THE EFFECT OF DIETARY DOCOSAHEXAENOIC ACID ON TESTIS FUNCTION IN RELATION TO LIPID COMPOSITION FROM FETUS TO ADULT RATS EXPOSED TO PRENATAL ETHANOL

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By

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Abstract

Testis unique lipid environment is vital for fertility potential. Prenatal ethanol (EtOH) exposure impairs, but dietary docosahexaenoic acid (DHA) supports testis functions. However, these impacts on the developmental profile are less investigated. This study examined the effect of dietary DHA on testis function in relation to testis lipid composition in rats exposed to prenatal EtOH. Female Sprague-Dawley rats were exposed to EtOH (3g/kg twice a day) or dextrose throughout the gestational period, while dams and their pups were fed with a semi-purified diet supplemented with and without DHA (1.4%, w/w fatty acids) throughout the study. Samples were collected at gestational day (GD) 20, postnatal day (PD) 4, PD21, PD49, and PD90 for further analysis. As the testis developed, n-6 polyunsaturated fatty acids (PUFA), mainly docosapentaenoic acid (DPA) and n-6 very long chain fatty acids (VLCFA) increased markedly replacing n-3 PUFA, predominately DHA and MUFA. The triacylglycerides (TAG) fraction contained the highest proportion of both n-6 DPA and total n-6 VLCFA, increasing from PD21 to PD90. Dietary DHA increased the levels of fetal serum testosterone and sperm with normal morphology in adulthood, regardless of EtOH exposure. The testis DHA levels increased in DHA-treated groups, regardless of EtOH exposure and testis maturation, replacing n-6 DPA and total n-6 VLCFA only in immature testis up to PD21. Overall, the early provision of DHA in the diet can be a positive factor for male fertility by affecting sperm quality in adulthood, likely by influencing testis DHA content and testosterone levels in the fetal period. Marked increases in testicular n-6 DPA and n-6 VLCFA accumulation as the testis matures, mostly in TAG, indicates their potential role in testis development. The minimal impact of moderate levels of prenatal EtOH exposure on testis function might be related to an energy-dense diet used in this study that warrants further attention in future.

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Dedications

I dedicate this work to my family

To my best friend and husband **Amir Hossein**

To my mother **Banoo** and my father **Ali Reza**

To my sisters **Rozita** and **Sara**

To my brother **Mohammad Reza**

To my brothers-in-law **Morteza, Ehsan, Iman, Sina**

To my nephew **Sina**

To my mother-in-law **Leyla** and my late father-in-law **Abdolkarim.**

Without your encouragement and continuous support, my success would not have been possible.

List of abbreviations

ALA	α-linolenic acid (C18:3n-3)
ANOVA	Analysis of variance
AA	Arachidonic acid (C20:4n-6)
BF_3	Boron trifluoride in methanol
CE	Cholesterol ester
CCHS	Canadian Community Health Survey
CCAC	Canadian Council on Animal Care
DHA	Docosahexaenoic acid (C22:6n-3)
DHT	Dihydrotestosterone
DPA	Docosapentaenoic acid (C22:5n-6)
EFA	Essential fatty acid(s)
ELOVL2	Elongation of very long chain fatty acids2
EPA	Eicosapentaenoic acid (C20:5n-3)
EtOH	Ethanol
FASD	Fetal alcohol spectrum disorder
FFA	Free fatty acid(s)
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
GD	Gestational day
H&E	Hematoxylin and Eosin
HDL	High-density lipoprotein
KOH	Potassium hydroxide
LA	Linoleic acid (C18:2n-6)
LDL	Low-density lipoprotein

LH	Luteinizing hormone
MUFA	Monounsaturated fatty acid(s)
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PL	Phospholipid(s)
PS	Phosphatidylserine
PI	Phosphatidylinositol
PUFA	Polyunsaturated fatty acid(s)
SD	Standard deviation
SEM	Standard error of mean
SFA	Saturated fatty acid(s)
SHBG	Sex hormone binding globulin
SGA	Small for gestational age
StAR	Steroidogenic acute regulatory
FADS	Fatty acid desaturase
FABP	Fatty acid binding protein
TAG	Triacylglyceride(s)
Tbp	TATA box binding protein
WHO	World Health Organization

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CHAPTER 1. LITERATURE REVIEW

1.1. Introduction

Infertility is defined by the World Health Organization (WHO, 2009) as being unsuccessful in pregnancy to conceive a child after 1 year or longer of unprotected sexual intercourse on a regular basis. Infertility rate concerns around 15% of couples in the world, with men contributing approximately 20% of infertility solely and 30% of cases (Smith, 2013). In Canada, the prevalence of the current infertility is 11.5-15.7% among couples according to the 2009-2010 Canadian Community Health Survey (CCHS) (Bushnik et al., 2012; Health Canada, 2009) which increased from 1984, with 5% of couples being infertile (Bushnik et al., 2012). Up to 16% of Canadian couples with women at the age of 18-44 years turned to assisted reproductive technology (Bushnik et al., 2012), with 3 and 2 out of 10 fertility problems being related to the male and both partners, respectively (Health Canada, 2012). In parallel with the increased rate of infertility, there was an alarming report of around 50% reduction in the sperm count during the past decades (Merzenich et al., 2010). Despite the significance, research on male reproductive function is still limited.

Normal testis functions are central to male fertility. The primary testicular functions are spermatogenesis and steroidogenesis. Semen analysis is a direct way to evaluate normal male reproductive functions. Sperm is developed in 6 stages over 65 days in humans (Widmaier et al., 2013), and any disturbance during this period could lead to reduced sperm count, disrupted motility and abnormal morphology of sperm resulting in male infertility (Sharpe, 2010). Although the exact causes of testis dysfunction are not known, commonly accepted elements are genetic, chemical and physical environmental toxins and lifestyle (Dondorp et al., 2010; Hammarberg et al., 2013). Among these potential risk factors, the quality of food we consume is becoming recognized as a major contributing part. The behavior-dependent lifestyle factors, such as smoking, alcohol, diet and sedentary life, have adverse effects on reproductive outcomes

(Vriese and Christophe, 2003). Alcohol consumption, as a lifestyle, has been associated with impaired male reproductive functions in both daily drinking males (Condorelli et al., 2014) and males exposed to alcohol prenatally (Ramlau-Hansen et al., 2010). The latter indicates that male fertility might be connected to fetal life, which is of interest. Therefore, changes in lifestyle, as modifying compartment, may positively affect male fertility, even from the pregnancy, but information is limited in the literature.

Testis and sperm are enriched in polyunsaturated fatty acids (PUFA) of C22 carbon chain in length. Docosapentaenoic acid (DPA, C22:5n-6) is the dominant fatty acid in rat testis and sperm (Castellano et al., 2011; Tam et al., 2008), which is normally a very minor component in other organs and tissues, whereas docosahexaenoic acid (DHA, C22:6n-3) is abundant in human (Poulos et al., 1973; Zalata et al., 1998) and monkey testis (Lin et al., 1993). The huge increase in the level of these PUFA in testis occurs during development in a close relationship with germ cells maturation (Davis et al., 1966), indicating the necessity of PUFA for sperm production. Decreased DHA was identified in males with undescended testes (Coniglio et al., 1975), in sperm of asthenozoospermic men with reduced sperm motility (Conquer et al., 1999), and oligospermic men with low concentrations of sperm (Aksoy et al., 2006; Zalata et al., 1998). In the rat, a lower level of n-6 DPA is associated with impairment in testis functions (Davis and Coniglio, 1967) and testis underdevelopment (Suh et al., 2011). Dietary intervention by n-3 PUFA, in particular diet supplemented with DHA appears to benefit both human (Comhaire et al., 2000; Robbins et al., 2012; Safarinejad, 2011) and rat (Li et al., 2013; Sebokova et al., 1990; Suh et al., 2011) by supporting male fertilizing capacity and testis maturation while increasing the proportion of DHA that has been shown in rat testis (Ayala and Brenner, 1980; Ayala et al., 1977). This evidence indicates that sperm fertile potential and testis maturation and spermatogenesis are closely associated with PUFA in testis. However, less is known about the effect of diet and lifestyle on PUFA metabolism in testis, especially during development.

A review of the background related to male fertility will concentrate on testis and sperm structure and functions, their lipid compositions, the effect of alcohol on the male reproductive system specifically during development, and the role of a provision of DHA on male fertility.

1.2. Male reproductive system

The male reproductive system consists of testis as a central organ for male fertility by producing male gametes and male sexual hormones (androgens). Endocrine regulation controls testicular structure and functions through the hypothalamus and pituitary gland, which are mediated and modulated by the mechanisms governed locally at the testicular level. Sperm parameters directly indicate male reproductive health. Normal structure and functions of the reproductive system to produce healthy sperm in adulthood are strongly based on normal development during fetal life and puberty (Weinbauer et al., 2010).

1.2.1. Testis

1.2.1.1. Structure

Proper structure of testis plays a prime role to its functions, resulting in normal male fertility. In adult men, the testis weighs about 20 g with a testicular volume of 15-20 cm^3 (Jones and Lopez, 2006; Myers, 1988). The testicular volume is known to be associated with the testicular function (Condorelli et al., 2013), which is determined by the thread-like seminiferous tubules occupying about 80% of the testis. The testis has two major functions: steroidogenesis and spermatogenesis. Cells responsible for steroidogenesis (Leydig cells) and spermatogenesis (Germ cells and Sertoli cells) are located in two discernable compartments that exist morphologically and functionally separated. The integrity of these two compartments is crucial for sperm characteristics (Jones and Lopez, 2006; Myers, 1988).

1.2.1.2. Functions

1.2.1.2.1. Spermatogenesis

Spermatogenesis refers to all the process involved in the production of spermatozoa *via* cell divisions that take place in the tubular compartment. Although the process of spermatogenesis normally is not initiated until puberty, there is a fetal determinant. An adequate pool of normal germ cells is required to facilitate normal spermatogenesis in adulthood. The sufficient number of germ cells is determined by the mitotically differentiation of primordial germ cells to spermatogonia or spermatogonial stem cells in fetal seminiferous cords (Sharpe, 2010). Therefore, any impairment during the fetal period might consequently disrupt spermatogenesis in adulthood. This will be explained more in the next section, "developing testis", on page 8.

The first cycle of spermatogenesis initiates at around puberty by the previously quiescent germ cells. During this period, the diploid (2N) spermatogonia as the sperm mother cells present in the germinal epithelium undergo a mitotic division. The majority of these daughter spermatogonia progress to become primary spermatocytes (2N). The remaining maintains the spermatogonial stem cells for continual sperm production throughout life. The primary spermatocytes undergo the first round of meiosis and divide into secondary spermatocytes which are haploid germ cells. These cells undergo the second round of meiosis to become haploid (N), smaller germ cells termed spermatids. Spermiogenesis is the morphological differentiation phase during which, non-motile spermatozoa are transformed into differentiated motile spermatozoa that release from the germinal epithelium into the tubular lumen. At this time, the spermatozoa are not capable of fertilizing an ovum yet (Jones and Lopez, 2006) **(Figure 1.1)**. After leaving the testis, spermatozoa undertake crucial alterations by passing through the epididymis, contributing to their maturation, making them potentially able to fertilize an egg (Hall et al., 1991). A new spermatogenic cycle starts every 16 days in humans (Jones and Lopez, 2006) and is 12.5 days in the Sprague-Dawley rat (Rosiepen et al., 1994). Four spermatogenic cycles are

required for development and maturation of spermatogonia into mature spermatozoa in both species. Thus, spermatogenesis requires at least 65 days entirely in humans (Jones and Lopez, 2006) and 53.5 days in rats (Clermont, 1972) to proceed from germ cells to mature sperm cells. Since the rate of cellular proliferation and differentiation are high during these periods, any external and internal insults with lifestyle changes could result in negative impacts on testis function. This is why the testis is considered to be one of the most vulnerable tissues in our body.

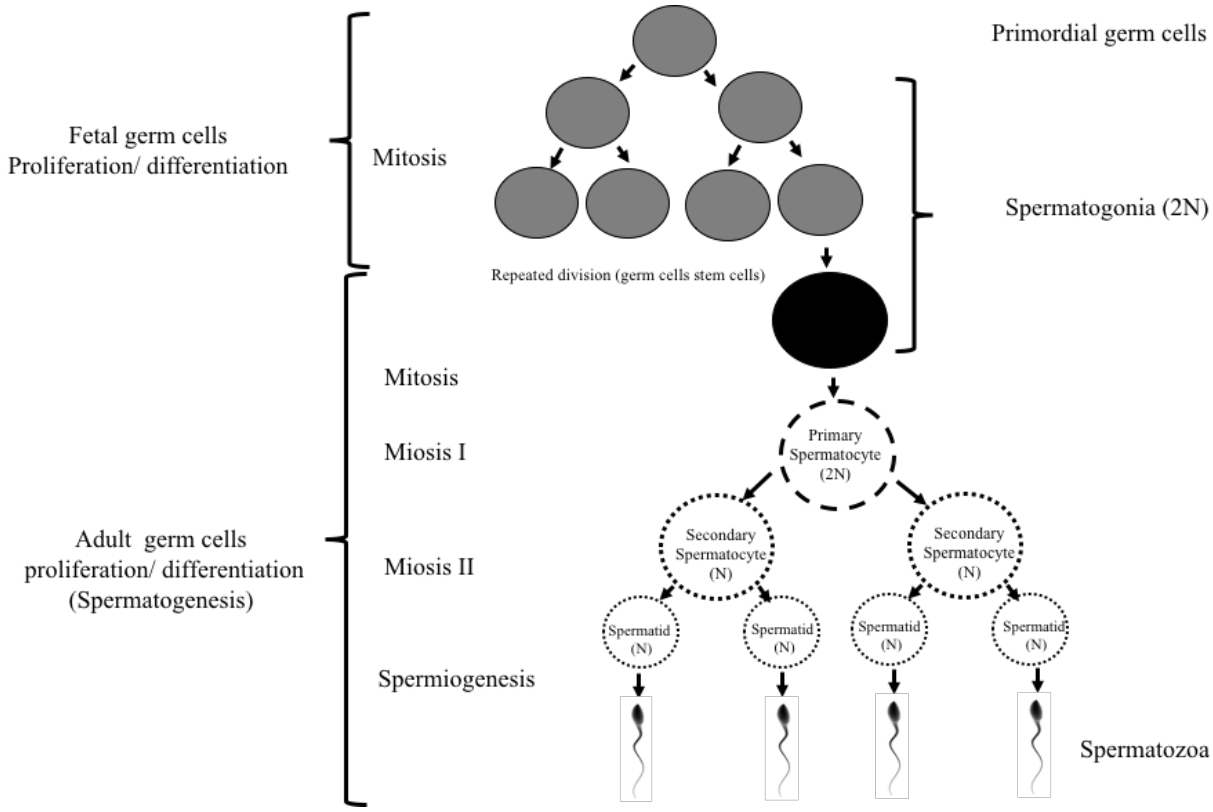

Figure 1.1. The process of Spermatogenesis
2N, diploid cells; N, haploid cells.

1.2.1.2.2. Steroidogenesis

Steroidogenesis includes all the enzymatic activities to produce androgen by Leydig cells (Weinbauer et al., 2010). Indeed, Leydig cells are responsible for both the synthesis and secretion of more than 95% of androgen. The androgen-producing cells are housed adjacent to the seminiferous tubules (Payne and Hardy, 2007; Weinbauer et al., 2010). Steroid hormones are produced via multiple enzymatic reactions occurring in the mitochondrial membrane. Cholesterol is the start-point of testosterone synthesis that can be either taken up from low-density lipoprotein (LDL) or might be synthesized *de novo* from acetyl-coenzyme A. In contrast to human, high-density lipoprotein (HDL)-derived cholesterol is the main substrate to produce androgen in rat testis (Andersen and Dietschy, 1978). Leydig cells are able to store cholesterol in an ester form in the cytoplasmic lipid droplets, which is regulated by pituitary luteinizing hormone (LH). Testosterone precursor transportation through the inner mitochondrial membrane is governed by steroidogenic acute regulatory (StAR) protein which is a rate-limiting step in testosterone production (Weinbauer et al., 2010). Testosterone is actively synthesized during fetal development that will be discussed in the next section, "developing testis", on page 8. It seems that 3β-hydroxysteroid dehydrogenase (3βHSD) enzyme can induce production of testosterone in Leydig cells in both human (Dufau et al., 1978; Tapanainen et al., 1981) and rat (Lording and De Kretser, 1972; Roosen-Runge, 1956; Tapanainen et al., 1984). As shown in **Figure 1.2** the first step in testosterone production is pregnenolone formation followed either through steroidogenic Δ4 or Δ5 pathway, with Δ5 being the predominant over Δ4 in human. 17-hydroxypregnenolone and dehydroepiandrosterone are the intermediates in steroidogenic Δ5 pathway. Along to steroidogenic Δ4 pathway, progesterone is produced from pregnenolone and the testosterone production occurs through the 17-hydroxyprogesterone.

