0	0.17	0.04	0.21	0.02	0.74	0.30	0.76	0.10	0.41	0.18	0.47	0.07	0.27	0.06	0.38*	0.00
16:0	34.66	1.93	28.94*	1.67	29.54	2.51	28.43	2.10	19.65	3.07	8.70*	0.30	30.06	0.87	26.02	4.99
18:0	8.08	0.39	12.15	0.96	4.40	0.98	6.79*	0.82	6.49	0.64	4.10*	1.11	6.58	2.12	10.76*	0.99
18:1n-9	9.39	1.04	9.44	2.02	9.98	1.99	11.85	1.42	7.83	1.35	5.53	1.03	10.57	0.80	9.13*	0.94
18:1n-7	1.52	0.12	2.29*	0.58	2.22	0.12	2.53	0.67	1.49	0.11	2.15	0.51	2.14	0.21	2.22	0.33
18:2n-6	2.85	0.65	4.36*	0.15	7.05	0.75	9.94	4.22	4.76	1.08	2.71	0.83	6.97	0.61	6.88	1.28
18:3n-6	0.10	0.03	0.14	0.03	0.23	0.13	0.18	0.08	0.20	0.04	0.15	0.05	0.17	0.02	0.17	0.01
18:3n-3	0.00	0.00	0.00	0.00	0.10	0.04	0.15	0.04	0.01	0.01	0.01	0.01	0.03	0.02	0.04	0.02
20:0	0.14	0.08	0.26	0.17	0.13	0.05	0.41*	0.13	0.23	0.30	0.23	0.28	0.09	0.08	0.20	0.04
20:3n-6	1.20	0.09	1.69*	0.20	1.31	0.24	2.07*	0.28	1.32	0.16	3.06	1.17	1.31	0.14	1.68*	0.12
20:4n-6	13.94	1.61	21.81*	3.10	5.69	1.24	6.64	2.09	14.93	3.97	11.51	5.44	19.16	0.80	21.89	5.05
20:5n-3	0.01	0.02	0.07	0.06	0.06	0.03	0.04	0.01	0.07	0.04	0.07	0.02	0.07	0.02	0.08	0.03
22:2n-6	0.07	0.07	0.07	0.01	0.06	0.07	0.22*	0.05	0.33	0.18	0.00*	0.00	0.08	0.12	0.13	0.05
22:4n-6	1.67	0.20	3.95*	1.04	5.07	0.56	6.11	1.11	3.28	0.03	5.85*	1.54	2.08	0.28	4.42*	1.28
22:5n-6	17.13	2.70	4.80*	1.53	18.70	3.13	11.21	4.72	14.56	0.34	16.45	7.22	11.76	0.60	7.65*	2.10
22:5n-3	0.07	0.05	0.12	0.04	0.16	0.02	0.23	0.06	0.86	0.42	3.18*	1.12	0.08	0.02	0.18*	0.01
22:6n-3	0.69	0.06	0.52	0.22	1.22	0.34	1.15	0.30	0.95	0.24	2.79	1.78	0.69	0.09	1.23*	0.43
VLCFA																
24:4n-6	0.95	0.05	1.29*	0.15	2.30	0.20	2.24	0.52	3.32	0.95	3.62	3.16	1.42	0.20	1.89*	0.33
24:5n-6	1.00	0.19	0.52*	0.07	4.29	0.36	1.84*	0.72	4.74	0.82	6.84	5.94	1.43	0.19	0.80*	0.33
24:5n-3	0.00	0.00	0.04	0.05	0.00	0.00	0.00	0.00	0.40	0.18	0.58	0.57	0.00	0.00	0.02*	0.02

24:6n-3	0.03	0.04	0.07	0.12	0.19	0.04	0.43*	0.10	0.24	0.37	10.73	9.30	0.06	0.03	0.20	0.27
26:4n-6	0.08	0.07	0.00	0.00	0.22	0.09	0.20	0.25	0.55	0.31	1.50	0.89	0.23	0.03	0.20	0.07
26:5n-6	0.09	0.02	0.04	0.05	0.75	0.16	0.33	0.29	1.58	0.42	1.21	0.89	0.32	0.06	0.23	0.11
26:5n-3	nd		nd		nd		nd		nd		nd		nd		nd	
26:6n-3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.15	0.27	0.00	0.00	0.00	0.00
28:4n-6	0.10	0.04	0.02*	0.03	0.18	0.06	0.04*	0.07	0.21	0.09	0.33	0.07	0.13	0.03	0.07*	0.02
28:5n-6	0.04	0.02	0.00*	0.00	0.82	0.07	0.24*	0.34	2.24	0.74	1.16	0.37	0.48	0.11	0.24	0.17
28:5n-3	nd		nd		nd		nd		nd		nd		nd		nd	
28:6n-3	0.01	0.02	0.04	0.04	0.00	0.00	0.02	0.03	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
30:5n-6	0.07	0.04	0.02	0.04	0.38	0.11	0.11*	0.10	0.46	0.44	0.32	0.07	0.29	0.09	0.12*	0.07
30:5n-3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.09	0.12	0.03	0.01	0.01	0.00	0.00
30:6n-3	0.11	0.09	0.10	0.09	0.04	0.07	0.02	0.04	0.07	0.12	0.05	0.05	0.02	0.02	0.00	0.01
32:5n-6	0.25	0.26	0.17	0.19	0.06	0.03	0.01*	0.02	0.06	0.03	0.10	0.01	0.03	0.02	0.00*	0.01
SFA	46.38	0.68	45.92	1.41	35.89	4.24	37.55	1.93	28.31	3.45	14.81*	2.84	38.53	1.94	37.87	4.52
MUFA	11.74	1.02	12.79	1.83	14.25	2.44	17.64	3.63	10.61	1.48	10.14	2.17	14.21	0.97	13.26	1.56
n-6 LCFA	37.01	1.94	37.03	3.58	38.24	3.28	36.69	2.97	39.40	5.20	39.94	15.11	41.64	1.00	43.25	5.68
n-3 LCFA	0.77	0.10	0.71	0.18	1.55	0.27	1.57	0.34	1.90	0.65	6.06	2.92	0.87	0.14	1.53*	0.49
n-6 VLCFA	2.59	0.36	2.07	0.39	8.98	0.70	5.01*	1.66	13.18	3.56	15.08	9.03	4.33	0.65	3.54	1.01
n-3 VLCFA	0.31	0.41	0.27	0.26	0.23	0.10	0.47*	0.10	0.83	0.63	11.63	9.98	0.09	0.03	0.23	0.24
n-6/n-3 LCFA	49.15	8.73	54.75	16.72	25.07	3.43	23.88	3.41	22.73	8.72	6.90*	1.70	49.07	8.10	29.38*	5.81
n-6/n-3 VLCFA	12.32	12.17	94.09	152.49	45.88	22.99	11.77	7.04	23.45	14.87	10.73	16.55	51.55	21.01	25.74	14.71
n-6 Tetra VLCFA	1.14	0.09	1.31	0.14	2.69	0.35	2.48	0.49	4.09	1.35	5.45	2.32	1.77	0.24	2.15	0.41
n-6 Penta VLCFA	1.45	0.27	0.76*	0.34	6.29	0.43	2.53*	1.17	9.09	2.27	9.62	6.72	2.27	0.36	1.27*	0.60

n-3 Penta VLCFA	0.00	0.00	0.04*	0.05	0.00	0.00	0.00	0.00	0.50	0.27	0.70	0.60	0.03	0.03	0.02	0.02
n-3 Hexa VLCFA	0.31	0.41	0.23	0.22	0.23	0.10	0.47*	0.10	0.32	0.49	10.94	9.43	0.08	0.02	0.20	0.26

Values are Mean±SD. Significant differences within each lipid class were identified using T-test. Asterisk in a column represents significantly different values within the same lipid class ($P<0.05$). SFA, total saturated fatty acids; MUFA, total monounsaturated fatty acids; LCFA, total long chain fatty acids; VLCFA, total very long chain fatty acids; n-3, omega 3 fatty acids; n-6, omega 6 fatty acids.