The major functional roles of testosterone, as an abundant androgen in the blood in adulthood, start from fetal life and continue recognizably during puberty and adulthood. Testosterone is involved in the masculinization of testis and brain, mediating of male sexual

behavior, masculinization of the bone-muscle apparatus, onset and regulation of spermatogenesis and libido (Weinbauer et al., 2010).

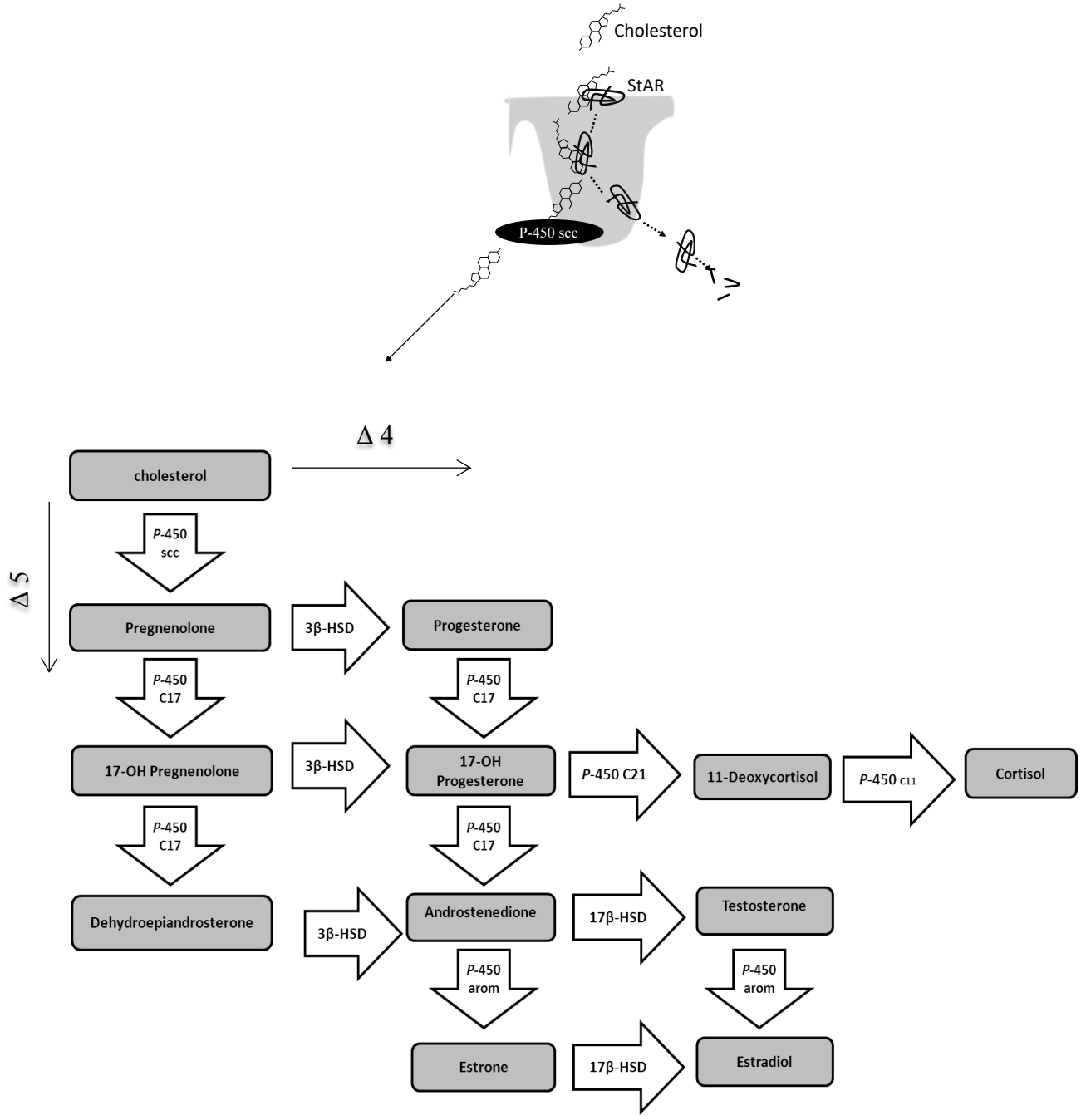

Figure 1.2. The process of steroidogenesis via two pathways
Scc, side chain cleavage; 3β-HSD, 3β-Hydroxysteroid dehydrogenase; C17, 17 Hydroxylase; C21, 21 Hydroxylase; C11, 11Hydroxylase; 17β-HSD, 17β- Hydroxysteroid dehydrogenase; arom, Aromatase. Δ5 pathway (17α-hydroxypregnenolone, dehydroepiandrosterone, testosterone); Δ4 (progesterone onwards).

Testosterone is a lipophilic steroid transferred within the cell via passive diffusion. In blood, testosterone is mainly carried via albumin or sex hormone binding globulin (SHBG), which is synthesized by hepatocytes (Weinbauer et al., 2010). Testosterone is a substrate for two antagonistic sex steroids, dihydrotestosterone (DHT) and estrogen. DHT is generated by enzymatic reduction of testosterone with 5α-reductase. By aromatizing the testosterone, it converts into estrogen that is a feminizing hormone (Jones and Lopez, 2006). Therefore, the level of testosterone in the blood is regulated by the rate of synthesis, stabilized by metabolic conversion and excretion (Weinbauer et al., 2010).

1.2.1.2.3. Neuroendocrine regulation of functional testis

Gonadotropin-releasing hormone (GnRH) released from the hypothalamus stimulate the production of follicle-stimulating hormone (FSH) and LH by the pituitary. FSH supports spermatogenesis, induces the formation of gonadotropin receptors in testis (Huhtaniemi et al., 1982), triggers the production of steroidogenic enzymes (Huhtaniemi et al., 1982) and testicular growth in overall (Ojeda et al., 1980). However, the stimulation of Leydig cells to produce and secrete testosterone is the responsibility of LH. Both LH and FSH control the level of testosterone and the first release of spermatogenesis (Weinbauer et al., 2010). Testosterone regulates both LH and FSH level by negative feedback on the hypothalamus (Marty et al., 2003). Therefore, there is a strong interaction between testosterone and its central regulators' production, as well as spermatogenesis.

1.2.2. Developing testis

The foundation of normal puberty and adult male reproductive structure and functions are determined during fetal period. Any developmental errors that occur during this period appear to be irreparable and cannot be corrected in adulthood. Impairment of the fetal genital system might lead to irreversible loss of germ cells and reduced capacity of the reproductive system in later life, emphasizing the importance of the fetal period for sexual organ development.

Rodents have been utilized as the most common animal models to study cellular and molecular mechanisms of testis functions. Sprague-Dawley rat is a popular strain used in the field of the reproductive system and developmental toxicology (Campion et al., 2013). However, how their experimental findings can be related to human is a challenge. The following section describes and compares the key events in the development of male testis and timing of these processes during prenatal and postnatal life in human and rat. The testis formation, fetal and postnatal proliferation/differentiation of testis cells, brain masculinization, puberty, spermatogenesis, and fetal and postnatal testosterone surges will be discussed briefly as below.

1.2.2.1. Sexual organ development

Human. Sexual development in human, as in most mammals can be separated into four stages: Pre-gonadal, biopotential and gonadal stages which the latter is divided into two stages: primary and secondary sex differentiation. The first three stages take place during the fetal period that is the first 8 weeks in human. However, secondary sex differentiation occurs partially in the fetus and continues at puberty (Mesiano, 2009). Testis formation is known as primary sex differentiation (Koopman et al., 1991) and occurs at the end of 6 weeks in human (Strauss and Barbieri, 2013) which does not require the local action of male hormones. However, testis masculinization occurs during secondary sex differentiation in the fetus that results from testosterone production after testis formation (Woodruff et al., 2010). The remaining secondary sex differentiation happens in postnatal life. These events during development have been presented from fetus to adulthood in **Figure 1.3**.

Rat. Similar to human, the process of the male reproductive system development in rat begins by pregonadal stage. Testis formation is completed by (GD)13 (Encinas et al., 2012; Wachtel, 2014) and after that, the secondary sex differentiation occurs. All the events can be sought during fetal development and postnatal life that includes four stages: i) “neonatal” the period between PD1-7, ii) “infantile” which is the stage between PD 8-21 iii) “juvenile” is PD21-

35; iv) “Peri-pubertal” stage that is extended from 35 to PD55-60, until the appearance of spermatozoa in vas deferens (Ojeda et al., 1980) **(Figure 1.4)**.

1.2.2.2. Sertoli cells

Human. Sertoli cells are the main organizer for the development of functional testis in the fetal period (Mackay, 2000). However, their roles are changed at the peri-pubertal stage to be central to spermatogenesis. This occurs when proliferative Sertoli cells stop dividing (Weinbauer et al., 2010) to differentiate into non-proliferative mature cells up to puberty (Wang et al., 1989). The population of Sertoli cells determine the testicular volume and seem to be positively connected to the spermatogenesis (Weinbauer et al., 2010). Mature Sertoli cells cannot divide. Thus, the number of Sertoli cells and testis volume are determined before adulthood.

Rat. Similar to human, Sertoli cells proliferation occurs during fetal, neonatal and prepubertal stages (Sharpe, 2010). They stop dividing entirely at around PD14-16 (Steinberger and Steinberger, 1971) and thereafter differentiate into non-proliferative cells up to puberty (Wang et al., 1989).

1.2.2.3. Germ cells

Human. Germ cells are responsible for transmitting genetic information between generations. They have symbiotic and cell-to-cell interaction with Sertoli cells to be protected from degeneration. The critical time during gametes development is the differentiation of germ cells into gonocytes occurring at the first mitosis at 8 weeks of pregnancy in human (Schoenwolf and Larsen, 2009; Weinbauer et al., 2010). In neonates, the number of spermatogonia increases by 6 times from birth up to 10 years of age, then increases exponentially at puberty, concurrent with elevation in the testis volume. The age at which the first ejaculation containing spermatozoa is known as Spermarche and occurs at puberty at around 13.4 years (Marty et al., 2003).

Rat. Gonocytes emerge at GD17-18 (Encinas et al., 2012) but stop dividing until early life at (PD3-4). At this time, spermatocytes restart mitosis to form the primary spermatogonia and then move from center to the edge of seminiferous cord (McGuinness and Orth, 1992). This is a critical time for establishing the spermatogenesis during the neonatal period. The formation of the lumen in the solid seminiferous tubule occurs at PD18 (Russell et al., 1989) and the seminiferous germinal epithelium is completely formed during PD48-60 (Hilscher and Hilscher, 1976). The first spermatozoa are detectable in seminiferous tubules on PD45 and in vas deferens on PD58-59 (Clegg, 1960) with reaching the highest concentrations of spermatozoa on PD91 (Sun and Flickinger, 1979). However, spermatozoa reach the highest maturity and motility on PD70 (Campion et al., 2013).

1.2.2.4. Leydig cells

Human. Leydig cells have two different populations that are responsible for testicular steroidogenesis in fetal and adult life. Leydig cell progenitors differentiate into Leydig cells (Gerald G, 2009) at around 7-8 weeks of gestation (Siiteri and Wilson, 1974; Tapanainen et al., 1981), with peak numbers achieved between week 14 and 18 of gestation (Strauss and Barbieri, 2013). They then deteriorate into the immature Leydig cells (Weinbauer et al., 2010). After a short increase in early postnatal life (Marty et al., 2003), the next period of Leydig cells proliferation begins at around puberty and the numbers reach a plateau in adulthood. Coincident with Leydig cells differentiation and proliferation during development, three surges of testosterone are recognizable in human (Strauss and Barbieri, 2013), including, fetal, neonatal and pubertal surges of testosterone. Both fetal and neonatal surges are profoundly essential for brain masculinization, which will be more discussed below in rat section. The last surge of testosterone is from 12 to 14 years of age (Claudio et al., 1999) governing puberty and the events happening during this critical period, particularly, spermatogenesis.

Rat. Leydig cells differentiation occurs at GD15 and reaches the highest amount at GD18-19 in rats that parallels the fetal surge of testosterone (Ward and Weisz, 1984; Warren et al., 1973). There is another testosterone surge a few hours after birth (Baum et al., 1988). The surge of testosterone is required for brain masculinization via affecting on the volume of the sexually dimorphic nucleus (SDN) in the preoptic area (POA) of the hypothalamus (Gorski et al., 1978). The masculinization and defeminization of the brain structure in early life (Dohle et al., 2003) regulate male sexual behavior in later life (Wu and Shah, 2011). The surge of testosterone is also necessary for sexual differentiation in the fetal and neonatal hypothalamus for maturation of LH release mechanism (Marty et al., 2003). Accompanying the increased number of fetal Leydig cells, the number of LH receptors elevate in testis regulating the testosterone production (Huhtaniemi, 1989). Adult Leydig cells proliferation initiate at PD14-28 and then from PD28-56 (Marty et al., 2003). Concurrently, the postnatal surge of testosterone slowly begins from PD28 (Marty et al., 2003) to reach a highest at PD51 (Korenbrot et al., 1977).

The above information on the key development of the male reproductive system in rat provides the basis of the pathophysiological development of testis challenged with nutrition, alcohol and other toxicological factors.

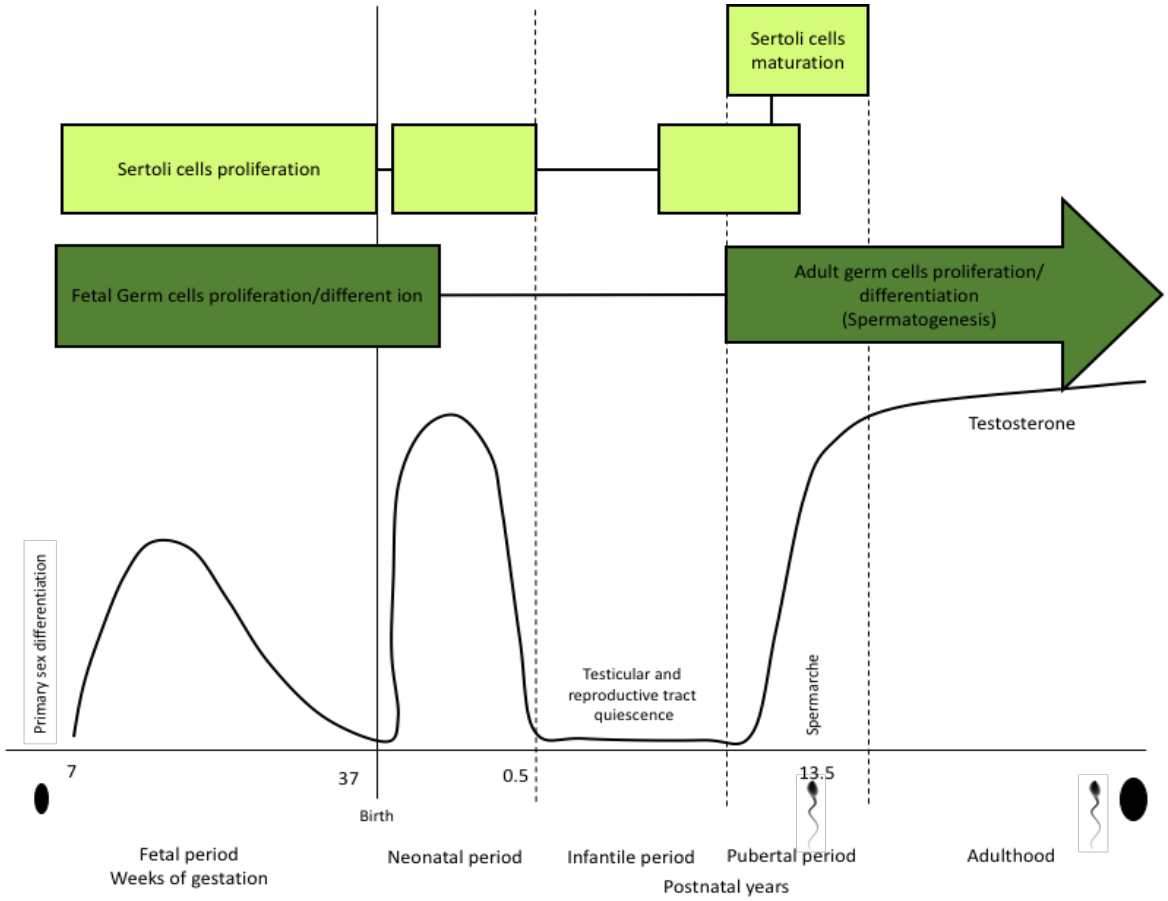

Figure 1.3. Time course related to the main events in testis development and functions in human

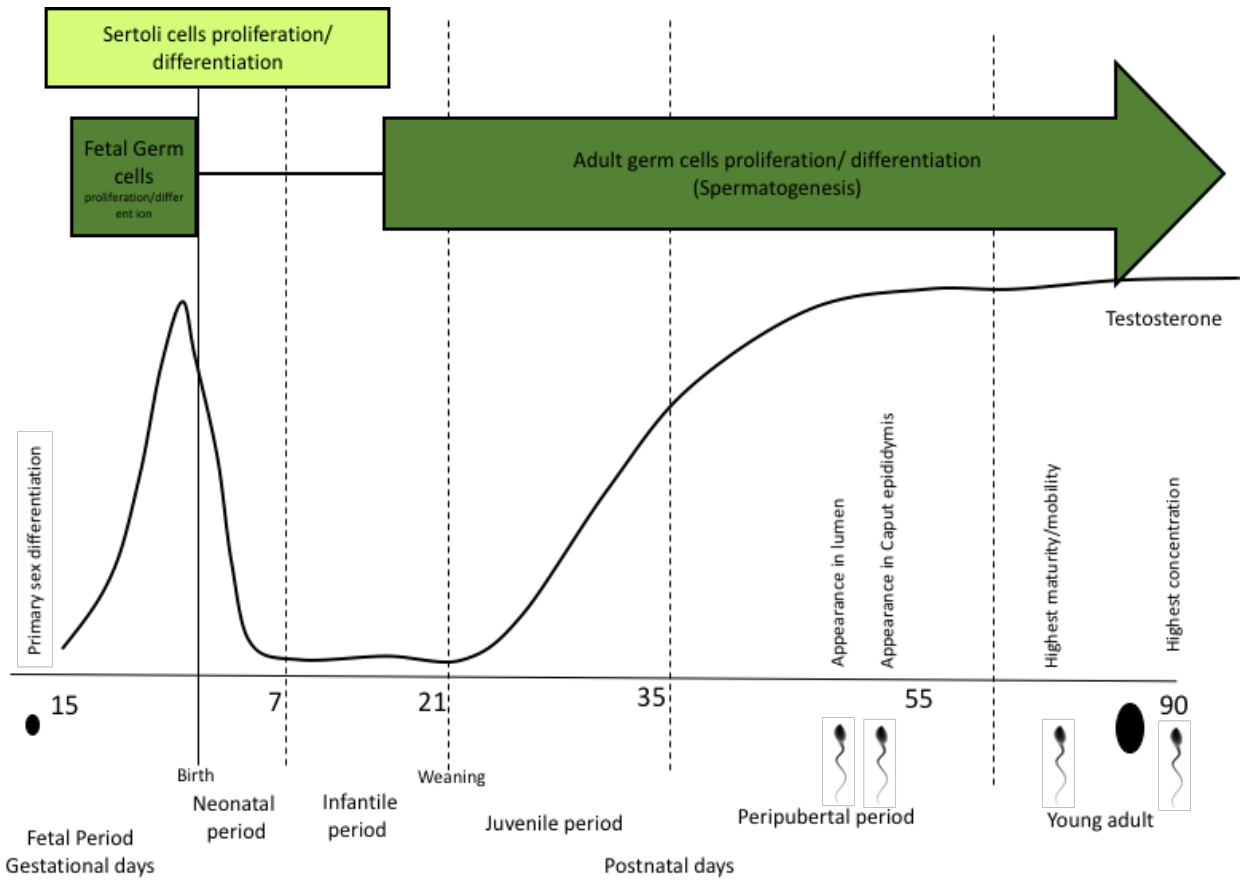

Figure 1.4. Time course related to the main events in testis development and functions in rat

1.2.3. Sperm

1.2.3.1. Structure

The spermatozoa are complex and highly specialized microscopic organisms illustrated in **(Figure 1.5).** The mature spermatozoa contain the head (including neck) and tail (including mid-piece and principal piece). Nucleus and acrosome are found in the head compartment and motility is provided by the tail of spermatozoa that is specialized for flagellar movement providing the required energy to move (Alberts et al., 2002). The structure of spermatozoa in the rat is similar to human, but the head of spermatozoa in the rat are resembled to hook. The length of spermatozoa in human are shorter than those of rats, 60 µm versus 160 µm (Prakash et al., 2014).

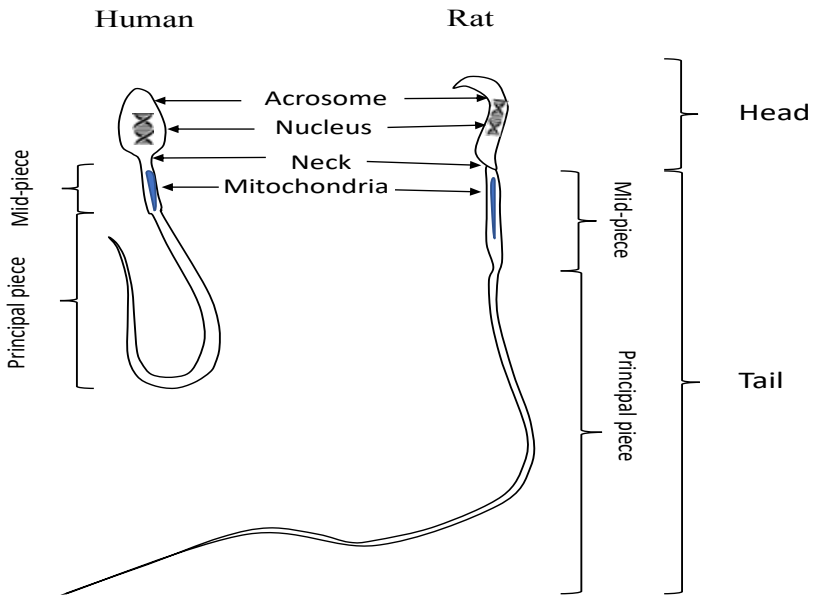

Figure 1.5. The structure of sperm in man and rat

1.2.3.2. Functions

The maturity of spermatozoa while transitioning through the epididymis is critical in the fertilization. Maturation leads to capacitation in the sperm that primes them for the acrosome reaction (Knobil and Neill, 2006). The acrosome is a secretory vesicle at the apical side of spermatozoa head evolved from the Golgi apparatus of the spermatocyte (Whitaker and Swann, 1993) **(Figure 1.5)**. The acrosome reaction is an external process with a numerous fusion between the acrosomal outer membrane and the plasma membrane of the zona pellucida of the oocyte to release the acrosomal fluid that contains the hydrolytic enzyme (Green, 1993). Normal motility is crucial for enabling sperm to the function which also develops by passing through the epididymis (Knobil and Neill, 2006). Importantly, all these preparation during spermatozoa maturation is concomitant with substantial alterations in the lipid contents of the plasma membrane, in particular, n-6 DPA level that increases markedly as has been shown in rat spermatozoa (Hall et al., 1991). The unique lipid compositions in the mature spermatozoa plasma membrane that might contribute to this specific sperm maturity will be focused in the next session, lipid composition on page 17.

1.2.3.3. Sperm quality and abnormality

Sperm quality is commonly accessed in the semen analysis, which is a key analysis for the evaluation of the testicular function. According to the WHO, four common terms are used to describe sperm quality in semen analysis (**Table 1.1**): i) oligozoospermia (oligospermia), a total number of sperm to be less than the lower reference limits of 15 million sperm/mL ejaculate; ii) azoospermia, defined as the absence of spermatozoa in the ejaculate; iii) asthenozoospermia, a semen sample testing motility, where less than 32% of sperm exhibit forward progression; iv) teratozoospermia, a semen condition of abnormal sperm morphology to have less than 4% morphologically normal sperm (WHO, 2010). In morphology, many different abnormalities can occur, including abnormalities in the head, neck or mid-piece, or tail. All of the above sperm characteristics including concentrations, motility, and morphology, are reproductive challenges that need to be overcome for fertilization to occur. Sperm is the only cell designed to leave the male body and join with the female ovum for fertility; thus any challenges leading to poor semen quality and abnormal morphology could impact fertility success.

Table 1.1. WHO semen analysis reference with lower limits

Sperm characteristics	Reference value (lower ref limits)	Infertility conditions	
Semen volume (ml)	1.5-5.0 (1.4-1.7)		
Sperm conc (ml)	>15 x 10^6 (12-16)	<12 x 10^6	oligozoospermia
Sperm conc (ejaculate)	>39 x 10^6 (33-46)	absence	azoospermia
Total motile sperm (%)	>40 (38-42)		
Progressive sperm (%)	>32 (31-34)	<31	asthenozoospermia
Vitality (live spermatozoa, %)	58(55-63)		
Normal morphology (%)	>4 (3.0-4.0)	<3	teratospermia

WHO (2010), Numbers in the bracket represents 5th centile and their 95% confidence intervals, Conc., concentrations; Used with permission.

1.3. Lipid compositions

1.3.1. Testis

The importance of lipids in testis development, structure and function has been recognized in the early 1930s in a rodent model (Ayala et al., 1977; Bieri et al., 1969; Burr and Burr, 1930; Coniglio, 1994). However, the lipids data from the human testis are still scarce. It has been reported that the total lipid in adult male testis is in the range of 26.7-28.5 (mg g^{-1} wet tissue) (Coniglio et al., 1975) which is almost similar to those in rat accounting for 18.1 (mg g^{-1} wet tissue) (Bieri and Prival, 1965). In both human and rat, the main lipids in testis are Phospholipids followed by triacylglycerides, sulfolipids (Goto-Inoue et al., 2009), PUFA (Coniglio, 1994) and very long chain fatty acids (Furland et al., 2007).

1.3.1.1. Phospholipids

Phospholipids (PL) as the predominant components of membranes are found in a large amount in the testis. The critical role of PL among different types of lipid is attributed to their structural properties in the plasma membranes. PL has a glycerol backbone, two fatty acids esterified at position *sn*-1 and *sn*-2 that provide two non-polar tails (hydrophobic). A polar head group (hydrophilic) in PL is due to the presence of a phosphate group at position *sn-3* attached to an alcohol base molecule (Saether, 2003). This structure makes PL an essential part of the structural matrix of all cell and subcellular membranes. The two which predominate in testicular membranes are phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Merrells et al., 2009). Others found in lesser amount but which have important role include phosphatidylserine (PS), sphingomyelin (SM) and phosphatidylinositol (PI) (Coniglio et al., 1975). Testicular total PL weighs 15.6-15.9 (mg g^{-1} wet tissues) representing nearly 40-60% (w/w) of the total lipids in human (Coniglio et al., 1975), which indicates that testis to be a PL enriched tissue. Therefore, the pattern of fatty acids in total PL reflects those of testis (Bieri and Prival, 1965). The lipid contents in the rat testis are occupied with the larger amount of PL accounting for 75% (Bieri

and Prival, 1965) to 80% (w/w) of total lipid (Oshima and Carpenter, 1968). The distribution of the PL in the adult rat testis is almost similar to that of human, with the largest and lowest being PC and PI, respectively (Oshima and Carpenter, 1968).

1.3.1.2. Fatty acids

The total lipids of adult human testis is composed of 38-45% saturated fatty acids (SFA), 19-24%, monounsaturated fatty acids (MUFA), 22-32% of n-6 PUFA, and 7-10% n-3 PUFA **(Table 1.2)** (Bieri and Prival, 1965; Coniglio et al., 1974, 1975; Hoffmann et al., 2005). In rat testis, the average of SFA, MUFA, n-6 PUFA and n-3 PUFA are 41.2, 14.9, 39.2 and 1.4% (w/w), respectively (Bieri and Prival, 1965; Coniglio et al., 1974; Davis et al., 1966). Palmitic acid (C16:0) and stearic acid (C18:0) as the main SFA and oleic acid (C18:1n-9) as the major monoenoic acid comprise 28.4, 11.2 and 18%, (w/w) of total lipid in human (Bieri and Prival, 1965; Coniglio et al., 1974, 1975; Hoffmann et al., 2005) and 35.6, 5.6 and 14% (w/w) of total lipid in rat testis, respectively (Bieri and Prival, 1965; Coniglio et al., 1974; Davis et al., 1966).

1.3.1.2.1. Polyunsaturated fatty acids

Among testis fatty acids, PUFA received more attention. PUFA is synthesized from essential fatty acids (EFAs), which are characterized as two families the n-6 and n-3 fatty acids. Linoleic acid (LA, C18:2n-6) and α-linolenic acid (ALA, C18:3n-3) are the parents for n-6 and n-3 fatty acids, respectively. They are desaturated and elongated to form a series of longer chain termed long-chain polyunsaturated fatty acids (Rustan and Drevon, 2001). Among PUFAs, arachidonic acid (AA, C20:4n-6, 11-13%, w/w total lipids) and DHA (8%, w/w total lipids) are the predominant fatty acids of n-6 and n-3 PUFA, respectively in the human testis (**Figure 1.6; Table 1.2**) (Bieri and Prival, 1965; Coniglio et al., 1974, 1975; Hoffmann et al., 2005). In the rat testis, n-6 DPA is the main PUFA in lipid membrane plasma comprising of around 16% of the

total lipid (Bieri and Prival, 1965; Coniglio et al., 1974; Davis et al., 1966). Therefore, the main fatty acid in the testis is species specific.

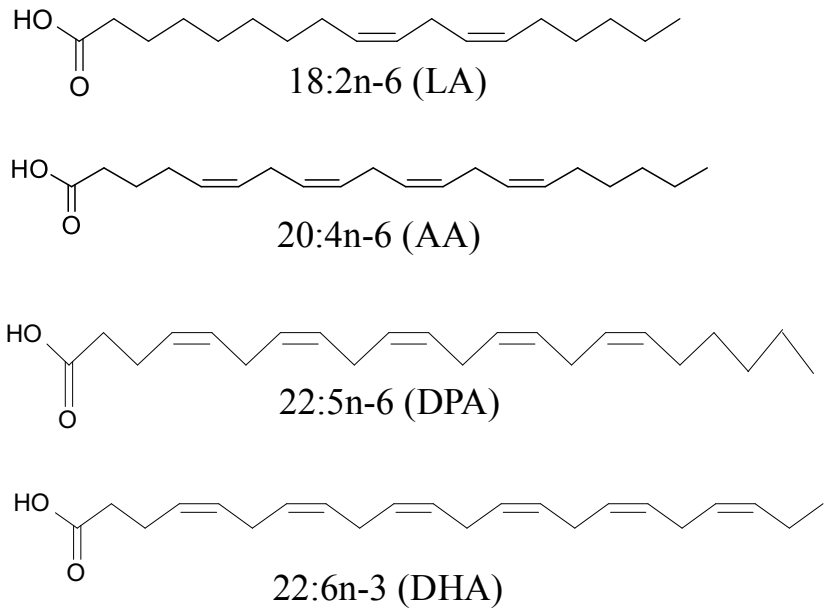

Figure 1.6. The major n-3 and n-6 polyunsaturated fatty acids found in human testis and sperm
LA, Linoleic acid; DPA, Docosapentaenoic acid; DHA, Docosahexaenoic acid; AA, Arachidonic acid; Used with permission.

1.3.1.2.2. Very long chain fatty acids

Very long chain fatty acids (VLCFA) are known for their scarce abundance. They have a chain length greater than 24 carbons with 3-6 double bonds. These fatty acids behave like saturated fatty acids in the mobile phase due to their special long chain structure. There is a 12-20 saturated-carbon chain in the proximal carboxylic region, and 3-6 conjugated methylene *cis* double bonds are presented in the distal region (Murray et al., 2014). VLCFA are not found as free fatty acids in tissue and form PL or sphingolipids and ceramides (Cer) when their amide bond linkage is modified. VLCFA and PUFA are located either at *sn-1* o*r sn-2* positions in glycerol backbone, and a polar head group can be added to the *sn-3* position, forming different

PL (Agbaga et al., 2010). VLCFA were estimated to make 9% of total PL contents exclusively detectable in SM of the testis (Poulos et al., 1987). In the prepubertal rat, the level of SM and Cer in testis are devoid of VLCFAs and increase in the commencement of the spermatogenesis to reach to 15% and 40% of total fatty acids, respectively (Furland et al., 2007).