n-6 Long chain fatty acids and testes development

Fatty acid profiles of normal and underdeveloped testes were compared in PL, TG, CE, and FFA. The PL fraction of the normal testes showed efficient conversion of LA to AA to DPA, however, in the underdeveloped testes there was an accumulation of LA ($P<0.0087$) as well as AA ($P<0.0029$), and subsequently a lowering of DPA ($P<0.0004$). This is also illustrated in Figure 3-2, showing a chromatogram of normal and underdeveloped testis. A similar trend, although not statistically significant, was observed in TG. The FFA profile also showed similar results with a specific lowering of DPA ($P<0.005$). These findings are consistent with a previous study conducted in our lab, where the underdeveloped testes showed a specific decrease in DPA in individual phospholipid classes, PC and PE (Suh et al., 2011). Other studies observed a similar decrease of DPA in testes; in cryptorchid rat testis (Furland et al., 2007a), and in adult rat testes with administration of antineoplastic drug doxorubicin (Zanetti et al., 2007). Thus, a decrease in DPA has been linked with testicular underdevelopment. The immediate precursor of DPA, 22:4n-6 was also higher in the underdeveloped testes compared to normal in PL, CE and FFA profiles. This indicates a lapse in conversion of 22:4n-6 to DPA, which may be a result of inhibited activity of Δ6 desaturase, the enzyme responsible for this conversion. Previous studies have indicated the importance of Δ6 desaturase in male reproduction, where Δ6 desaturase FADS2 null mice were unable to synthesize PUFAs and were sterile (Stoffel et al., 2008). However, while most studies have observed the loss of PUFA in membrane phospholipids, our data suggests that there may also be a loss of these major fatty acids from nonmembrane associated TG and FFA, and thus be a contributing factor in testes underdevelopment. Interestingly, this trend was not exhibited in CE, where no significant difference was found between normal and

underdeveloped testes fatty acid profile. But we also observed significant variations in CE fraction based on the higher standard deviations.

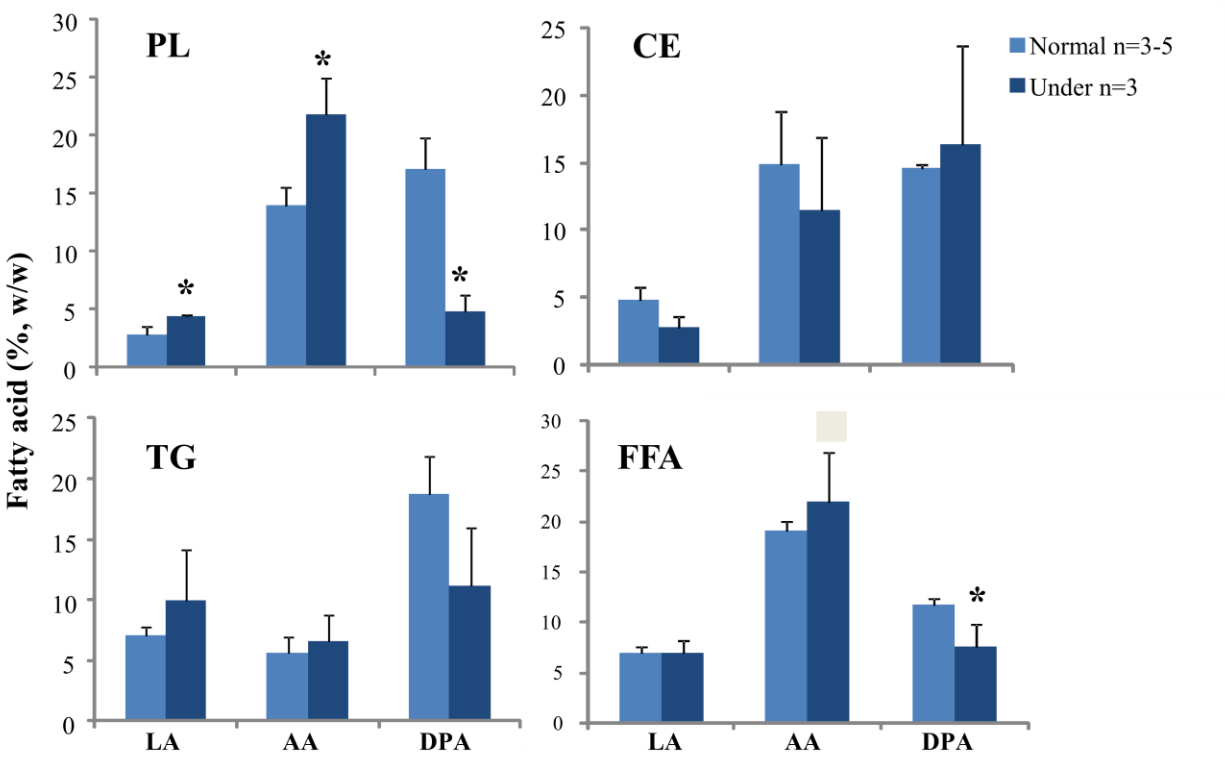

Figure 3-3: Comparison of n-6 LCFA in normal and underdeveloped testes in major testicular lipid classes
Bars are Mean (n=3-5) ± SD. Significant differences were identified using T- test. Columns with asterisk indicate significant differences in a fatty acid between normal and underdeveloped testes ($P<0.05$). PL, phospholipids; TG, triacylglycerides; CE, cholesterol esters; FFA, free fatty acids; LA, linoleic acid; AA, arachidonic acid; DPA, docosapentaenoic acid

Very long chain fatty acids and testes development

Along with LCPUFA, the profiles of VLCFA were also studied in all lipid classes. To fully understand metabolic differences in n-6 metabolism between normal and underdeveloped testes, conversion of n-6 tetraenoic to pentanoeic VLCFA was examined in all four lipid classes.The level of n-6 penta VLCFA is significantly lower in

underdeveloped testes PL, TG and FFA, despite the level of tetra VLCFA being similar in normal and underdeveloped testes ($P<0.0188$). Similar trend is seen in the TG and FFA, both showing a significantly lower level of n-6 pentaenoic VLCFA in underdeveloped testes compared to normal testes (Figure 3-4). The CE fraction however, did not follow this trend, and showed similar levels of tetranoic and pentanoic fatty acids in normal and underdeveloped testes. Thus, along with DPA, changes in n-6 VLCFA indicate that conversion of n-6 tetranoic to pentanoic VLCFA may be impaired in the underdeveloped testes in comparison to the normal testes. The n6 fatty acids specifically 22:5n-6 and 28:4n-6 are attached to germ cells in rat testes. Cryptorchid testes show a decrease in the levels of these fatty acids (Furland et al., 2007a). This study shows similar results in underdeveloped testes in obesity, further establishing a link between obesity and impaired lipid metabolism in the testes. Another aspect of n-6 metabolism that is highlighted in this study is the difference in fatty acid composition between CE and other lipid classes. While all other classes showed a loss of DPA as well as n-6 penta VLCFA, there was no significant difference in the levels of these fatty acids in the underdeveloped testes compared to the normal testes. A study looking at the loss of testicular lipids following X-ray exposure in rats found primary loss of DPA and VLCFA in sphingomyelins and ceramides, but with a simultaneous increase in DPA-rich CE in the seminiferous tubules (Oresti et al., 2010). This study indicates that perhaps a similar pattern of changes may be taking place in the underdeveloped testes. Impaired DPA and n-6 VLCFA metabolism points at inhibited activity of $\Delta 6$ desaturase enzyme in the underdeveloped testes. It remains to be understood whether this inhibited activity is the cause of depletion of n-6 fatty acids from major lipid classes, or whether there is transport of these n-6 fatty acids from other lipid classes to CE. Further research can be aimed at understanding fatty acid metabolism and distribution in the underdeveloped testes.