Germ cells contain higher concentrations of VLCFA compared to Sertoli cells in seminiferous tubules (Kihara, 2012), with predominantly accumulation in the head spermatozoa (Furland et al., 2007). VLCFA have to be synthesized in *situ* from shorter fatty acids and only in tissues with high concentrations such as retina, brain, and testis that are enriched in the enzyme that catalysis the elongation of very long chain fatty acid (ELOVL) to which ELOVL1-2 and 5 belong to testis (Sandhoff et al., 2010). The precursors of VLCFA, LA, eicosapentaenoic acid (EPA, 20:5n-3), DPA, and DHA are derived from diet or synthesized in situ continually (except for LA) are carried from liver to the target tissues (Agbaga et al., 2008; Suh and Clandinin, 2005; Yu et al., 2012) and VLCFA are generated after a series of elongation and desaturation processes (Suh and Clandinin, 2005; Suh et al., 2000).

There is little known regarding VLCFA functions. It has been suggested that VLCFA are involved in spermatogenesis progression by affecting sperm production and sperm maturation, in particular, during meiotic division of spermatocytes into spermatids (Kihara, 2012; Robinson et al., 1992). Experimental studies revealed that ELOVL2 knockout mice had blocked spermatogenesis at the first meiosis to have only primary spermatocytes (Zadravec et al., 2011). Moreover, in animals with impaired spermatogenic germ cells, induced by post-ischemic reperfusion or x-ray irradiation, reduced quantities of testicular VLCFA, indicating their requirement for spermatogenesis and male fertility (Furland et al., 2007; Kihara, 2012; Sandhoff, 2010). Recently, Dr. Suh's lab found that underdeveloped rat testis had selectively low concentrations of n-6 DPA accompanied with a selective loss of pentaenoic n-6 VLCFA in testis while increasing tetraenoic n-6 VLCFA (Datar, 2015). This is puzzling what cellular and molecular mechanisms are involved in this selective loss in addition to the lack of clear

information on the exact function of these fatty acids in male reproduction. All in all, the aforementioned evidence indicates the highly important role of VLCFA for spermatogenesis and male fertility. Unlike the PUFA, information still lacks on VLCFA in the male reproductive system. It also has not been studied intensively if lifestyle, such as dietary lipid and or alcohol, can impact the contents and functions of VLCFA thereby affecting male fertility.

1.3.1.2.3. Seminolipid

Seminolipid is the main and specific glycosulpholipid in the mammalian germ cells (Handa et al., 1974; Ishizuka et al., 1973; Kornblatt et al., 1972) including human (Ishizuka and Yamakawa, 1974). Seminolipid is found exclusively in mammalian testis, accounting for more than 90% of glycolipids (Goto-Inoue et al., 2009). In the rat, the biosynthesis of seminolipid takes place during sexual maturity, on PD15-20 that the first cycle of spermatogenesis happens. It is accompanied by the production of primary spermatocytes (Handa et al., 1974; Kornblatt et al., 1974; Lingwood, 1985), with accumulation mainly in head spermatozoa (White et al., 2000). Seminolipid chemical structure is anionic, amphiphilic containing 3 regions including high, intermediate and low polarity (Alvarez et al., 1990; Vos et al., 1994). It is suggested that seminolipids are structural component in the lipid raft (Vos et al., 1994) that are located in the extracellular leaflet of plasma membrane regulating the production and differentiation of sperm, cell-cell adhesion and recognition events (Ishizuka, 1997; White et al., 2000) as well as spermatozoa membrane integrity and stability (Attar et al., 2000; Bou Khalil et al., 2006). The absence of seminolipid can result in the arrest of spermatogenesis in the first meiosis (Fujimoto et al., 2000; Honke et al., 2002) with impairment in differentiation stage in spermatogenesis (Honke et al., 2002; Kornblatt et al., 1972). Cerebroside sulfotransferase (cst) is one of the major enzymes involved in seminolipid synthesis in testis and sulfatide synthesis in the brain (Ishizuka, 1997; White et al., 2000). Injection of wild-type spermatogonia into seminiferous tubules in the mouse with lack of cst enzyme resulted in the rapid synthesis of seminolipid in testis,

normalizing spermatogenesis and cellular differentiation. Whether seminolipid is altered with diet and environment challenge is not much known. Besides, the information lacks regarding the pattern of seminolipid development during testicular maturation.

1.3.2. Developing testis

Similar to other tissues, lipid compositions of mammalian testis change with age. Data from autopsy and orchiectomy showed a marked decline in the lipid contents of testis during maturation in human testis. Testis lipid contents reduce significantly short after birth, from 45 to 28 mg/g during the first 3 days after birth and by 4 months, reach 23 mg/g of the wet tissue which is more comparable to those of adult (Coniglio et al., 1975). In contrast, the level of DHA increased considerably during maturation, with an increase from 0.5 to 2.6% (w/w) in 4 month-old infants reaching to 8.5% (w/w) of total fatty acid in mature testis (Coniglio et al., 1975). This indicates the crucial role of DHA in testis functions in human. The regulation of fatty acid pattern during development in testis is suggested to be directed by optimal capacity of testis for PUFA metabolism and specialization of the enzyme in testis to incorporate PUFA into spermatozoa (Retterstøl et al., 2001; Sæther et al., 2007). Then the question arises what could be provided to maintain optimal PUFA metabolism during life stages.

Earlier studies from lipid composition in developing testis in rat revealed significant alterations in testis lipid profile. Almost 2.4% (w/w) of wet weight of organ tissue in both mature and immature testis is composed of lipid, from which PL accounting for the most significant proportion (Davis et al., 1966; Oshima and Carpenter, 1968). During testicular development from PD28 to 90, SFA, mainly stearic acid and MUFA, majorly oleic acid, decreased by testis maturation in total lipid as well as some lipid classes, including triacylglyceride (TAG) and PL classes (Davis et al., 1966). In contrast, n-6 DPA replaced SFA and MUFA contents in mature testis (Davis et al., 1966; Oshima and Carpenter, 1968), emphasizing the importance of n-6 DPA in testis functions. No significant changes in the level of n-6 DPA precursors, LA and AA, have

been reported during the same period (Davis et al., 1966). According to the morphological studies, the time during which testis fatty acid compositions undergo considerable changes is coincident with testis maturation, predominately between PD28 to PD49 that described in the previous section. The main events during these period include the maturation and differentiation of Sertoli cell (Wang et al., 1989), increased activity of new generation of Leydig cells followed by the surge of testosterone at puberty (Marty et al., 2003), the production of spermatids and maturation of spermatozoa releasing in the lumen (Clegg, 1960). Concurrently, PUFA content, predominantly n-6 DPA, increased 2-folds, from 0.83 to 1.81 mg g^{-1} wet weight of tissue. The pattern of n-6 DPA alterations in the total lipid of the testis is similar to those of PL (Davis et al., 1966). Less is known about the trend of testicular lipid composition from fetus to adulthood.

Further studies from underdeveloped testis also supported that the DHA content of undescended testis was comparable to those value of 4-month-old boys (2.6% w/w of total lipid) in human. Male testis with marked atrophy also revealed a non-significant reduction in the level of DHA (Coniglio et al., 1975). Interestingly, only DHA, not AA, has been found to decrease in testis during degeneration of testis (Coniglio et al., 1975), indicating DHA be a significant factor for the development of germinal cells in the human. Similarly, factors that induce testis degeneration, such as hypophysectomy (Nakamura et al., 1968), surgical cryptorchidism together with administration of cadmium chloride (Davis and Coniglio, 1967), diminished the level of n-6 DPA in rat testis. Studies reported that the underdeveloped testis contained significantly lower level of n-6 DPA in rat testis (Suh et al., 2011; Zanetti et al., 2007). Therefore, the level of DHA in human and n-6 DPA in rat might be used as a marker of maturity in testis associated to its function.

1.3.3. Sperm

1.3.3.1. In healthy sperm

The fatty acid compositions in the membrane PL of mammalian sperm are different from other cell types. Sperm is comprised of 60% of the testicular volume and therefore, the specific level of the predominant fatty acids in spermatozoa is similar to those of the testis (Roosen-Runge, 1956). Sperm lipid profiles, generally determined on ejaculated sperm, undergo epididymal maturation, thus the lipid profile may differ from testicular spermatozoa which have yet to mature. Sperm lipid profiles are composed of a heterogeneous mixture of glycolipids, sterols, PL, SFA, MUFA, and PUFA, which are believed to play an important role in the viability, maturation, motility and fertilizing ability of the spermatozoa. PL is the integral components of the sperm membrane representing the most lipid fraction (Mann and Lutwak-Mann, 1981). In addition to supply sperm as a nutrient by easily oxidizing (Poulos et al., 1973), PL is responsible for maintaining sperm structural and functional integrity (Gomathi et al., 1993). Among phosphatide fractions, PE and PI have been reported to facilitate sperm motility and fertilization and PC as a main source of energy for sperm (Fleming and Yanagimachi, 1981). However no further studies confirmed this. Among the lipids, the fatty acid composition of sperm has received significant attention due to its direct relationship with fertility. Depending on the species, the PL-bound fatty acids may be comprised of a great number of polyenoic derivatives, mainly DHA and n-6 DPA (Poulos et al., 1973). Several studies have carried out detailed fatty acid profiling of human spermatozoa (**Table 1.2**). The major SFA and MUFA fatty acids are palmitic acid (30.2%) and stearic acid (15.3%), and oleic acid (9.9%), respectively. The PUFA are mainly LA (5.8%), AA (4.4%) and then remarkably high amounts of DHA (21.3%), ranging from 14-32% (w/w) of total fatty acid in PL (Martínez-Soto et al., 2013; Tavilani et al., 2007; Zalata et al., 1998). Most studies agree with the distinctively high level of DHA in sperm, which is thus considered as a marker for infertility (Aksoy et al., 2006; Gulaya et al., 2001; Zalata et al., 1998). Interestingly, DHA is not evenly distributed in sperm. Learning from

monkeys, DHA is significantly enriched in the tail of sperm (19.6%, w/w total fatty acids) in comparison to the head (1.1%, w/w total fatty acids) (Connor et al., 1998), suggesting that DHA is involved in sperm movement by its contribution to the membrane fluidity (Gulaya et al., 1993; Nissen and Kreysel, 1983; Zalata et al., 1998) (**Figure 1.7**). Fluidity is also crucial for fertilization via the membrane fusion events and signals transduction pathway. Any changes in fluidity can impede the aggregation and activation of this critical signaling process in the sperm membrane (Wathes et al., 2007). Additionally, the presence of DHA in head sperm suggests another role for DHA in sperm fertilizing ability that is acrosome reaction, an event that is related to structural integrity in sperm membrane (Vriese and Christophe, 2003).

1.3.3.2. In infertile sperm

Lipid composition in infertile sperm is considerably different from healthy sperm. Sperm parameters, including sperm density and normal morphology (Aksoy et al., 2006) have been attributed to the DHA in sperm. Unlike normozoospermic males who have above the lower reference limits in sperm characteristics described in **Table 1.1,** men with oligospermia, asthenozoospermia, and oligoasthenozoospermia had significantly lower level of DHA in their spermatozoa (Connor et al., 1998; Safarinejad et al., 2010; Tavilani et al., 2007) **(Table 1.2).** Men with these conditions were found to have increased level of SFA and MUFA, and lower PUFA level (Aksoy et al., 2006; Coniglio et al., 1975; Zalata et al., 1998), compared with the normozoospermic men. An alteration in the level of PL classes also has been noted in infertile spermatozoa, with a reduction in the level of PE and a rise in the level of PS (Gulaya et al., 2001). This indicates that decreased sperm fertility potential is closely associated with alteration of sperm lipid contents.

In the rat spermatozoa, the proportion of palmitic, stearic acid and oleic acid are 10.4, 14.9 and 13.8%, (w/w) of total fatty acids, respectively. Similar to testis, rat sperm important fatty acid involved in fertility is n-6 DPA, with a 3 fold increase during sperm maturation

increases from spermatocyte to spermatid (Davis et al., 1966). The sperm level of n-6 DPA also increased from 21.7 to 25.5%, (w/w) total fatty acids during their transition through epididymis from caput to cauda, respectively, indicating the vital role of n-6 DPA in mature and fertile spermatozoa.

Table 1.2. The overall fatty acid composition in testis and spermatozoa from normal and infertile males

	Testis[1]	Spermatozoa[2]	
Fatty Acid		Normozoospermic	Infertile men[3]
Palmitic acid (C16:0)	28.4 ± 2.6	30.2 ± 1.1	33.4 ± 5.1
Stearic acid (C18:0)	11.2 ± 0.6	9.9 ± 5.0	15.3 ± 1.2
Oleic acid (C18:1n-9)	18.0 ± 2.1	9.9 ± 0.5	11.8 ± 0.6
Linoleic acid (C18:2n-6)	6.0 ± 1.1	5.8 ± 3.1	4.0 ± 0.5
Arachidonic acid (C20:4n-6)	13.3 ± 2.8	4.4 ± 3.8	3.3 ± 1.7
Docosahexanoic acid (C22:6n-3)	7.4 ± 0.9	21.3 ± 7.3	13.4 ± 3.0
Total SFA	40.5 ± 2.8	48.9 ± 4.4	51.0 ± 4.2
Total MUFA	21.3 ± 1.6	17.2 ± 2.6	19.1 ± 2.7
Total n-6 PUFA	25.3 ± 3.8	14.4 ± 6.7	13.9 ± 3.7
Total n-3 PUFA	8.5 ± 1.3	17.4 ± 2.4	15.8 ± 2.9
Ratio of n-6/n-3 PUFA	3.1 ± 0.9	0.7 ± 0.4	1.0 ± 0.3

Values are mean ± SD.
[1]An average of 4 studies (%, w/w testis total lipids) (Coniglio et al., 1975; Hoffman et al., 2005, Coniglio et al., 1974, Bieri and Proval, 1965)
[2] An average of 3 studies (mole% of spermatozoa) (Zalata et al., 1998, Martinez-Soto et al., 2013, and Tavilani et al., 2007). Zalata and Talivani used phospholipids.
[3] Infertile men includes Fatty acids were combined for men with oligospermia, asthenozoospermia and oligoasthenozoospermia; Used with permission.

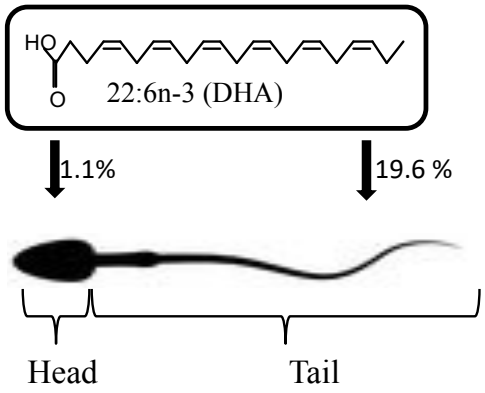

Figure 1.7. Docosahexaenoic acid distribution in the spermatozoa in monkey
Used with permission.

1.4. Lifestyle factors affecting male reproductive function

Genetic, environmental exposure and lifestyle have been recognized as the main factors for detrimental effects on natural reproductive potential. The lifestyle factors include dietary intake, physical activity and consumption of alcohol and tobacco, affecting natural reproductive potential. Although other factors, such as oxidative excess, have been shown to be associated with male infertility (Braga et al., 2012; Dondorp et al., 2010; Vriese and Christophe, 2003), lipid composition provided in the diet (Vriese and Christophe, 2003) is the hidden part of the underlying factors in male fertility that needs to be considerate more.

1.4.1. Influence of dietary lipid

Testicular lipids are metabolically active and come from both dietary intake and *de novo* synthesis in testis (Coniglio, 1994). Furthermore, their levels in the testis and spermatozoa can be modified according to the dietary lipid compositions (Bieri et al., 1969; Brinsko et al., 2005; Yan et al., 2013; Zaniboni and Cerolini, 2009). Dietary fat intake thus can affect the male reproductive function by modulating the fatty acid composition of testis and spermatozoa.