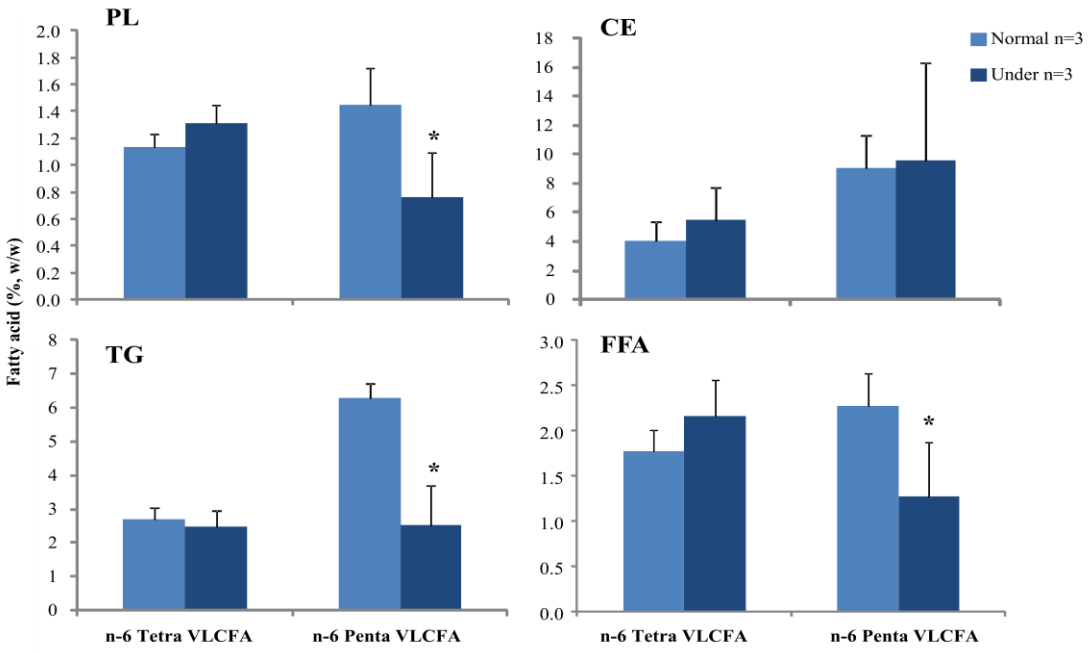

Figure 3-4: Comparison of n-6 VLCFA metabolism in normal and underdeveloped testes

Bars are Mean (n=3-5) ± SD. Significant differences were identified using T- test. Columns with an asterisk indicate significant differences in fatty acid type between normal and underdeveloped testes ($P<0.05$). PL, phospholipids; TG, triacylglycerides; CE, cholesterol esters; FFA, free fatty acids; LA, linoleic acid; AA, arachidonic acid; DPA, docosapentaenoic acid

Conclusion

The study results show that testes underdevelopment in an obese state is closely linked to lipid metabolism, more specifically altered LCPUFA and VLCFA metabolism. Looking at the fatty acid composition of all four lipid classes has enabled us to have a clearer and more complete picture of the n-6 lipid metabolism changes in an underdeveloped testes compared with normal testes. Underdeveloped testes seem to have a loss of n-6 fatty acids from membrane lipids, but not CE. To address the decline of DPA and n-6 pentaenoic fatty acids, the elongase and desaturase enzymes controlling these pathways need to be further studied. Specifically, activity of Δ6 desaturase enzyme may hold the key to improving lipid metabolism in an underdeveloped testes as shown in (Figure 3-6).

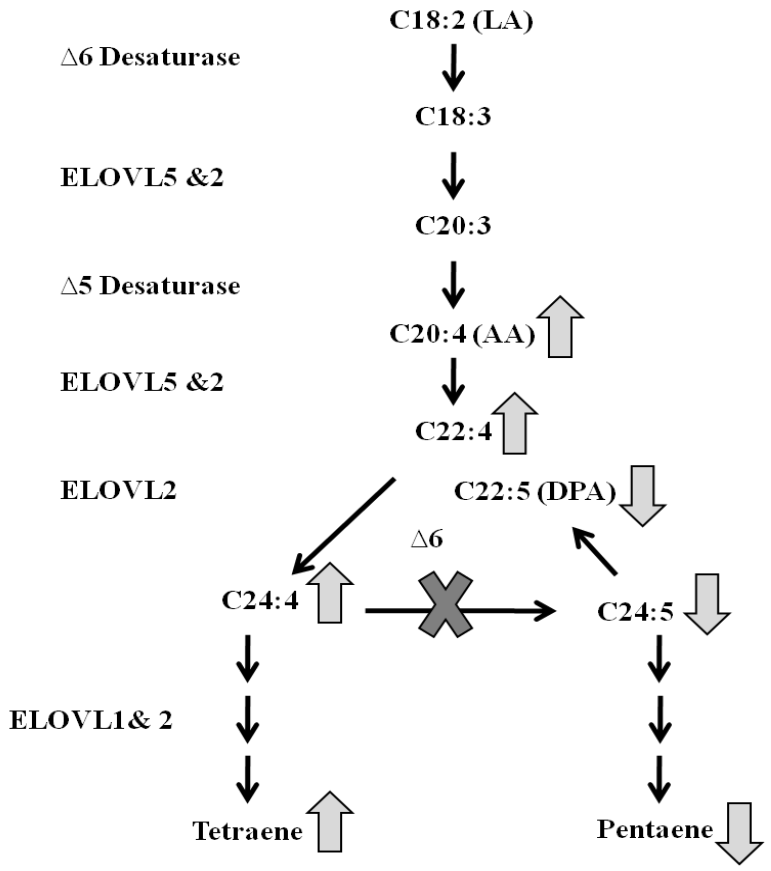

Figure 3-5: Potential defective n-6 essential fatty acid metabolism in underdeveloped testis

ELOVL- elongation of very long chain fatty acids; Tetraene- fatty acids containing four double bonds; Pentaene- fatty acids containing five double bonds. Tetraenoic long chain fatty acids and very long chain fatty acids are increased in underdeveloped testis, suggesting Δ6 desaturates may be defect in this underdeveloped testis.

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Taylor and Dr. Zahradka, Dr Kim was responsible for help in preparation of the manuscript. Dr. Suh was the principal investigator and responsible for design of the project, financial support, data analysis, and preparation of this paper.

CHAPTER IV: GLOBAL TRANSCRIPTOME PROFILE ANALYSIS IN NORMAL AND UNDERDEVELOPED TESTES OF OBESE (*fa/fa*) ZUCKER RATS

Authors: Jutika Datar, Alemu Regassa, Woo- Kyun Kim, Carla G. Taylor, Peter Zahradka, Miyoung Suh

Abstract

Zucker obese rats often have non-identical paired testes different in size, leaving one testis underdeveloped. Our earlier study found that the underdeveloped testis have a selective decrease in docosapentaenoic acid (DPA, C22:5n-6), a dominant fatty acid in the testes. This study was conducted to examine global testicular transcriptome profile in underdeveloped testis relative to developed ones. Testes were obtained from 14 week old, sexually mature male obese *fa/fa* Zucker rats. A testis was considered underdeveloped if the size difference between the paired testes was over 30%. Global transcriptome profile was studied using Affymetrix Rat Gene 2.0 ST Array (Affymetrix, Santa Clara, USA). Out of the transcripts differentially expressed, 1121 and 309 were overexpressed in the underdeveloped and normal testis, respectively (fold change cut off $\geq$1 and P-value $\leq$0.05). The Ingenuity Pathway Analysis (IPA) indicated that transcripts that were overexpressed in normal testes, relative to under developed testes, are involved in triacylglycerol biosynthesis, sphingomyelin metabolism and phosphatidylglycerol biosynthesis. Transcripts that were overexpressed in underdeveloped testes, relative to normal testes, are involved in the production of nitric oxide and reactive oxygen species, and nuclear factor (erythroid-derived 2)-like 2 (NRF2) mediated oxidative stress response. In conclusion, the study results show that lipid metabolism and oxidative stress play a crucial role in testicular development.

Keywords: Obesity, Testes, Infertility, Lipids, Microarray, Transcriptomics

Introduction

Male infertility is increasing over recent decades and contributes to about 30% of cases in infertile couples (Resolve, 2014). It is accepted that environmental toxins and lifestyle factors including obesity are associated with male infertility. Obesity in men is associated with poor semen quality, low semen volume and low sperm count (Jensen et al., 2004; Sallmen et al., 2006). According to Health Canada, the prevalence of obesity in adult Canadians was one in four in 2011-2012, while the proportion of obese Canadians increased by 17.5% in the last 8 years (Navaneelan & Janz, 2014). Despite this evidence, research on male reproductive function is still limited. The *fa/fa* Zucker rat is an obese animal model having a defect in the leptin receptor causing the rats to become hyperphagic, and subsequently obese. It is thus possible to have an idea of male reproductive system in an obese condition using this animal model. In comparison to their lean counterparts, these animals show germ cell loss (Vendramini et al., 2014), a shorter epididymis, and often have unpaired testes, with one of the testes smaller than the other (Suh et al., 2011). This can affect sperm concentration and maturation (Jones, 2006; Widmaier, 2006). Moreover, docosapentanoic acid (DPA, C22:5n-6), a major fatty acid in the testes is specifically lower in the underdeveloped testes phospholipids (Suh et al., 2011), despite of its precursor arachidonic acid (AA, C20:4n-6) showing no decrease. This indicates a direct involvement of lipid in testes development and a change in the enzyme function, which could change these pathways. Only a few studies have investigated genes controlling testes function and have identified certain genes related to lipid metabolism to be important in normal testes function. Mice with a knockout of fatty acid elongase, ELOVL-$2^{-/-}$ have impaired reproductive function (Zadravec et al., 2011), and the absence of long chain fatty acids in FADS-2 (-/-) mice showed hypogonadism and sterility. Gene *Wt1* proved to be crucial to normal reproductive function, as its

deletion resulted in disruption of levels of several hormones and impaired spermatogenesis in mice (Chen et al., 2014). Mice that were deficient in UDP-galactose *cgt* were unable to synthesize seminolipid, a lipid moiety unique to the testes, and showed anatomical defects in their reproductive organs, with impaired spermatogenesis (Fujimoto et al., 2000). These studies show that a number of genes are crucial to normal testes function, and some are also involved in synthesis of certain types of lipid classes. However, the expression of genes controlling these pathways in an obese condition is not known. Additionally, there may be other genes that might be differentially expressed in an obese state. By using obese Zucker rats, this study aims at obtaining the expression profiles of testes in an obese state as well as examining the changes in gene expression between normal and underdeveloped testes. This would ultimately help to understand some of the reproductive complications associated with obesity.

Materials and methods

Animals and diets

Five weeks old male *fa/fa* Zucker rats were obtained from Charles River Laboratories (St. Constant, PQ), acclimatized for 1 week, then raised on a semi synthetic diet with safflower oil (7.2% w/w), enriched in n-6 fatty acids as described in Chapter II. The animals were allowed to feed *ad libitum*. The same diet was fed to the lean Zucker rats. When animals reached maximum sexual maturity (Robb et al., 1978), at 14 weeks old, they were sacrificed by CO_2 asphyxiation followed by decapitation. Testes, were immediately collected and frozen in liquid nitrogen and stored at -80°C for further analysis. A testes was considered underdeveloped if the difference in size was over 30% between the paired testes as shown previously (Suh et al., 2011). The testes weight of lean (n=2) and obese (n=4) animals were 1.8±0 g and 1.7±0.8 g respectively. The testes

weight of normal (n=4) and underdeveloped (n=4) testes was 1.7±0.44 g and 0.9±0.1 g respectively.

RNA isolation and microarray processing

Frozen testes were decapsulated and homogenized using a PowerGen Model 125 Homogenizer. Total RNA was extracted using TRIzol (Invitrogen, Canada), as per the manufacturer's protocol. For 100 mg tissue, 1 ml TRIzol was added to precipitate the RNA. The extracted RNA was resuspended in 20 µl RNAase free water and incubated for 10 min at 60 °C. RNA yield and integrity in each sample was assessed using a NanoDrop (Thermo Scientific, Canada) and an Agilent 2100 bioanalyzer (Agilent Technologies, USA), respectively. Targets were prepared using WT PLUS Reagent Kit (Affymetrix, USA). All sample RNA concentrations were adjusted to 250 ng in order to synthesize first strand and second strand cDNA. The second strand cDNA was in vitro transcribed to produce cRNA. Then, 15 µg of cRNA was used to synthesize 2nd cycle single strand cDNA and for preparation of biotinylated targets. The biotinylated targets were hybridized on GeneChip® Rat Gene 2.0 ST Array (Affymetrix, USA) using a hybridization oven (Affymetrix, USA) at 45ºC for 16 hours. Following hybridization, staining and washing of chips was done as per the manufacturer's protocol on the Fluidics Station 450 instrument (Affymetrix, USA). Stained arrays were then scanned using a GeneChip™ 3000 laser confocal slide scanner (Affymetrix, USA). Raw images were quantified with Affymetrix GeneChip Command Console Software (Affymetrix, USA). Probe level data were background corrected, normalized and summarized using GC RMA algorithm under FlexArray software. Summarized data were annotated using the rat annotation file (RaGene-2_0-st-v1.na33.2.rn4.probeset.csv.zip) and differentially expressed genes were identified using t-test with fold change cut off ≥1 and P value ≤0.05. The list of genes overexpressed in each group were uploaded into Ingenuity

Pathway Analysis (IPA). Uploaded datasets were compared based on 1) obesity: testes of lean animal vs testes of obese animal and 2) testes development: normal testes vs underdeveloped testes, using comparison analysis in IPA. From IPA, pathways related to lipid metabolism were identified, and gene networks related to lipid metabolism and reproductive function were studied.

Validation of microarray data using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

A select set of genes were chosen from the microarray dataset for array validation. The same RNA samples that were used for array hybridization were used for cDNA synthesis. First strand cDNA was synthesized using a high capacity reverse transcription kit following the supplier's protocol (Applied Biosystems, Canada). Pairs of primers for each gene were designed from the mRNA sequence of the target gene using the National Centre for Biotechnology Information (NCBI). Quantitative real-time RT-PCR was performed in duplicate reactions including nuclease free water, the forward and reverse primers of each gene, template cDNA and SYBR Green as a detector using a CFX Connect TM Real-Time PCR Detection System (Life Science Research, Bio-Rad, Canada). Data were generated using the ΔΔCt method by normalizing the expression of the target gene to a housekeeping gene (RPL13A), and the values were reported as fold changes of the expression of the target genes in normal testes compared to underdeveloped testes. Primer pairs used for qRT-PCR assay and their sequences are presented in Table 5-1.