Based on a few observational studies, higher intake of SFA is associated with lower sperm count and concentrations, whereas higher intake of n-3 PUFA is related to higher normal sperm morphology with lowering the sperm head defect (Attaman et al., 2012) **(Table 1.3).**

Even in healthy young men, higher intake of fish as a part of a heart-healthy diet was positively associated with sperm motility (Gaskins et al., 2012). In sub-fertile and asthenozoospermic men, higher intake of n-3 PUFA, fish and seafood was positively associated with sperm motility, counts, and sperm morphology (Afeiche et al., 2014; Attaman et al., 2012; Eslamian et al., 2012; Vujkovic et al., 2009). It is also inversely and dose-dependently associated with the risk of asthenozoospermia in men (Eslamian et al., 2015). Dietary n-3 PUFA also increased testicular volume (MInguez-Alarcón et al., 2017), which contributes to normal testis function. In contrast, an inverse correlation has been reported between n-6 PUFA intake and testicular functions. A recent study involving 209 Spanish healthy men presented that there is an inverse association between high consumption of n-6 PUFA and testicular volume (MInguez-Alarcón et al., 2017). Considering testicular volume is decided by the seminiferous tubules, which contain spermatogenic cells, it raises the possibility for the adverse effect of n-6 PUFA on testicular function, specifically sperm production. This can be further supported by the findings from assessing a higher ratio of n-6 to n-3 PUFA in membrane lipid composition of spermatozoa in sub-fertile or infertile men (approximately n-6 to n-3 PUFA ratio of 1.0 versus 0.7 in normozoospermic men) as mentioned in the previous section (**Table 1.2**). If this finding is indeed true, this could be worsened by our common dietary patterns in Western countries, where intake of n-6 fatty acids is much higher (Blasbalg et al., 2011), which contributes to an n-6 to n-3 fatty acid ratio of 10-20:1 versus 1:1 in our ancestral diets (Molendi-Coste et al., 2011). Dietary consumption of canola and soybean oils has been significantly increased over the past few decades due to higher production outputs.

In terms of metabolism, high consumption of n-6 PUFA reduces the endogenous synthesis of n-3 PUFA including DHA, leading to the high production of n-6 PUFA, as explained in **Figure 1.8.** Two desaturases, Δ6-desaturase and Δ5-desaturase coded by the fatty acid desaturase 2 (FADS2) and fatty acid desaturase 1 (FADS1) genes, respectively, are required for the formation of the long-chain PUFA of both n-6 and n-3 fatty acid series.

Due to the same enzymes involved in desaturation of the LA and ALA, proper amount and the n-6 to n-3 fatty acid ratio in diets is pivotal as a high level of n-6 or n-3 fatty acid in one can inhibit the desaturation of the other. Although Health Canada's recommendation for the ratio of n-6 to n-3 PUFAs in the diet is 1-4:1 (Simopoulos, 2002), more investigations are required to find the optimum dietary ratio required for proper maintenance of male fertility functions.

Less is known about the role of dietary lipid during the developmental stage. Although EFA-deficient diet-induced histological damage in rat during development has been investigated (Bieri et al., 1969), the association between dietary lipids specifically DHA on testis maturation, function and sperm parameters is limited.

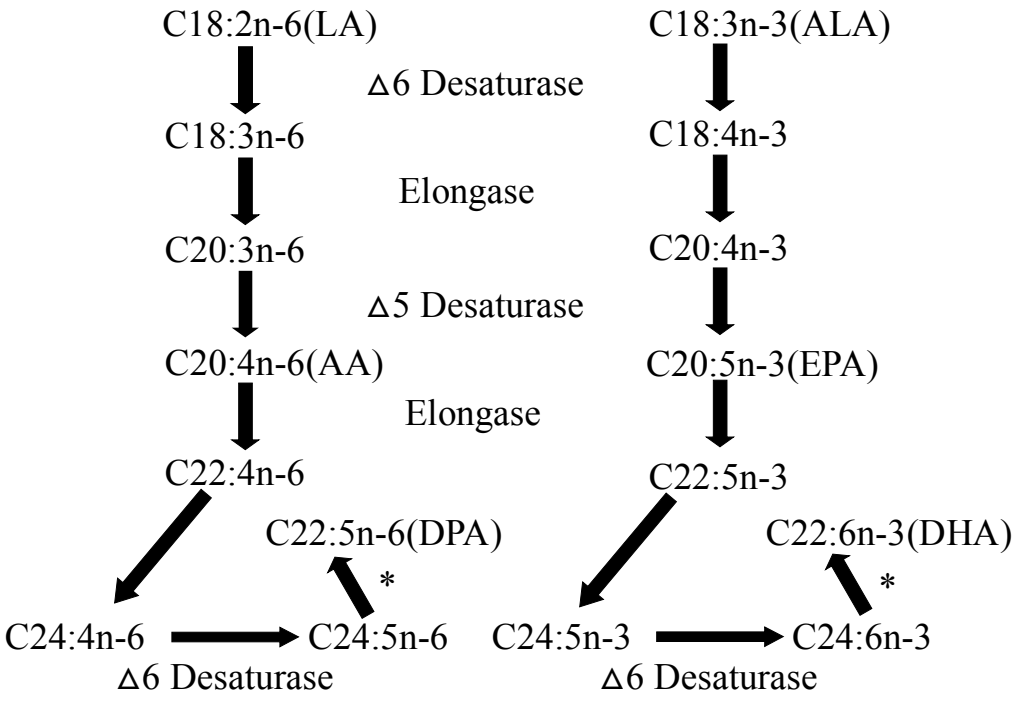

Figure 1.8. The n-3 and n-6 polyunsaturated fatty acid synthesis from the dietary essential fatty acids

Linoleic acid (LA) and α-linolenic acid from the diet are enzymatically desaturated and elongated to longer-chain, more unsaturated fatty acids via the same desaturases and elongases. EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid. * Presents one-step β-oxidation; Used with permission.

1.4.1.1. n-3 fatty acid supplementation in male fertility

1.4.1.1.1. Human studies

In healthy men. A limited number of interventional studies exist in assessing diets high in n-3 fatty acids for semen quality in both healthy men and men with infertility conditions (**Table 1.3**). For healthy men, 75 g of walnut a day (a source of ALA) for 12 weeks improved sperm morphology, motility, and vitality (Robbins et al., 2012). On the other hand, healthy men receiving 50 mL menhaden fish oil for 4 weeks did not find a significant difference in sperm quality (Knapp, 1990). Consistent to this finding, another study investigating the effect of 1.5 g DHA per day on sperm parameters, did not observe any difference after 10 weeks treatment. However, DHA supplementation decreased sperm DNA fragmentation (Martinez-Soto et al., 2010). Limited evidence is available to determine if dietary supplementation with n-3 PUFA, as fish oil or DHA, improves sperm parameters in males with fertility issues **(Table 1.4)**.

In infertile men. Evidence from a relatively large study of infertile males (n=238) with idiopathic oligoasthenoteratospermia, a condition including oligospermia, asthenozoospermia and teratozoospermia, showed that 1.84 g supplementation of fish oil (1.84 g: 0.72 g of EPA (20:5n-3 + 0.48 g of DHA) per day for 32 weeks improved sperm concentrations, motility and morphology, which suggests fish oil as a cheap and non-aggressive treatment strategy for these patients (Safarinejad, 2011). Another study performed with 27 infertile men receiving 1 g DHA in addition to 0.35 g n-6 fatty acids and vitamins for 24 weeks, showed that the sperm concentrations were increased with a concomitant increase in pregnancy in their female partner (Comhaire et al., 2000). However, not all studied agreed with these positive effects of n-3 PUFA consumption. Asthenozoospermic men supplemented with 0.4 or 0.8 g DHA per day for 12 weeks did not show any changes in their sperm parameters (Conquer et al., 2000). Although the level of DHA in serum and seminal plasma increased dose-dependently, dietary DHA was unable to incorporate into spermatozoa PL in asthenozoospermic men. It raises a question of

whether the quality of sperm can be improved if the intervention period is longer as other studies in the above.

1.4.1.1.2. Animal studies

In healthy animal. Animal experiments also support the beneficial effect of n-3 PUFA supplementation on the male reproductive system via increasing the level of n-3 PUFA in the testis lipid composition. In studies on boars fed supplemented diet with hydrogenated animal fat (AF) and fish oils, including menhaden oil (MO) and tuna oil (TO) containing 18 and 33% (w/w) DHA plus vitamin E for 28 weeks, showed reduced level of testosterone only in those fed by diet containing higher amount of DHA (TO) compared to MO and AF. Testis lipid composition underwent some alterations with PE level that replaced SM in a dose-dependent manner. It also has been demonstrated that the level of n-3 fatty acids replaced n-6 fatty acids level in both testes (in all phospholipid classes) and sperm (total lipid), reducing significantly the ratio of n-6/n-3 fatty acid in both, with a ratio of 0.22 in sperm, in those fed by fish oil compared to AF (Castellano et al., 2011). These changes in lipid composition of the sperm plasma membrane did not result in any difference in sperm parameters (Castellano et al., 2010). Moreover, the results obtained from TO-consuming boars receiving 8.5% (w/w) DHA from the diet for a shorter feeding period of 6 weeks revealed the same replacement of n-6 by n-3 PUFA in the sperm lipid composition, with decreased n-6/n-3 fatty acid ratio from 4.5 to 1.6. These alterations in sperm lipid composition were coincident with an increased proportion of sperm with normal morphology and higher motility (Rooke et al*.,* 2001). Thus this might indicate that the dietary DHA influences the male reproductive functions in a dose and time-dependent manner. In a study conducted by Sebokova et al., (1990), it has been shown that dietary supplementation with 20% fish oil in Sprague-Dawley rats replaced the level of n-6 DPA by DHA in testis plasma membrane leading to a marked increase in both basal and LH-stimulated testosterone synthesis.

These alterations in the testosterone synthesis have been attributed to n-3 PUFA-induced modifications on the LH-receptors binding capacity in the plasma membrane of testis tissue.

In infertile animal. In addition to healthy condition, n-3 PUFA benefits the male reproductive system in those with reproductive impairment. Investigations in male obese Zucker rats with underdeveloped testis containing a lower level of n-6 DPA than those of their lean counterparts were fed supplemented diet by 0.5 % (w/w) n-3 PUFA for 8 weeks. The results indicated that obese rat responded more to dietary intervention, with 2-fold more DHA incorporation in their testis than those in the lean group (Suh et al., 2011). Administration of n-3 PUFA for 4 weeks mitigate the impairment of reproductive function in rats suffering from the non-alcoholic fatty liver disease (NAFLA) induced by a high-fat diet (45% Kcal fat). In this study, n-3 PUFA increased sperm motility and testosterone level in comparison with those received no n-3 PUFA (Li et al., 2013). Data from Δ6 desaturase knockout mice that were not capable to synthesize PUFA, in particular, AA, n-6 DPA and DHA, were suffering from infertility due to defective spermatogenesis. However, spermatogenesis were completely restored when diet was supplemented with DHA (0.2% w/w) after weaning. DHA administration also increased sperm count in these animals compared to those fed by AA and n-6 DPA enriched diets. Interestingly, the n-6 DPA level was remained diminished indicating that a higher level of DHA in testis lipid composition functionally repaired the disruption of the male reproductive system in this population (Roqueta-Rivera et al., 2010). These studies showed that testis is well-responded to dietary lipids and n-3 PUFA intervention can improve reproductive function and compensate the lack of some special fatty acids in this organ in species with either DHA or n-6 DPA as the predominant testicular fatty acids.

1.4.1.1.3. During development

Limited information is available in regards to dietary supplementation of DHA during development and its role on testosterone level, spermatogenesis, and testis structure and sperm

parameters. The study conducted by Ayala et al., (1977) explored the alterations in testis lipid composition and spermatogenesis during development at PD49 and PD63 in rat offspring by feeding on fish oil containing 17% DHA of total fatty acids (w/w) from the time of weaning (PD21). It was observed that compared to sunflower oil, dietary fish oil increased the testis level of DHA at both ages replacing n-6 DPA level of the same fraction of testis lipid, including PC, PE, and TAG. Interestingly, fish oil-consuming rats achieved earlier maturation of germinal cells when compared to those fed by n-6 PUFA enriched diet. It has been concluded that higher amount of n-6 DPA in rat testis might be resulted from providing higher n-6 PUFA in the laboratory diet and thus alterations in the dietary pattern of PUFA can result in similar changes in testicular lipid composition in a way that both DHA and n-6 DPA coexist. Indeed, DHA can functionally replace n-6 DPA in germinal tissue in maturing rats (Ayala and Brenner, 1980; Ayala et al., 1977). Therefore, in agreement with those studies performed in adulthood, dietary DHA supplementation during development improved male reproductive functions. However, less is known about the effect of dietary DHA during development in relation to testosterone level and sperm parameters. Moreover, how the early provision of dietary DHA from the fetal period might influence testicular development and function has not been investigated yet.

Table 1.3. Observational studies showing the association between dietary n-3 PUFA and semen quality

Reference	Population	Semen quality
Vujkovic et al., (2010)	Sub-fertile men (n=161)	Intake of fish and other seafood were associated with higher sperm motility
Gaskins et al., (2012)	Healthy men (n=188)	Higher intake of fish as a part of a dietary pattern with high intake of chicken, fruit, vegetables, legumes and whole grains was positively associated with sperm motility
Attaman et al., (2012)	Sub-fertile men (n=99)	Higher intake of n-3 PUFA was associated with improved sperm morphology
Eslamian et al., (2012)	Asthenozoospermic men (n=72) vs. controls (n=169)	Higher intake of sea food was associated with lower risk of asthenozoospermia
Afeiche et al., (2014)	Sub-fertile men (n=155)	Intake of fish is positively associated with sperm count and sperm morphology
Eslamian et al., (2015)	Asthenozoospermic men (n=107) vs. controls (n=235)	Intake of n-3 PUFA and DHA were inversely and dose-dependently associated with the risk of asthenozoospermia

DHA, docosahexaenoic acid; n-3 PU FA, omega-3 polyunsaturated fatty acids; Used with permission.

Table 1.4. Intervention studies showing the effect of n-3 PUFA supplementation on healthy and infertile population

Reference	n-3 PUFA treatment	Study length	Population	Semen quality
Robbins et al., (2012)	75g/d Walnut vs. usual diet without consumption of tree nuts (control)	12 wks	Healthy men; walnut groups (n=59) vs. control (n=58)	Increased sperm motility, normal morphology and vitality in those receiving walnut than the control group consuming their normal diet
Knapp (1990)	50 mL/d menhaden oil	4 wks	Healthy men (n=10)	There was no significant effect with or without menhaden fish oil consumption on sperm motility and count
Conquer et al., (2000)	DHA (0.4g/d and 0.8 g/d) and no DHA (control)	12 wks	Asthenozoospermic men (n=28) in three groups	No significant effect on sperm parameters between 2 DHA interventions and control
Comhaire et al., (2000)	1g DHA, 0.25 g LA and 0.10g AA	24 wks	Infertile men (n=27)	Increased sperm concentrations, with improved sperm membrane fluidity and acrosome reaction
Safarinejad (2011)	Fish oil: [1.12 g/d of EPA and 0.72 g/d of DHA] vs. corn oil (control)	32 wks	Oligoasthenoteratospermia Fish oil group (n=119) vs. controls (n=119)	Increased sperm concentrations, motility and morphology in fish oil treatment group than controls

DHA, docosahexaenoic acid; ALA, α-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; LA, linoleic acid, wks, weeks; Used with permission.

1.4.2. Influence of alcohol consumption

Like a diet, alcohol consumption is a modifiable risk factor, which could result in medical conditions in male fertility. The association of chronic alcohol consumption with male reproductive disorders in both men and laboratory animals is well-investigated (Anderson et al., 1983; Gordon et al., 1976, 1979; Haider et al., 1985). However, the information related to the alcohol-induced lipid changes in testis and spermatozoa, influencing male reproductive function is limited.