Table 4-1: List of primers used for qRT-PCR validation

Gene Name	Forward Primer	Reverse Primer	Product length (base pair)	Annealing temperature (°C)
SAT1	ATGTCTCTTAGTCCGGGGAGG	GGAGGTGGTTCCTCATTCGT	181	59
PLA2G2D	TGTTGCTATGCCCACCTGAA	GTAGTACCGCAGTCGCTTGT	195	59
APP	TCCAACCGTGGCATCCTTTT	AGTGGTCAGTCCTCGGTCA	104	58
PPT1	CCGCCTGGGGCTAAAGAAAA	ACGAGAGAAGGACTGGTTGC	198	58
CD36	TGTACTCTCTCCTCGGATGGC	ATGCTTTCTATGTGGCCTGGT	160	59
MAP2K4	TGGACAGCTCGTGGACTCTA	AACTCCAGACATCAGAGCGG	125	58
MYO7A	AAGACTCAAGCTGGGGCAAA	ATCCACACGTAGTCCCCCTT	199	59
SMPD4	CCTACAGGGACGTGTGAACC	AGAGGACTGTCAGGGAGGAC	186	59
LPIN1	CAGACAGAGAGTGGTCCCCC	GAAGCATGGAAGTGGCAAGAC	121	59
AGPAT2	CTGCTGCTGTTGCTGCTTG	GCGAAGGCCATACACGTACT	198	59
AGPAT1	ATCCCCAAATCCTGTCGCC	GCCACAGCTCCATTCTGGTC	145	59
LIPE	GATTAGAGCCCAAAGGGAGGT	TGGGTAGTCTGTGAGGGTCT	193	58
PLA2G6	TGCGGAAGTTAGGAGTGCTG	AACGAGGCGTCCAAAGAACT	106	59
TXNDC2	TGTGGCCCTTGCAGAAAGAT	GGAAAGTCGGGAGGTGGAAG	139	58

Results

Changes in gene expression between lean and obese testes

Global expression analysis between lean and obese animal testes showed that out of 60 genes differentially expressed, only 6 genes were differentially expressed between the groups. In the lean animal testes, olfactory receptor 1691 (Olr1691), olfactory receptor 1701 (Olr1701), solute carrier family 25, member 52 (Slc25a52, also known as Mcart2) and solute carrier family 25, member 51 (SLC25A51) were overexpressed. On the other hand, in the obese group Kremen1 and Slco1a2 genes were found to be overexpressed (Fold change ≥ 1 and P value ≤ 0.05).

Differential expression of genes between normal and underdeveloped testes

Canonical pathways analysis

Out of the differentially expressed transcripts, 1121 were overexpressed in underdeveloped testes, and 309 were overexpressed in the normal testes. Genes overexpressed in the normal testes compared to underdeveloped testes are those involved in triacylglycerol biosynthesis such as 1-acylglycerol-3-phosphate O-acyltransferase 1 (AGPAT1), 1-acylglycerol-3-phosphate O-acyltransferase 2 (AGPAT2), and lysophosphatidylcholine acyltransferase 4 (LPCAT4). Expression of genes regulating sphingomyelin metabolism such as sphingomyelin synthase 1 (SGMS1), sphingomyelin phosphodiesterase 2, and neutral membrane (SMPD2) were also significantly upregulated in normal testes compared to underdeveloped testes (P-value ≤ 0.05). CDP diacylglycerol biosynthesis was also a significant pathway in the normal testes where genes such as 1-acylglycerol-3-phosphate O-acyltransferase 3 (AGPAT3) were found to be overexpressed (P-value ≤ 0.05). Phosphatidylglycerol biosynthesis II (Non-plastidic) is another

significantly changed pathway and genes involved in the pathway such as AGPAT1, AGPAT2, AGPAT3 and LCAT4 were upregulated in the normal testes (P-value ≤0.05). On the other hand, genes overexpressed in the underdeveloped testes were related with acute phase response signaling, such as alpha-2-macroglobulin (A2M), interleukin-33 (IL33), and oncostatin M receptor (OSMR) (P-value ≤0.05). Another significantly changed pathway was the complement system such as complement component 7 (C7), and complement component 1, s subcomponent (C1S) (P-value ≤0.05). Other significant pathways affected in underdeveloped testes were nitric oxide and reactive oxygen species production in macrophages controlled by genes such as v-akt murine thymoma viral oncogene homolog 1 (AKT1), apolipoprotein E (APOE) and germ cell-sertoli cell junction signaling, regulated by cadherin 2, type 1, N-cadherin (neuronal) (CDH2), gelsolin (GSN), and tumor necrosis factor receptor superfamily, member 1A (TNFRSF1A) genes (P-value ≤0.05). Significance of these pathways was calculated in IPA using Fisher's exact test right-tailed.

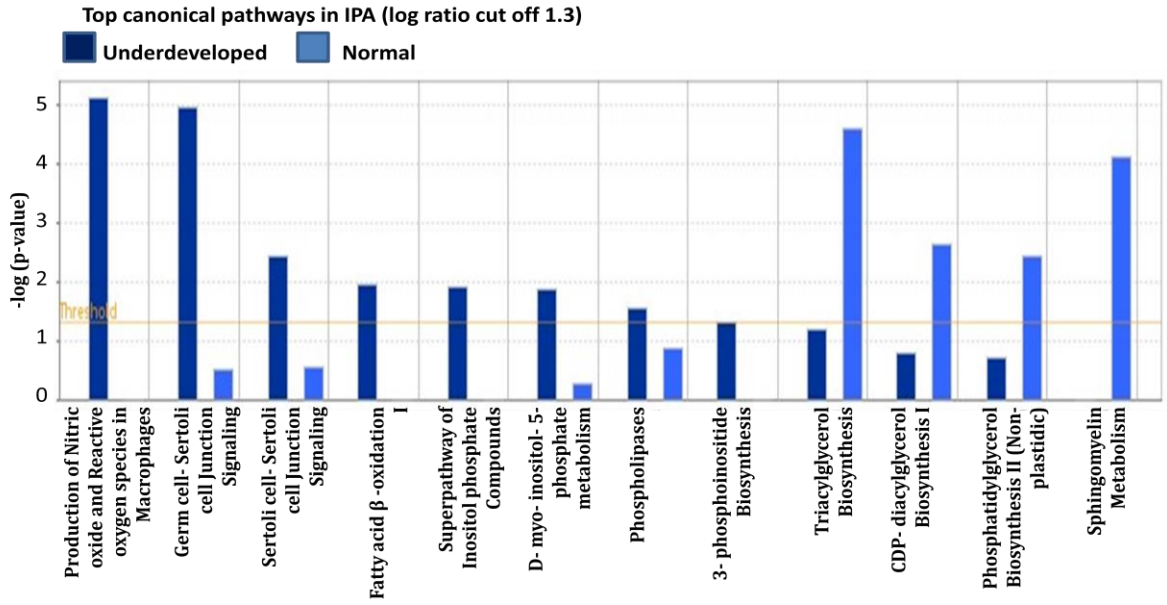

Figure 4-1: Top canonical pathways in Ingenuity Pathway Analysis
Segregation of genes in canonical pathways and comparison of genes overexpressed in underdeveloped and normal testes, P value ≤ 0.05, represented by the yellow line.

Upstream regulators

A look at the upstream regulators showed the predicted activity of these regulators based on the observed gene expression of their targets and the current literature available on these genes. A z score algorithm was used by IPA for these predictions and to remove errors by random data. Fisher's exact test is used for calculation of p value. In the normal testes, upstream regulators having predicted inhibition were retinoblastoma-like 2 (RBL2), mRNA let-7, mature mRNA let-7a-5p (and other miRNAs w/seed GAGGUAG) (Mammalian) (P-value ≤0.05). The genes T-box 2 (TBX2) and TAF7-like RNA polymerase II (TAF7L), on the other hand were predicted to have been activated based on the expression of the genes present in its network (P-value ≤0.05). In the underdeveloped testes, there were a number of upstream regulators such as the tumor

necrosis factor (TNF), beta estradiol and interleukin 6, while APOE was predicted to have been inhibited (P-value ≤0.05).