In both human and animal, chronic alcohol consumption can affect testicular function manifested by testicular atrophy and negatively altering sperm parameters, such as reducing sperm motility, total sperm concentrations, sperm viability, and the number of sperm with normal morphology exhibited mainly as tail defects (Anderson et al., 2010; Donnelly et al., 1999; Marinelli et al., 2004; Muthusami and Chinnaswamy, 2005). These adverse effects could be related to alcohol-induced changes in testosterone production. Alcohol consumption reduces testosterone synthesis leading to decrease in the testicular and serum level of testosterone in both acute and chronic alcohol users (Adler, 1992; Bannister et al., 1987; Mendelson et al., 1978; Välimäki et al., 1984). Many mechanisms exist on alcohol-induced testosterone reduction: there might be a considerable conversion of testosterone to estradiol (Gomathi et al., 1993) due to the increased activity of aromatase enzyme induced by alcohol consumption (Purohit, 2000); it may be related to the direct toxic effects of alcohol on Leydig cells (Anderson et al., 1980; Mello et al., 1985) to inhibit the enzymes involved in testosterone synthesis (Gordon et al., 1980); alcohol and its metabolite, acetaldehyde, inhibit the binding of LH to their receptors located on the Leydig cells and therefore, suppress the activity of the required enzymes for testosterone formation (Adler, 1992; Bannister et al., 1987); In addition, the metabolism of testosterone in the liver is appeared to be influenced by alcohol leading to an abnormal level of testosterone in alcoholics (Dees and Kozlowski, 1984; Gordon et al., 1976); The central regulation of testis function also is reported to be blocked, with a reduction the GnRH and LH resulted from alcohol

impacts on the hypothalamic-pituitary-gonadal axis (Anderson et al., 2010; Muthusami and Chinnaswamy, 2005). It is of interest to know whether these mechanisms also exist in prenatally exposed to alcohol affecting later in testis function.

Alcohol is a potential lipid modulator and as described in earlier, testis and sperm lipid composition plays a critical role in male fertility. Little studies perused the effect of alcohol intake on lipid composition in sperm or testis, especially during development. Males with chronic alcohol consumption experienced a significant decrease in sperm total PL, mainly in SM, PC and PE with a marked elevation in the sperm cholesterol contents. These alterations in the sperm lipid composition were associated with abnormal semen quality, including lower sperm motility and concentrations (Gomathi et al., 1993). Moreover, experimental models investigating the alterations in testicular lipid composition in relation to chronic alcohol consumption showed that PUFA were replaced by SFA accompanied with higher malonaldehyde formation and lower level of glutathione in testicular mitochondria, suggesting higher lipid peroxidation in testis of alcohol-fed rats (Rosenblum et al., 1985). These findings indicate the direct effects of alcohol on testis lipid metabolism. Although the exact mechanisms are not known, the above results provide evidence to support the adverse effect of alcohol consumption on the male reproductive system. Further studies are required to pursue the effect of alcohol on particular testis fatty acids, including DHA, n-6 DPA, and VLCFA.

1.4.2.1. Prenatal alcohol exposure

The abnormal testicular environment in fetal life leads to impairment in testis development and function (Sharpe, 2001). Similar to adulthood, prenatal alcohol exposure influences adversely male reproductive system. These testicular disruptions were demonstrated in both Leydig cells and Sertoli cells manifested in their main functions, spermatogenesis and steroidogenesis, respectively. Fetal alcohol exposure impacts the development and activity of fetal reproductive organ and its central regulations through readily crossing the placenta (Sharpe,

2010), breeding to an irreversible process (Håkonsen et al., 2014; Ramlau-Hansen et al., 2010). Although available during the fetal period, alcohol influences might exhibit in later life. The toxicity of prenatal alcohol exposure affects cell replication, growth, differentiation, and immigration of developmental cells (Michaelis, 1990). These alterations might lead to impairments in pre and postnatal intra-testicular events required for normal spermatogenesis and steroidogenesis. This section details the impact of fetal alcohol exposure on the male reproductive system, fetal DHA status as well as the evidence related to n-3 supplementation in fetal alcohol-exposed offspring.

1.4.2.2. Prenatal alcohol exposure on male reproductive organ

Coincide with the developmental retardation in fetal alcohol-exposed offspring, reduction in their testicular weight has been reported at both early and late developmental stages. In the study conducted by Fakoya and Caxton-Martins's et al., (2004) the exposure to prenatal alcohol-induced 66% reduction in the testis weight accompanied by persistent growth retardation in adult rats (Fakoya and Caxton-Martins, 2004). In addition to testis, the weight of accessory glands, including prostate and seminal vesicles, have been also reported to be affected by prenatal alcohol exposure (Parker et al., 1984). Anogenital distance as a marker of sexual differentiation at birth can be influenced in this population, with a considerable reduction (Chen and Smith, 1979; McGivern et al., 1988, 1992; Parker et al., 1984; Rudeen, 1986; Udani et al., 1985).

1.4.2.2.1. Spermatogenesis

Alcohol exposure during the gestational period can suppress or delay the spermatogenic process during development. Findings from experimental models showed the inhibition of spermatogenesis that had been arrested at spermatogenic phase with no spermatozoa in rats whose mother exposed to alcohol via gastric intubation (Fakoya and Caxton-Martins, 2004). It has been also shown that prenatal alcohol exhibited retardation in the onset of spermatogenesis

aligned with a delay in the reproductive development in offspring. Histological analysis demonstrated a delay in the gonocytes cell division and movement from center to the edge of seminiferous cords short after birth, a decreased percentage of open lumen, with the tubules containing lower number of spermatocytes at PD18 and round spermatids at PD 25 leading to a lower percentage of released spermatozoa into the lumen at PD55 in alcohol-exposed offspring during gestational period (Lan et al., 2013). These studies support that the spermatogenic process might be affected by prenatal alcohol exposure, although the underlying mechanism is not known yet.

1.4.2.2.2. Steroidogenesis

As a main regulator of testicular function, hormonal balance in the male reproductive system is pivotal and therefore, any changes might cause physiological and functional dysregulations in testis. Alcohol-related impairments in both testes cellular morphology and function that are involved in testosterone production as a main male hormone can be associated with long-term abnormalities in the male reproductive system, including pubertal development and sexual dimorphism behavior.

Studies conducted on rodent models have shown that the surges of testosterone are blocked at both fetal and neonatal level in those subjected to fetal alcohol (McGivern et al., 1988, 1993, 1998). Interestingly, the inhibitory effect of fetal alcohol exposure on the surge of testosterone was observed even if mothers were exposed to alcohol only before pregnancy (McGivern 1998). The fetal surge of testosterone in the final week of pregnancy through the first week after birth in the rat is critical for normal brain sexual differentiation that occurred at the same time (Ward and Weisz, 1984). Prenatal alcohol-exposed offspring at birth exhibited lower testosterone values in their plasma and brain than those of controls (Kakihana et al., 1980). Neuroanatomical data from brain morphology in adult prenatal alcohol-subjected rats (PD70-80) indicated a clear reduction in the size of SDN in POA in the hypothalamus that is normally larger

in males compared to females (Barron et al., 1988; Rudeen, 1986). This might be related to the incomplete brain masculinization in early life, leading to impairment in male sexual behaviour that are attributed to fetal alcohol exposure in many studies (Blanchard and Hannigan, 1994; Hård et al., 1984; McGivern et al., 1988; Udani et al., 1985; Ward et al., 1996).

In the study reported by Ward et al., (2003) maternal alcohol exposure combined with stress but not alcohol alone, considerably delayed the surge of testosterone by one day, indicating that the adverse effect of fetal alcohol exposure might be related to the other potential factors. Although the level of LH and testosterone lowered at peri-puberty (PD55) in offspring exposed to fetal and early postnatal alcohol, the normal level of testosterone in adult rats suggest a delay in reaching the adult values of testosterone in this animal (Udani et al., 1985). Additionally, in the study in which only the effect of fetal but not early postnatal alcohol exposure was pursued, the level of serum testosterone was higher at PD55 in offspring compared to those in control group (Jungkuntz-Burgett et al., 1990). Similarly, the level of testosterone in salivary samples of 14-years-old boys whose mothers consumed alcohol during pregnancy were considerably elevated (Carter et al., 1990). The higher level of circulating testosterone can be interpreted as a delay in the pubertal surge of testosterone that normally begins at PD51 in rats (Korenbrot et al., 1977) or 12-14 years old in human (Claudio et al., 1999) leading to a developmental delay in the onset of puberty.

The data from epidemiological studies showed boys prenatally exposed to binge drinking are prone to a delayed pubertal development (Håkonsen et al., 2014). Although animal experiments failed to confirm retardation in the onset of puberty in male offspring rats exposed to prenatal alcohol, their female counterparts exhibited delayed puberty due to fetal alcohol exposure (McGivern et al., 1992). Another possibility for a higher level of testosterone might be related to its central dysregulations resulted from alcohol-induced alterations in LH pattern. Adult rats subjected to alcohol in utero had a blunted response of hypothalamus to testosterone (Jungkuntz-Burgett et al., 1990). This decline in the response of the hypothalamus to the

negative feedback of the testosterone suggests that a higher level of testosterone may be required to stimulate the neuropeptide of LH releasing hormone.

Alcohol exposure in uterine appears to disrupt testosterone production at the organ level as well. Deficits in the testicular steroidogenic enzyme activity (Kelce et al., 1989) as well as the reduced number of Leydig cells with a lower sensitivity of LH-receptors, suggests gonadal impairments in testosterone production in offspring obtained from alcohol-exposed dams at early life. Therefore, in the case that gonads are not able to produce a higher level of testosterone as a result of alcohol-induced testicular damage during the fetal period, exogenous testosterone might correct the consequent disruption as shown previously by Ward et al., (1996). Indeed, the impairment in male sexual behavior was repaired by administration of exogenous testosterone (Ward et al., 1996). These reports support that prenatal alcohol can lead to alterations in Leydig cells maturation and therefore the testosterone production and regulation.

1.4.2.2.3. Sperm parameters

The number of spermatogonia enters the meiosis during spermatogenesis determines the sperm density measured in semen analysis as a marker for male fertility. However, only limited investigations showed lower sperm concentrations in the adults exposed to alcohol prenatally. The results from a human cohort study on 347 adult male in Denmark showed 32% reduction in sperm concentrations in those whose mother consumed more than 4 drinks per week during pregnancy (Ramlau-Hansen et al., 2010).

Taken together, the above studies indicate that alcohol exposure during pregnancy can affect the male reproductive system leading to testis dysfunction, dysregulation or both and low sperm quantity. However, not all studies agree on these findings. Some animal models reported no adverse effect of prenatal alcohol on anogenital distance (McGivern et al., 1988, 1992) and testis weight at birth. Animal experiments failed to see the alterations in sperm count in the rat model (McGivern et al., 1992) and sperm motility and sperm morphology in men exposed to

prenatal alcohol (Ramlau-Hansen et al., 2010). Testosterone level did not decrease either in fetal (Ward et al., 2003) or adult rats (Ramlau-Hansen et al., 2010; Udani et al., 1985; Ward et al., 1996). Human studies did not support each other regarding the association between cryptorchidism and prenatal alcohol exposure (Damgaard et al., 2007). This variability might be attributed to numerous factors, including the pattern of alcohol consumption, the dose of and duration of alcohol, developmental timing, and nutritional factors. Although it is hard, more longitudinal studies could answer these controversies.

1.4.2.2.4. Alcohol effect on DHA status of plasma and tissue

Alcohol is a potent lipid modulator in many organs with significant alterations in their fatty acid profiles. It has been shown that chronic alcohol exposure diminished level of PUFAs, including AA, DHA, or both in the liver (Salem et al., 1996), brain (Harris et al., 1984; Pawlosky and Salem, 1995) and blood (Salem and Olsson, 1997) in the animal experiments. In a similar trend, lower DHA level was observed in the fetal brain and liver of pigs born to alcohol-consumed dams with no significant changes in their plasma DHA level (Burdge et al., 1995). The data have gained support from the study conducted by Denkins et al., (2000) on rats with remarkably a higher level of DHA in serum and liver but lower in the brain in the alcohol-exposed fetus at GD20. These findings probably reflect a limited capacity of organs to uptake and store this pivotal substrate from blood due to alcohol-induced alterations in the membrane fluidity. Investigation on alcohol-consumed mothers ($\geq$ 23.6 g/d) also showed 30% increase in DHA level in the cord blood in comparison to those values of controls (who consumed <1.6 g/d) (Denkins et al., 2000). Study on the umbilical cord vessels of mothers consume alcohol during pregnancy (<60 g/d; moderate drinkers and >60 g/d; heavy drinkers) also revealed that DHA and AA level in arterial umbilical vessels increased in moderate and heavy drinkers, respectively, than that of abstainers. This effect of maternal alcohol on fetal DHA status was independent of the maternal plasma fatty acid (Beblo et al., 2005). The DHA level of erythrocytes also increased

considerably in neonates exposed to alcohol and smoking during pregnancy compared to those whose mother abstained from alcohol and smoking (Smuts et al., 1999). All these studies show that DHA deficiency in tissues may be a possible mechanism for the adverse effect of alcohol on developmental fetus specifically regarding neurodevelopment.

Erythrocytes have important roles in the transport of DHA from mother into the fetus (Ruyle et al., 1990). During the third trimester in pregnancy, almost 50 to 60 mg per day DHA is transferred to the fetus via placenta (Clandinin et al., 1980a, 1980b). The mechanism by which prenatal alcohol induces the DHA deficiency might be related to increased DHA requirement in offspring due to a decline in DHA synthesis via inhibition of the desaturase and elongase enzymes (Nervi et al., 1980). Lipid peroxidation also may contribute to the DHA reduction in prenatal alcohol-exposed animal (Pawlosky et al., 2001). Suboptimal nutrition that is common among alcoholic pregnant mothers and alcohol-induced malabsorption (Lieber 2003; Young et al., 2003) and interference with placental transfer of nutrients (Syme et al., 2004) might impact DHA status of offspring whose mother consumed alcohol during pregnancy. If there is any disruption in DHA production, peroxidation and transportation exist due to alcohol effect, probably can be compensated by a high level of DHA.

DHA reduction in some tissues like brain and liver of prenatal alcohol exposed-offspring suggests that DHA deficiency might be a partial reason for the detrimental effects of prenatal alcohol on offspring development. There are limited studies that have to lighten more information on this hypothesis. DHA-enriched tuna oil in prenatal alcohol-exposed pigs exhibited increased DHA level in the brain in developing fetus leading to partially improvement in the neurodevelopmental dysfunction (Burdge et al., 1997). It also has been reported that n-3 supplementation can modify the adverse effect of prenatal alcohol on lipid composition in the mouse fetal brain (Wainwright et al., 1989). Considering the important role of DHA in both brain and testis, DHA supplementation may improve the malfunction in the reproductive system caused by prenatal alcohol, which needs to be tested.

1.5. Docosahexaenoic acid dietary source and recommendations

Unlike brain and retina enriched in DHA, testis is continuously drained of DHA due to the releasing of spermatozoa to the epididymis (Sæther et al., 2007) and testis, therefore, requires a constant supply of DHA. In order to have adequate dietary DHA and its precursors, knowledge regarding their dietary sources is required. Dietary n-3 fatty acids can be found in three main forms in the diet: ALA, EPA and DHA. In addition to the plant-source of DHA precursor, a food-based approach for achieving EPA and DHA is recommended. Seafood, particularly marine fish and shellfish, are the main sources of DHA. The American Dietetic Association/Dietitians of Canada recommended two servings of 8 oz of fatty fish (herring, salmon, sardine; accounting > 0.5 g DHA/ 100 g) per week for health promotion and reduced risk of various chronic diseases (Kris-Etherton et al., 2007). DHA also can be found in the liver of some special lean fish (cod) (Kris-Etherton et al., 2000). Halibut and Pollock are known as moderate sources of DHA corresponding to 0.3-0.5 g of DHA/100 g.

Nutritional supplements or fortified food with fish oil or DHA, such as eggs containing 0.15 g and algae supplements, are also needed to meet current recommendations for some individuals with limited or lack of fish intake in their diet. Although vegetable oils are not a good source of EPA and DHA, some are rich sources of ALA, such as flax oil.

The general male population has been encouraged to consume n-3 fatty acids-rich foods to benefit their reproductive tract health. Institute of Medicine of the National Academies issued a Dietary References Intake (DRI) for ALA to be 1.6 g d^{-1} providing 0.6-1.2% of energy, for men 19 to >70 years (Otten et al., 2006). DHA is then synthesized endogenously from ALA in the body, but its dietary intake is also recommended due to the low efficiency in DHA production (<0.1%) (Lin et al., 2010; Plourde and Cunnane, 2007). There is no DRI for DHA or EPA, but some recommendations have been issued for different population groups based on evidence demonstrating the various health benefits of these n-3 PUFA. The American Dietetic Association/Dietitians of Canada recommended 500 mg day^{-1} of EPA+DHA (Kris-Etherton et

al., 2007). Although this amount is required for normal growth and neural development, as well as preventing nutrient deficiencies, it is questionable if it is enough for males with reproductive health issues. Regardless, to achieve the recommendation for DHA intake, it is required to increase the dietary intake of DHA in a preformed state in addition to its plant-derived precursors.

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CHAPTER 2. RESEARCH PLAN

Rationale

The testis is a central organ responsible for male fertility. Abnormal testis function results in decreased sperm parameters quality, low sperm count, or both, which are associated with the majority of male infertility cases. These functions are well-associated with lipid composition in testis. The testis and sperm are structurally and biochemically enriched with polyunsaturated fatty acids (PUFA) of 22 carbon chain in length relative to other tissues, DHA in human and n-6 DPA in the rodent. The huge increase in the level of these fatty acids occurs during testis development that is coincident with the sperm germ cells maturation (Davis et al., 1966), indicating their particular roles in testis maturation and spermatogenesis. The significant decrease in these fatty acids have been noticed in the degenerated (Nakamura et al., 1968; Davis and Coniglio, 1967) and underdeveloped testis in the rat (Suh et al., 2011; Zanetti et al., 2007) and undescended testis in the human (Coniglio et al., 1975). In the rodent model, n-6 DPA is also considerably increased during passing through the epididymis (Hall et al., 1991), indicating their involvement in normal maturation of spermatozoa. Some evidence showed that sperm from infertile men had a major reduction in the level of DHA (Aksoy et al., 2006; Gulaya et al., 2001; Zalata et al., 1998). Thus, maintenance of an optimal level of PUFAs of 22 carbons might be crucial for germ cell maturation, normal development, and function of the testis and the quality and quantity of spermatozoa.

Although species-differences are obvious as the predominant fatty acid in the animal and human testis, dietary supplementation of DHA appears to benefit both humans (Conquer et al 2000, Safarinejad 2011) and rodents (Li et al., 2013; Sebokova et al., 1990). Observational studies presented that the consumption of n-3 PUFA is positively correlated with male reproductive functions in both healthy males and those with fertility problems. Dietary n-3 PUFA alter testis fatty acid composition (Safarinejad et al., 2011) influencing the steroid pathways (Gromadzka-Ostrowska et al 2002, Hurtado de Catalfo et al 2008, Nagata et al 2000,

Sebokova et al 1990) as well as spermatogenesis and sperm parameters (Estienne et al., 2008; Mitre et al., 2004; Rooke et al., 2001). As shown, the effects of dietary DHA on male fertility in adults has been well-investigated, but studies are limited in examining the effect of provision of DHA in very early life stage on male reproductive functions.

VLCFA are also unique molecules, which are found only in the brain, retina, testis, and spermatozoa. They are required to be synthesized from their PUFA precursors and cannot be obtained from diet. While the functional roles of VLCFA are not well-defined, testis VLCFA with up to C34 carbons increases in the commencement of the spermatogenesis to reach to the maximum during puberty (Furland et al., 2007). This implies that VLCFA may be involved in testicular germ cell maturation (Kihara, 2012; Robinson et al., 1992), together with C22 PUFA. However, its occurrence in testis from early life stage to adults with and without dietary DHA challenge has not been explored.

Prenatal alcohol exposure impacts on male reproductive system resulting in life-long disruptions in male reproductive functions. Cohort studies in human revealed that in adult men who were prenatally exposed to alcohol showed to have low sperm concentrations (Ramlau-Hansen et al., 2010), hormonal imbalance and a tendency towards a delayed pubertal development. Studies utilizing rodent models with prenatal alcohol exposure, showed impairment in testis development, delayed (Lan et al., 2013) or inhibited spermatogenesis, decreased testis size (Fakoya and Caxton-Martins, 2004) and shortened anogenital distance (McGivern et al., 1988; Udani et al., 1985; Ward et al., 1999), blocked testosterone surge (Kelce et al., 1989, 1990; Udani et al., 1985; Ward et al., 1999) and abnormal brain sexual differentiation (Barron et al., 1988; Rudeen et al., 1986). While the impacts of early alcohol exposure are clear, these findings may be related to alcohol-induced altered lipid metabolism. Alcohol is known to be a potent lipid modulator and has shown to reduce the level of PUFAs, including AA, DHA, or both in many organs, including liver, brain, and retina (Harris et al.,

1984; Pawlosky and Salem, 1995; Salem and Olsson, 1997). Thus, it is reasonable to accept that DHA might be decreased in the testis as well with prenatal alcohol exposure.

Overall, considering the fact that fetal alcohol exposure-induced reproductive dysfunctions might be associated with altered DHA metabolism, it is worth exploring if DHA supplementation can mitigate the negative effect of alcohol on the testis function in relation to the testis lipid. A long-term follow-up on the effects of prenatal alcohol on the development and function of testis, in relation to testis developmental lipid profiles in each life stages, has not been thoroughly investigated.

Objectives

The overall objective of this study was to determine if DHA provision in diet affects testis development and function in relation to lipid composition in rats exposed to prenatal ethanol (EtOH). Focus was given to diet-induced changes in lipids (PUFA and VLCFA) in relation to testis development and function.

Specific objectives

I. To measure the effect of the dietary DHA on overall offspring development in fetus (GD20), neonates (PD4), weaning (PD21), peri-puberty (PD49), and adult (PD90) rats exposed to prenatal EtOH:

To determine the fetal DHA status in serum in relation to offspring development

To measure placental weight in fetus

To measure fetal and postnatal body weight

To measure fetal and postnatal testis weight

II. To measure the effect of DHA on testis developmental markers in relation to testis structure in rats exposed to prenatal EtOH:

To examine the number and diameter of seminiferous cord at GD20

To measure the number of seminiferous tubules with a full spermatogenic cell series (spermatozoa) at PD49

To measure seminiferous diameter and height of germinal epithelium at PD49 and PD90

III. To test the effect of dietary DHA on testosterone during development from GD20 to PD90 in rats exposed to prenatal EtOH:

To measure serum testosterone levels

To determine mRNA expression of gene involved in testosterone production at PD49 and PD90

IV. To test the effect of dietary DHA on sperm parameters in adult rats exposed to prenatal EtOH:

To determine sperm motility

To determine sperm count

To determine the number of sperm with normal morphology

V. To examine the effect of dietary DHA on testis total fatty acid content, in particular PUFA and VLCFA, during development from GD20 to PD90 in rats exposed to prenatal EtOH:

To examine the effect of dietary DHA on gene expression of enzymes involved in lipid metabolism at PD49 and PD90

To determine the developmental profiles of testis fatty acid

VI. To characterize fatty acid profiles in testis lipid classes (phospholipids, triacylglycerides and free fatty acids) from PD21 to PD90

To examine the effect of DHA on fatty acids distribution in major lipid classes in testis

To measure the developmental profiles of fatty acids in testis lipid classes

Hypotheses

The hypothesis of this study was that dietary DHA will induce changes in testis lipid

profiles and affect testis development and function from GD20 to PD90 in rats exposed to prenatal EtOH. More specifically, it was hypothesized that

I. Dietary DHA will improve the overall offspring development in relation to the fetal DHA status in those exposed to prenatal EtOH

II. Dietary DHA will improve testis developmental markers in offspring exposed to prenatal EtOH

III. Dietary DHA will increase the level of testosterone during development in offspring exposed to prenatal EtOH

IV. Dietary DHA will increase sperm parameters in adult rats exposed to prenatal EtOH

V. Testis total fatty acid level will be altered by dietary DHA as testis develops in offspring exposed to prenatal EtOH

VI. Fatty acid level of major lipid classes of testis will be altered by dietary DHA by increasing DHA level during development from PD21 to PD90

Chapter composition:

The following chapters will cover testing these hypotheses as listed below:

1- Hypothesis I will be covered in Chapter 3
2- Hypothesis II, III and IV will be covered in Chapter 4
3- Hypothesis V will be covered in Chapter 5
4- Hypothesis VI will be covered in Chapter 6

CHAPTER 3. IMPACTS OF DIETARY DOCOSAHEXAENOIC ACID PROVISION IN AN ENERGY-DENSE DIET ON THE OFFSPRING DEVELOPMENT CHRONICALLY EXPOSED TO MODERATE PRENATAL ETHANOL

Will be submitted to "Applied physiology, Nutrition and metabolism" (in preparation)

Abstract

Background and Objective: Prenatal ethanol (EtOH) exposure is known to decrease docosahexaenoic acid (DHA) in major organs, which may be associated with the developmental abnormalities in offspring. This study tested the effect of DHA provision in the diet on fetus and pups development at different ages after exposure to the moderate level of prenatal EtOH.

Methods: Female Sprague-Dawley rats were randomly assigned to receive EtOH (3g/kg twice a day) or dextrose for the entire pregnancy. Upon being pregnant, dams were fed an energy-dense diet supplemented with (Cont+DHA and EtOH+DHA) or without DHA (1.4%, w/w total fatty acids) (Cont and EtOH), with pups being continued their dam's diet after weaning. Samples were collected during development at different stages. Serum lipid concentrations were analyzed in dams and fetus at gestational day 20 (GD20).

Results: Maternal outcomes remained unchanged amongst the groups. EtOH only affected liver size, with a significant enlargement only in GD20 females. DHA serum level was increased in DHA-consuming dams and their offspring. Fetal body weight, brain and testis size were lower in EtOH+DHA than EtOH or Cont+DHA groups. A prompt catch-up growth occurred shortly after birth for the female but not in male neonates. In the same group and only in males, the placental relative weight was the highest among the groups.

Conclusion: Overall, dietary DHA in rats exposed to prenatal EtOH resulted in minimal developmental effect on offspring. An energy-dense semi-purified diet could have helped mitigate the effect of prenatal EtOH exposure that needs to be further analyzed. The sex-dependent differences also need to be more investigated.

Keywords: docosahexaenoic acid, prenatal ethanol exposure, pregnancy nutrition, fetal development, fetal development, fetal diet intake, energy-dense diet

Introduction

It has been estimated that 12-15% of women continue to consume ethanol (EtOH) during pregnancy and the rate of chronic EtOH use (defined as 7 drinks/week) or binge drinking (5 drinks per occasion) is about 3%, as reported recently (Li et al., 2012). Prevalence of prenatal EtOH-associated complications is varied but higher in South Africa, Italy, Canada, Croatia, New Zealand, and the United States. Global prevalence rates for fetal alcohol syndrome (FAS), partial FAS (pFAS), an alcohol-related neurodevelopmental disorder, and alcohol-related birth defects (ARBD) were 2.89, 11.22, 5.19, and 3.52 per 1000 birth, respectively (Roozen et al., 2016). EtOH is a teratogenic compound that can pass through the placenta, causing damage to the brain and other organs, resulting in developmental disabilities in the affected children. There is no safe amount of EtOH that can be consumed during pregnancy. Thus, a child prenatally exposed to EtOH may be born with cognitive and intellectual disabilities as well as physical impairments, termed fetal alcohol spectrum disorder (FASD) (Jones and Smith, 1973) with a prevalence of 22.77 per 1000 birth (Roozen et al., 2016).

The evidence for the effect of maternal EtOH on fetal and postnatal development have been shown by human epidemiologic and clinical experiments (Popova et al., 2018), as well as in animal experiments (Murawski et al., 2015). Due to the challenges in conducting human studies, most of the data generated on FASD-related physiological and biochemical changes during gestation, are through animal models that showed the similar features observed in human, such as retardation in pre- and postnatal growth development and abnormalities in neurodevelopment and sexual maturity (Abel, 1978; McGivern et al., 1995; Li et al., 2013). Rodent models are the most preferred due to their short gestational period, well-established and specific timeline of the development of each organ (Wilson and Cudd, 2011).

Nutrition is identified as a critical factor determining the extent of prenatal EtOH-induced damage. Moreover, pregnant women addicted to EtOH have poor nutritional status (May et al., 2016). Evidence exists that the minimal growth development is due to prenatal EtOH exposure in offspring born to mothers who consume an energy-dense diet during pregnancy in rats (Wiener et al., 1981; Goad et al., 1984). Therefore, the nutritional status of the mother could help to minimize the detrimental effects of EtOH on the developing fetus.

Omega-3 and omega-6 polyunsaturated fatty acids (n-3 and n-6 PUFA) are necessary to maintain cell-membrane fluidity, inhibit inflammatory processes, improve vascular functions and cardiac disorders, inhibit platelet aggregation, and decrease liver triacylglyceride synthesis (Lauritzen et al., 2016). The brain is a lipid-rich organ with a high level of PUFA, especially docosahexaenoic acid (DHA, C22:6n-3), that maintains the health of neurons (Wiktorowska-Owczarek et al., 2015). Fetal and postnatal DHA status plays a fundamental role in the normal growth and the development of brain functions, by affecting neurological function and modulating the membrane integrity, signal transduction involved in neurotransmission, neuro-plasticity, and inflammation in neurons. It has been reported that the average n-3 PUFA intake of Canadian pregnant women is 82 mg per day, with about 90% of women consume less than 300 mg (Denomme et al., 2005) that is the dietary recommendation of DHA for pregnant women (200-300 mg/day) (Koletzko et al., 2007). It might be worse in those consuming EtOH with suboptimal nutrition intake or EtOH-related impairments in digestion (Lieber 2003; Young et al. 2014). In addition, it has been shown that level of PUFA, including arachidonic acid, DHA, or both decreased by the EtOH exposure in the liver (Salem et al., 1996), brain (Harris et al., 1984; Pawlosky and Salem, 1995) and blood (Salem and Olsson, 1997) in the animal experiments. A lower accumulation of DHA was observed in the fetal brain and liver of pigs born to EtOH-

consuming dams with no significant changes in DHA plasma (Burdge and Postle, 1995) or an elevation in the serum DHA level due to EtOH consumption in human neonates (Denkins et al., 2000). This might indicate a reduction in the DHA transportation from blood to tissues in the fetus due to prenatal EtOH exposure. Provision of DHA as a part of diet may reverse the reduction of DHA status, thereby mitigating the adverse effects of prenatal EtOH on overall growth and development of major organs.

Therefore, this study was designed to determine if optimal nutrition along with a provision of DHA in the diet during pregnancy can mitigate the effects of EtOH on fetal and postnatal development to adult.

Material and Method

Experimental design

The experimental design of the study is shown schematically in **Figure 3.1.** This study was approved by the University of Manitoba Animal Care Committee and is in agreement with the Canadian Council on Animal Care (CCAC) and Use of Experimental Animals (CCAC, 1993).

Pre-conception adaptation and mating

Female Sprague-Dawley rats (n=37) at the age of 9-10 weeks old were purchased from Charles Rivers Breeding Laboratories (Sherbrooke, Quebec, Canada). After acclimatization for one week, animals (n=14) were randomly selected and slowly adapted to 50% (w/v) EtOH, by increasing the dose of EtOH from 1/3 to 3/3 in 3 days to reach the final expected dose (3g/kg BW via oral gavage, twice a day), representing a moderate dose of EtOH exposure. This level of EtOH is equivalent to the amount reached by women taking 1-2 drinks within an hour (Schambra et al., 2015). The remaining animals (n=23) received 80% (w/v) dextrose solution isocaloric to

EtOH. Dextrose was used as it does not have an adverse effect on oxidative stress (Bloomer et al., 2010) and thus the central nervous system. After 3 days of EtOH adaptation, two female rats were housed with one male in a cage for mating. Pregnancy was confirmed with visual inspection for vaginal plug and/or vaginal swabs. The day of confirmation of pregnancy was defined as a gestational day (GD) 0. For fetus analyses, dams were terminated on GD20 and fetal tissues were collected. For analyses of postnatal development, pups were terminated at postnatal day (PD) 4, 21, 49 and 90. During the adaptation and mating period, animals were fed with the control diet (no-DHA) as described below.

Diets

Upon confirmation of pregnancy, the half of EtOH-treated pregnant animals was randomly assigned to receive either the control or DHA (1.4%, w/w fatty acids) supplemented diet. Thus, the experimental groups were i) control without EtOH (Cont, n=8); ii) control with DHA (Cont+DHA, n=8) iii) EtOH without DHA (EtOH, n=8); iv) EtOH with DHA (EtOH+DHA, n=6); v) pair-fed (PF) with no EtOH but diet amount matched the EtOH group consumed (PF, n=7). Since EtOH-exposed group may have a reduced diet intake, thereby reducing the body weight, the inclusion of PF group is necessary for the interpretation of the outcome results, if the results are from the effect of EtOH or reduced intake. The control and EtOH groups were gavaged twice a day, 8 hours apart, with dextrose or EtOH during the entire gestational period.