Table 4-2: Upstream regulators in normal and underdeveloped testes

	Upstream Regulator	Predicted Activation State	Activation z-score	p-value of overlap
Normal	Uric acid	Activated	2.000	0.0137
	PTEN	Activated	2.333	100.0
	Let-7	Activated	3.121	0.0130
	Let-7a-5p (and other miRNAs w/seed GAGGUAG)	Activated	3.081	0.0204
	ADCYAP1	Inhibited	-2.442	100.0
Underdeveloped	Lipopolysaccharide	Activated	8.417	$1.83x10^{-44}$
	TNF	Activated	7.884	$4.21x10^{-43}$
	Beta-estradiol	Activated	3.203	$1.11x10^{-29}$
	APOE	Inhibited	-3.629	$3.94x10^{-16}$
	IL6	Activated	7.103	$1.81x10^{-15}$
	progesterone	Activated	2.863	$6.60x10^{-14}$

PTEN, Phosphatase and tensin homolog; ADCYAP1, adenylate cyclase activating polypeptide 1; TNF, Tumor necrosis factor; APOE, Apolipoprotein E; IL6, Interleukin 6. Predicted activity is based on observed gene expression of targets and the current literature available. A z score algorithm is used for predictions and to remove errors by random data. Fisher's exact test is used for calculation of p value.

Downstream analysis

Downstream analysis showed the genes in each dataset and their association with disease conditions. Genes associated with cancer, metabolic and inflammatory diseases, reproductive system disorders and endocrine system disorders were overexpressed in the

underdeveloped testes relative to the normal testes. These associations are made using a z score algorithm.

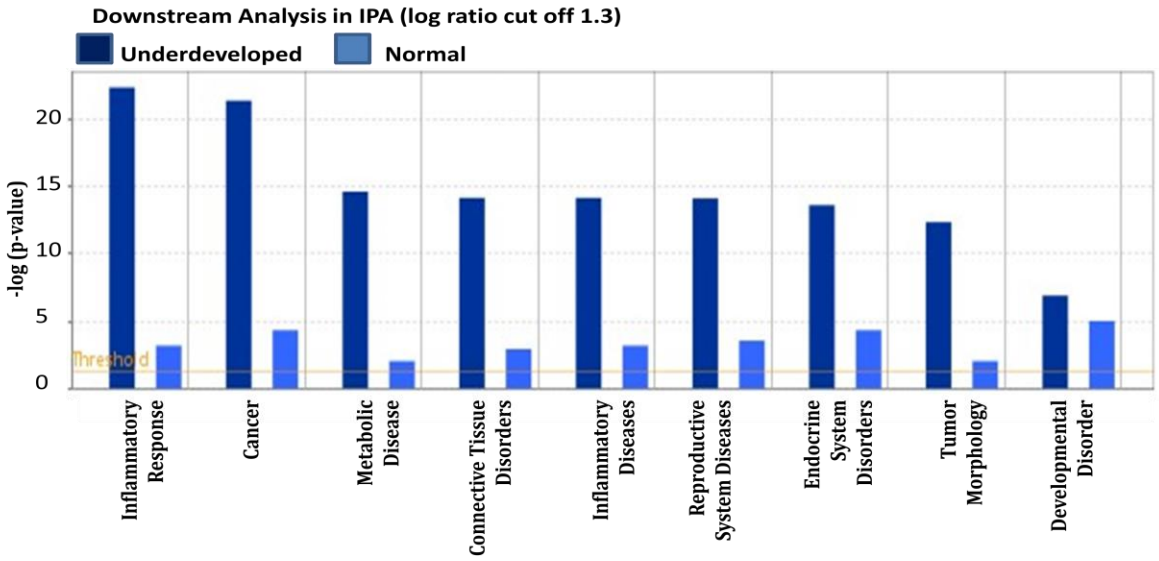

Figure 4-2: Downstream analysis in Ingenuity Pathway Analysis
Significant disease functions involved with overexpression of genes in underdeveloped testes, P value ≤ 0.05, represented by the yellow line.

Gene networks

Genes were grouped based on their functions in metabolic pathways, and networks were generated. Several networks were affected in the normal and underdeveloped groups. Pathways related to lipid metabolism and reproductive functions were overlaid on these networks. Interestingly in the normal testes, certain genes were upregulated in the triacylglycerol and sphingomyelin pathways, DHA signaling, sertoli cell, sertoli cell junction signaling and sperm motility. On the other hand, a lipid metabolism related gene network associated with the underdeveloped testes showed an upregulation of genes associated with prostate cancer signaling, leptin signaling in obesity, and molecular

mechanisms of cancer. Figures 5-3 and 5-4 show examples of gene networks that are associated with normal and underdeveloped testes respectively.

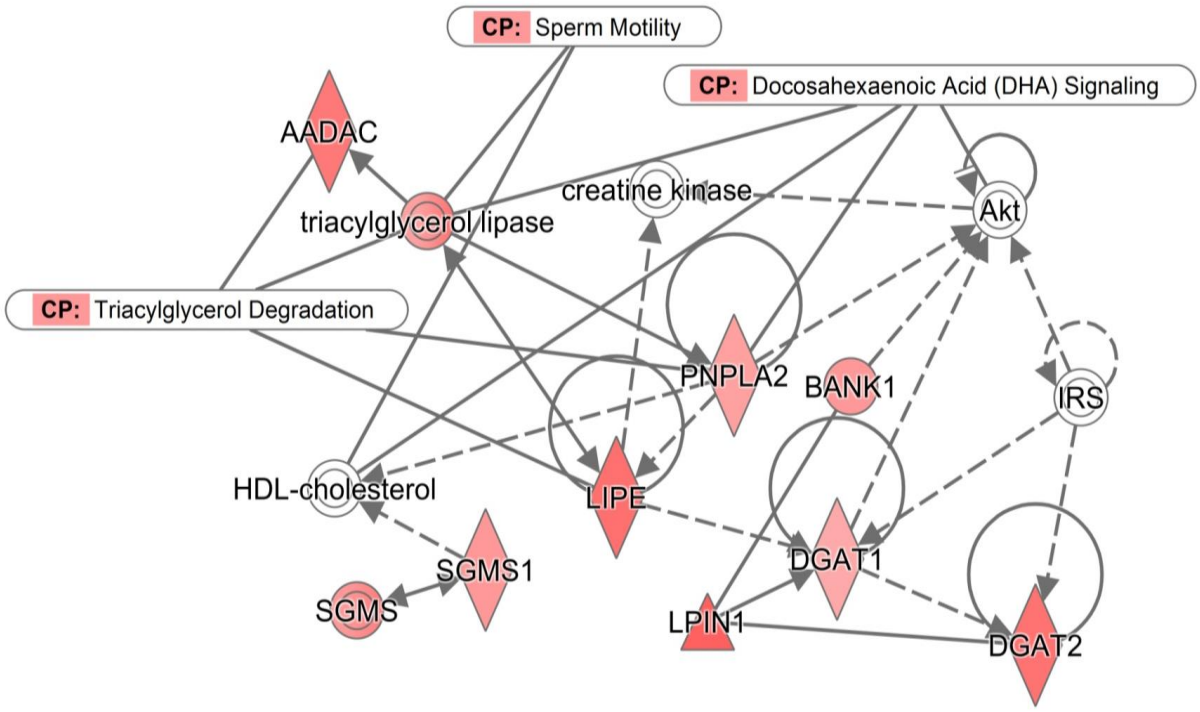

Figure 4-3: Example of gene network in normal testes
Network showing relations between genes upregulated in the normal testes. Direct relationships between molecules are shown by a solid line, while indirect relationships are represented by a dotted line. Shapes are designated according to the nature of the molecule- rhombus, triangular-phosphatases, rhombus- family of enzymes, rectangular-growth factor, trapezium- transporter, oval- transmembrane receptors, circular- other families. Pathways overlaid indicate which genes are associated with them. Color intensity indicates degree of upregulation.

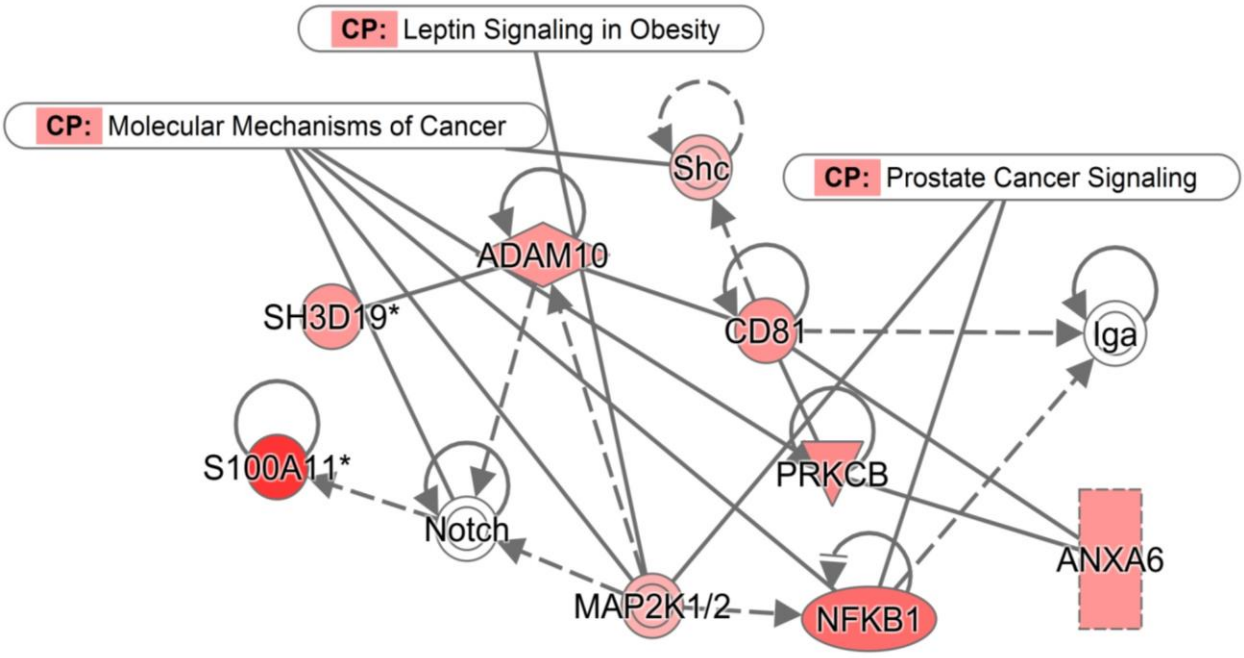

Figure 4-4: Example of gene network in underdeveloped testes
Network showing relations between genes upregulated in the underdeveloped testes. Direct relationships between molecules are shown by a solid line, while indirect relationships are represented by a dotted line. Shapes are designated according to the nature of the molecule- rhombus, triangular-phosphatases, rhombus- family of enzymes, rectangular- growth factor, trapezium- transporter, oval- transmembrane receptors, circular- other families. Pathways overlaid indicate which genes are associated with them. Color intensity indicates degree of upregulation.

Validation of microarray data using qRT-PCR

A group of genes with different fold changes were selected from the microarray data and quantified using qRT-PCR and the results were consistent with that of the microarray data (Figure 5-5) as the fold change expression between the normal and underdeveloped testes were significantly different ($P < 0.05$).

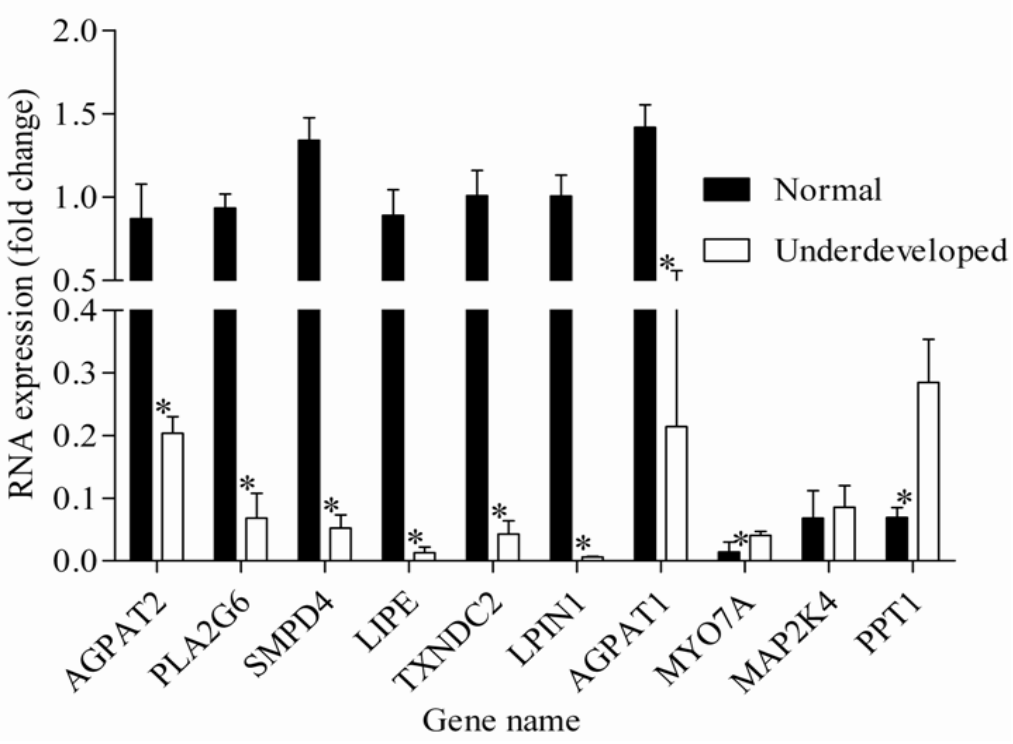

Figure 4-5: qRT-PCR validation of selected genes
RNA expression levels of selected genes that were differentially expressed between normal and underdeveloped testes. AGPAT2, 1-acylglycerol-3-phosphate O-acyltransferase 2; PLA2G6, phospholipase A2 group VI; SMPD4, sphingomyelin phosphodiesterase 4; LIPE, lipase hormone sensitive; TXNDC2, thioredoxin domain containing 2; LPIN 1, lipin 1; AGPAT1, 1-acylglycerol-3-phosphate O-acyltransferase 1; MYO7A, myosin VIIA; MAP2K4, mitogen-activated protein kinase kinase 4; PPT1, palmitoyl-protein thioesterase 1

Discussion

The role of microarray to decipher transcriptome profile in testes

Although the technique of transcriptome analysis is relatively new, some studies have analyzed transcriptome profiles in testes. Lervik et al (2015) looked at the developmental changes in boar testes from 12 to 27 weeks, and found that transcripts regulating steroidogenesis act at specific developmental stages (Lervik et al., 2015). Genes with crucial roles in sperm formation, maturation and fertility were identified, and with the

help of network interaction it was possible to surmise that these genes have similar regulation across species (Petit et al., 2015). Another study on boar testes measured the gene expression of boars fed methylated micronutrients and their effect on testes and sperm cells (Bruggmann et al., 2013). Thus microarray is a useful tool to identify gene clusters that are important in basic testes function.

Transcriptome profile as affected by testicular health

The microarray data indicated a large number of transcripts that were associated with testicular health and anomaly. However, the majority of these transcripts are of unknown functions and hence only those with known functions are discussed here. Transcripts involved in triacylglycerol biosynthesis, sphingomyelin metabolism and CDP diacylglycerol biosynthesis were among those overexpressed in normal testes. The upregulation of molecules involved in triacylglycerol biosynthesis, sphingomyelin metabolism and CDP diacylglycerol biosynthesis in the normal testes emphasizes the role of these metabolic pathways in normal testicular function. Studies have linked these metabolic pathways to testes development. Upregulation of fatty acid binding proteins (FABP) was seen in different stages of testicular development, with increased expression of fatty acid binding protein 9 (FABP9), diacylglycerol acyltransferase (DGAT2), and lipid droplet protein perilipin (PLIN2) (Oresti et al., 2013). These metabolic pathways and the downregulation of the associated genes in the underdeveloped testes emphasize the importance of lipid metabolism in testes growth. The underdeveloped testes on the other hand showed signs of an inflammatory immune response, with genes such as A2M, IL33, and C7 being upregulated, indicating a dysfunctional metabolism. Oxidative stress was another condition indicated by the underdeveloped dataset, which has been associated with obesity (Dandona et al., 2004). It is also known that polyunsaturated fatty acids (PUFAs) are prone to lipid peroxidation (Agarwal et al., 2008), indicating the

underdeveloped testes may have an impaired PUFA metabolism compared to normal testes. It is also interesting that one of the most significant pathways affected in the underdeveloped testes is the germ cell-sertoli cell junction signaling pathway. Genes such as CDH2 linked to this pathway show different expression between normal and cryptorchid testes (Rode et al., 2015). GSN is another gene upregulated in this pathway, which has previously been studied a part of the gelsolin phosphoinositide pathway (Guttman et al., 2002). These genes need to be further studied to understand their roles in testicular pathology. This differential gene regulation between normal and underdeveloped testes had the most impact on disease conditions. This was reflected by downstream analysis where genes over expressed in underdeveloped testes were associated with endocrine system disorders, reproductive system disorders and cancer. Networks generated by IPA have highlighted genes present in lipid metabolic pathways that are upregulated in both groups. An upregulation of triacylglycerol lipase and its relationship to sperm motility further emphasizes the importance of triglyceride metabolism in normal testes function (Figure 5-3). It was also interesting to see the presence of the DHA signaling pathway and its relationship to triacylglycerol lipase and patatin-like phospholipase domain containing 2 (PLPLA2) in the normal testes. This indicates that pathways related to n3 and fatty acid metabolism are significantly affected in the normal testes. Networks in the underdeveloped testes on the other hand were connected with cancer, specifically prostate cancer signaling, and leptin signaling in obesity. Polymorphisms in nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NFKB1) have been associated with development of prostate cancer (Han et al., 2015; Yang et al., 2014).

Differential gene expression with diet and obesity

Dietary supplementation with fatty acids, especially n6 fatty acids has an effect on gene expression. Supplementation with PUFA upregulates the expression of hormone receptors in roosters, thereby increasing reproductive function (Feng et al., 2015). However, Bruggmann et al. (2013) did not find any significant difference in the expression levels of RNA molecules longer than 100nt, indicating that these RNA molecules may be less susceptible to nutritional changes (Bruggmann et al., 2013). Hence, further studies will have to be conducted using a greater number of tissues to determine dietary influences on mRNA expression. When comparing lean and obese animal testes, we found that Olr1691, Olr1701, Mcart2 and SLC25A51 genes were overexpressed in the lean animal testes. The expression of olfactory receptors in non olfactory tissues has been studied. Immunohistochemistry of mouse tissues showed the presence of olfactory marker protein (OMP) in the Leydig cells of mouse testes (Kang et al., 2015). Previously, olfactory receptor hOR17-4 was found to be crucial in sperm chemotaxis and the fertilization process in human sperm (Spehr et al., 2003). Fukuda et al. (2006) also found expression of multiple transcripts from olfactory receptors in different stages of spermatocyte development (Fukuda & Touhara, 2006). Upregulation of these genes in the lean testes compared to obese testes could thus indicate a more efficient spermatogenesis and sperm development in the lean animal compared to the obese animal.

Conclusion:

The objective of this study was to observe the gene expression of testes changes in testes in an obese state. Previous studies conducted in our lab showed obese animals had phenotypic differences in testes and accessory organs compared to the lean animal.

Additionally, some obese animals had unpaired testes, leaving one of the testes underdeveloped. Expression profiles of these testes indicate that obesity has a definite impact on gene regulation. In addition to this, the underdeveloped testes have a changed gene expression profile, with an increased propensity to develop cancer, endocrine system disorders, and other reproductive complications. The microarray data show that lipid metabolism could be hampered in underdeveloped testes, which further emphasizes the role of lipids in normal testes development. Certain genes involved in lipid metabolic pathways have also been identified. Further studies focusing on lipid metabolic pathways and the genes regulating them would improve our overall understanding of the male reproductive system and the etiology of male infertility.

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CHAPTER V: DISCUSSION AND CONCLUSION

Summary

This study has provided an overview of the impact of obesity on testes development and pathology, and the response of testicular lipid environment to dietary lipids. To achieve this, the present study used the *fa/fa* Zucker rat, which provided an ideal animal model to mimic the obese condition seen in humans. The diets were designed to provide a means of comparison between n-3 and n-6 diets, and within the n-3 diets, a comparison between ALA, EPA and DHA. Genotypic and lipid composition differences were observed when lipid profiles of the testes were compared. Overall the obese animals had a different PUFA metabolism compared to the lean animal, and n-3 fatty acid diet supplementation seemed to decrease n-6:n-3 PUFA ratio in obese rats. Testes imbalance was further studied and a comparison of lipid profile of normal and underdeveloped testes revealed that the PUFA metabolism, specifically n-6 metabolism was impaired in the underdeveloped testes compared to the normal testes. To understand changes in lipid metabolism and to get an overall understanding of testes function, a microarray was performed on testes and a full transcriptome profile was obtained. Comparison of transcriptome profiles revealed that there were genes differentially expressed on the basis of lean and obese condition, as well as testes development. Data from gene expression revealed genes involved in lipid metabolism were differentially regulated in normal and underdeveloped testes.

Strengths and limitations

This study permitted a multifaceted approach in studying testes function. The Zucker rat provided a lean and obese model to be able to detect genotypic differences in testes function. Providing n-6 and n-3 supplemented diets, which changed the PUFA content

and kept the SFA and MUFA content constant, offered us with an ideal platform to study PUFA metabolic changes in the testes. Specifically, a comparison of the dietary administration of ALA, EPA and DHA made it possible to understand the stepwise metabolism related changes in obese animal testes. The condition of testicular imbalance could be further studied due to the characteristic underdeveloped testes of the obese Zucker rat. Previous studies have looked at specific genes and their expression in testes. Microarray analysis provided a whole transcriptome profile which has identified new areas for further study.

The limitations of this study are perhaps the ability to extrapolate these results to human studies. Although the Zucker rat has been used as a model for obesity, it has a deficient leptin receptor, a condition not necessarily present in obese humans. With regards to diet, although the n-6 supplemented diet was compared between lean and obese group, there was no complete comparison between lean and obese groups with n-3 dietary supplementation. Gene expression profiles of diet groups were not compared. Numbers of testes used for analysis of gene expression were low, indicating perhaps a larger number of tissues can be used to confirm the findings.

Recommendations for future research

Future research can be aimed at a closer look at the enzymes and genes present on the PUFA metabolic pathway. Lipid analysis has indicated a specific decrease in DPA, a predominant fatty acid found in the testes. Further research can also include dietary supplementation in different growth stages, and whether early supplementation alters lipid metabolism compared to supplementation in the adult stage. Although significant differences in sperm morphology with respect to diet supplementation were not observed, further investigation into the impact on sperm morphology by varying DHA and EPA

supplementation can be carried out. Transcriptome analyses can also include the gene expression in semen samples, coupled with long and short term n-3 fatty acid supplementation, thus helping in bridging the knowledge gap between animal and human studies.

Conclusion

This research has cast light upon the less understood and not as extensively studied subject of male infertility. A strong link has been indicated in testicular development and lipid metabolism. Looking at the fatty acid profiles of four major lipid classes has permitted us to have a more precise and complete picture of the lipid metabolism in the testes and the fluctuations in lipid homeostasis observed with genotypic and diet influences. Animals studies indicate that nutrition, especially dietary fats may be the key to improving not just male fertility, but general male reproductive health. Microarray analysis has provided a lot of new genes which could work synergistically in spermatogenesis and steroidogenesis. Further exploration of these genes, as well as gene diet interactions will help in a better understanding of male fertility.

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