The experimental diets and fatty acid compositions are shown in **Table 3.1**. These were formulated isocalorically in a semi-purified and nutritionally complete diet. The fatty acid compositions were measured in our laboratory **(Table 3.1).** The total energy density was 4.17 Kcal/g diet composed of carbohydrate (50%), protein (17.5%) and fat (32.5%), reflecting the nutrient intake of the pregnant mother in North America (Denomme et al., 2005; Friesen and Innis, 2010; Hui et al., 2014). The only difference between the two experimental diets was the

DHA level (0 vs. 1.4%, w/w fatty acids), which has been reported to be a sufficient amount of DHA for pregnant rats and growing pups (Wang et al., 2018; Weiser et al., 2015). Animals had access to diet and water, *ad libitum*. The amount of food consumed by each dam was recorded 3 days a week and dam's weights on a daily basis. Experimental diets were continued throughout the entire pregnancy and lactation period. After weaned at postnatal day (PD) 21, the pups were fed the same diet as their mothers until the end of the experimental period at PD90.

On GD20, half of the dams in each group were anesthetized via isoflurane and the blood was obtained via cardiac puncture. The uterine horn was removed and placed on ice. Fetuses were weighed and then decapitated with sharp scissors as soon as removed from the uterus. The remaining pregnant dams were allowed a parturition to follow up on offspring. At PD4, litter was randomly culled to 8 pups/dam with an equal male to female sex ratio to allow equal access to dam's milk. Pups were remained with their natural mothers until weaning. Offspring were terminated at different time points at GD20 (fetus), PD4 (neonate), PD21(weanling), PD49 (peri-puberty), and PD90 (adult). For lipid analysis, trunk blood samples were collected from the fetuses and by cardiac puncture in dams. Brain, liver, testis, and placenta were removed, weighed and flashed frozen in liquid nitrogen. They were stored at -80 °C for further analyses. Depending on the litter size and sex difference, the numbers of animal used per test were varied.

Fatty acid analysis

Total serum fatty acid profiles were analyzed using a one-step method (Kang and Wang, 2005) without lipid extraction. Serum (50 μl) from dam and fetus were directly methylated using 14% boron trifluoride in methanol (BF_3) (Suh et al., 2009). Separation of fatty acid methyl esters was carried out using a microcapillary column (15 m × 0.1 mm inner diameter and 0.2 m film thickness; BPX70; NC, USA), and a chromatography system (Vista 6010 GLC and Vista 402

data system; Varian Instruments, Mississauga, ON, Canada). Hydrogen was used as a carrier gas under a flow rate of 0.5 mL/min. The initial temperature was 130°C, then increased to 175°C at 20°C/min held for 1 min, then was increased to 200°C at 6°C/min, held for 0.5 min, raised again to 200°C at 6°C/min with no hold and finally increased to 280°C at 30°C/min. The injector/detector temperatures were 270°C and 290°C, respectively. Standard FAME 461 (Nucheck Prep Inc. Waterville, MN, USA) was used for peak identification.

Statistical analysis

Statistical analysis for all data were analyzed using two-way analysis of variance (ANOVA) for the main factors, EtOH and diet. Multiple comparisons were done using Duncan post-hoc test when an interaction exists. When there was no interaction effect identified, the ANOVA was re-tested for the main factors, which enabled us to test against the correct degree of freedom of error. Student's *t*-test was used to compare the data from EtOH and PF groups. Statistical significance was set at $P < 0.05$. All data are expressed as mean ± standard deviation (SD).

Results

Maternal weight gain and intake during pregnancy

The effect of DHA and EtOH exposure on maternal weight and dietary intake was measured. In this study, EtOH exposure to pregnant dams did not induce any preterm labor and did not affect the litter sizes, ranging from 14.0 to 15.5 in the fetus **Table 3.2.** The maternal body weight and weekly diet intake during pregnancy were not significantly different among the groups, although DHA-treated groups tended to have a higher food intake (**Figure 3.2**). There were not any statistically significant differences in the dam's body weight during pregnancy

among different groups (**Table 3.2**). There were no significant differences in dams or their offspring outcomes between PF and EtOH groups.

Placental weight

The placental absolute and relative weight in both males and females are shown in **Table 3.3.** The interaction between EtOH and diet was significant for both placental absolute ($P<0.0004$) and relative weight ($P<0.0001$). EtOH-exposed male fetus showed a significant increase in the placental size, only if their mothers were fed the DHA diet (EtOH+DHA group) compared to that of male offspring from dams consumed control diet (EtOH group) and among all other groups in placental absolute and relative weight, respectively. However, the female placenta was not affected by EtOH, dietary DHA or the interaction between them.

Body weight during development from GD20 to PD90

The body weights of animals during development from GD20 to PD90 for both males and females are presented in **Table 3.4**. The weekly diet intake of dams during lactation (data not shown) was not significantly different among the groups, including EtOH-exposed groups. There was a significant interaction between EtOH and diet ($P<0.0001$) in the body weight of male and female fetus at GD20. Offspring in Cont and EtOH+DHA groups weighed approximately 0.25 g less than those in Cont+DHA and EtOH groups. Although female neonates at PD4 in both groups achieved the compensatory growth shortly after birth, their male counterparts in EtOH+DHA group still weighed lower ($P<0.04$) than other groups. However, their body weight reached that of the other groups by PD21. Dietary DHA induced a significant ($P<0.02$) weight gain in adult (PD90) females, who were exposed to prenatal EtOH (EtOH+DHA) when compared to those animals fed control diet (Cont and EtOH groups). There was no significant main effect of EtOH on body weight at any of the time points. Newborns also were estimated as small for gestational age (SGA) when weight was smaller than the mean values of the controls

(mean – 1.0 × SD) (Saito et al., 2010). According to this definition, 8, 15, 0 and 12% of females and 15, 21, 14 and 31% of males were SGA in Cont, Cont+DHA, EtOH and EtOH+DHA groups, respectively.

Organs weight during development from GD20 to PD90

The effects of DHA on brain, liver, and testis were measured during development in offspring exposed to prenatal EtOH.

Brain. The data related to the brain absolute and relative weight are shown in **Table 3.5**. Similar to the body weight, the interaction between EtOH and diet at GD20 was significant for brain absolute weight in both females ($P<0.01$) and males ($P<0.04$). The brain weight in EtOH+DHA group was lower than both Cont+DHA and EtOH groups in females and lower than Cont+DHA in males. Dietary DHA reduced the brain absolute weight in both female and male neonates in DHA-treated groups. In addition to the early life, the brain weight in late life for both sexes (females at PD49 and males at PD90) was influenced in the offspring fed the DHA diet, with a reduction in DHA-treated groups. However, there were no significant changes to the brain weight normalised to the body weight in any of the groups. Female brain relative weight at PD90 decreased ($P<0.01$) with DHA diet in EtOH+DHA group among other groups. There were no significant independent effects of EtOH on brain size at any time points.

Testis. The data related to testis absolute (a) and relative (b) weight are shown in **Figure 3.3**. Consistent with the brain weight, a significant interaction between EtOH and diet was identified in testis absolute weight, with testis in EtOH+DHA weighed less ($P<0.004$) than those of EtOH and Cont+DHA groups at GD20 and less ($P<0.01$) than EtOH group at PD4. In contrast, the interaction between EtOH and diet was significant ($P<0.006$) in adulthood, with testis weight was higher in EtOH+DHA than Cont+DHA group. All the changes in testis weight

disappeared when they were normalised to the body weight. No significant main effect of diet or EtOH was observed amongst the groups at any of the time points.

Liver. The liver absolute and relative weight are shown in **Table 3.6**. EtOH independently induced a significant increase ($P<0.0001$) in the fetal liver size (absolute and relative weight), only in females, in both EtOH-treated animals compared to their non-EtOH-treated counterparts. That was consistent with the lower liver weight ($P<0.05$) in PF than EtOH group. Dietary DHA only increased the liver size of both males and females in adulthood compared to those without DHA treatment ($P<0.05$).

Serum polyunsaturated fatty acids in dam and fetus

The selected fatty acids in the serum of dam and fetus are presented in **Table 3.7** and the data related to the serum total fatty acid composition of fetus and dam are presented in Appendix I and II, respectively. By adding DHA to the diet, the level of DHA increased significantly in the serum of both dam and fetus ($P<0.001$) while decreasing docosapentaenoic acid (DPA, C22:5n-6) level ($P<0.0003$). There was no significant independent effect of EtOH on fatty acids in serum of dam or fetus.

Discussion

This study investigated the effect of dietary DHA on offspring development in rats exposed to prenatal EtOH. This study found that maternal DHA did not affect outcomes in dam, including the body weight, dietary intake and litter size, except for the serum DHA level, increasing in DHA-treated dam similar to that of fetus. EtOH administration during pregnancy did not lower DHA level in dam and fetus serum. The offspring in EtOH+DHA group weighed lower in both sexes compared to Cont+DHA and EtOH groups, with a catch-up growth shortly after birth and a compensatory weight gain late in adulthood in females. A significant rise in

placental relative weight only in males and in liver size only in females suggest a sex-dependent response to EtOH, DHA diet or both of them in offspring during development. A nutritionally balanced and energy-dense semi-purified diet used in this study may have helped to maintain a better physiological state that minimizes the effects of prenatal EtOH exposure.

Maternal outcomes during pregnancy and offspring placental and body weight during development from GD20 to PD90

Under our experimental conditions of the moderate dose of EtOH, a nutritionally balanced and energy-dense diet with or without DHA, there were no significant maternal dietary intake, body weight gain and litter size differences. The fetus exposed to prenatal EtOH weighed significantly more than those in Cont or EtOH+DHA groups in both males and females. Some epidemiological studies also reported fewer low-birth-weight newborns associated with low to moderate dose of prenatal EtOH consumption (Lundsberg et al., 2015). It has been shown that mothers with a low dose of EtOH intake, during pregnancy had fewer low-birth-weight infants than those with no or more than this amount EtOH exposure (McDonald et al. 1992). These reports were in agreement with our results, with the lowest percentage of SGA in EtOH group, pronounced more in the female fetus. Contrary to these observations, many other studies showed significant body weight changes in fetus exposed to prenatal EtOH (Lochry et al. 1980; Vaglenova and Petkov 1998; Chen and Nyomba 2003). There are several factors that affect maternal and offspring outcomes in EtOH-exposed condition, including animal model, the forms, palatability and energy density of the diet, EtOH dose and administration, animal age and the number of fetus per dam, etc.

In this study, the lack of differences in maternal food intake when compared to many other studies could be due to the differences in the circadian rhythm of rats fed liquid diet versus

a solid diet, with the latter being consumed mainly during the lights-off period (Wiener et al. 1981). The pattern of liquid diet intake depends on the presence of EtOH, which was used mainly during the day in what appeared to be eating spurts (Wiener et al., 1981). Another possible explanation for the minimal EtOH-induced impairment observed in the present study might be related to restricting the cycling EtOH exposure to 8 hours apart to allow the mucus membranes of the dams to recover, with normal eating behavior (Salem et al. 1996). Moreover, the energy density of experimental diets could also be a factor in determining the changes in maternal body weights. Earlier studies supported the role of calorically dense diet on maternal outcomes and their offspring development in those exposed to prenatal EtOH (Wiener et al. 1981; Goad et al. 1984). Therefore, it can be speculated that the lack of body weight loss induced by EtOH may be due to the high-energy density of the diet (4.17 kcal/g) consumed by dams in this study relative to rat chow (3.36 kcal/g; Lab Diet 5001, MO, USA) that is generally used in other rodent studies. Therefore, the severity of EtOH-induced damage might be closely associated with the quality and quantity of maternal diet, the pattern of EtOH and the form of diet, influencing the effect of EtOH on maternal outcomes and the offspring development.

As expected, the fetus in DHA-treated group (Cont+DHA) weighed more than the controls when they were not exposed to EtOH. However, the fetal body weight of both females and males in EtOH+DHA group weighed significantly less compared to those with either prenatal EtOH exposure (EtOH) or dietary DHA treatment (Cont+DHA). Moreover, the proportion of offspring with SGA in this group was the highest amongst the groups, with males more than females. A prompt catch-up growth occurred shortly after birth in female neonates and slowly for males, suggesting sex-related differences in catch-up growth. In the same group it was only in male offspring that the placental size increased significantly. These results indicate that

maternal EtOH ingestion during pregnancy influences placental weight and function, leading to alterations in the fetal and postnatal development. The consumption of large amounts of EtOH during pregnancy is associated with the reduced placental capacity for nutrient transportation in humans and experimental animals (Turan and Arzu 2005). In our findings, only a minor effect of EtOH combined with DHA was observed in placental size in male offspring in which the placental weight was not in proportion to the fetal body weight at GD20. This huge placental relative weight in males likely affected their postnatal body weight gain in EtOH+DHA group. It has been reported that the prevalence and incidence of FASD are sex-related with males outnumbering females (Thanh et al. 2014). The study of behavioral changes resulting from EtOH exposure during development reported sex-related changes that appeared only in adult males (Vaglenova and Petkov 1998). Therefore, more consideration should be given to the sex-related differences during prenatal EtOH exposure.

Offspring organ weights during development from GD20 to PD90

Brain and testis. Although the brain and testis absolute weight were affected by dietary DHA in fetal, early and postnatal life, these changes appeared to be in proportion to the body weight as they were not observed in brain and testis relative weights. The lower brain relative weight in EtOH+DHA group in adult females might be due to the significant compensatory gain weight that was not in proportion to their brain weight. The testicular size increased as testis develops and the highest testis relative weight at PD49 might support the critical role of this age in sexual maturation.

Liver. Changes in liver weights are the marker for EtOH exposure. Most studies reported that a decrease in body weight with EtOH administration also showed a decrease in liver weights (Gallo and Weinberg 1986; Meyers et al. 2002; Huebner et al. 2016). However, in this study, the

fetus fed EtOH had an enlarged liver, only in female at GD20. The enlarged liver was observed only at fetal stages suggesting that withdrawal of EtOH with the diet provided resulted in normalization of the liver weights.

Plasma total fatty acids in dams and fetuses

Experimental diets used in this study were formulated with and without DHA (1.4% of total fatty acids). This level of DHA is considered as sufficient dietary DHA (Wang et al., 2018) and hence, relevant to clinical observations. As predicted, serum DHA level increased considerably in both dam and fetus, regardless of the effect of EtOH. In this study, the serum DHA was not affected by EtOH in the dam and fetus among the different groups consistent with a previous study by Denkins et al. (2000). In this case it is interesting to find how dietary DHA influenced DHA status in the tissues as reported in other studies (Salem et al. 1996; Harris et al. 1984; Pawlosky and Salem 1995).

Conclusion

The moderate does of EtOH during pregnancy had a minimal impact on the dams or offspring outcomes likely due to the calorically dense semi-purified diet used in this study that need to be further analyzed. While DHA diet increased serum DHA, it only affected fetal body weight not at postnatal developmental stage. There was a sex-dependent response to either prenatal EtOH or diet during fetal and postnatal development that warrants further attention in future studies.

Table 3.1. Fatty acid composition of the experimental diets

		Diet	
		Control	DHA
Fat mix (g/100g)	Beef tallow	68.0	65.9
	Canola oil	4.9	4.8
	Corn oil	26.5	25.7
	Flaxseed oil	0.4	0.4
	AASCO	0.2	0.2
	DHASCO	-	4.7
Fatty acids (%, w/w Fat)			
	Myristic (C14:0)	2.1	2.5
	Palmitic (C16:0)	20.0	19.8
	Stearic (C18:0)	10.6	10.3
	Linoleic (LA, C18:2n-6)	17.5	17.0
	α -Linolenic (ALA, C18:3n-3)	1.0	1.0
	Arachidonic (AA, C20:4n-6)	0.1	0.1
	Docosahexaenoic (DHA, C22:6n-3)	-	1.4
	LA/ALA ratio	17.8	17.9
	PUFA/SFA ratio	0.6	0.6
	n-6/n-3 ratio	18.0	8.2

All the basal diet ingredients and also beef tallow purchased from Dyets Inc (Bethlehem, PA, USA). Canola oil, corn oil and flaxseed oil obtained from Loblaw's (Brampton, ON, Canada), ACH Food Companies Inc (Oakbrook, IL, USA), Omega Nutrition (Vancouver, BC, Canada), respectively. AASCO and DHASCO donated from DSM (Cupertino, CA, USA).

Table 3.2. The effect of dietary DHA on litter size and maternal diet intake in dams consuming EtOH during pregnancy

	Cont	Cont+DHA	EtOH	EtOH+DHA	PF
Litter numbers (% Male)	15.3 ± 1.5 (36)	15.5 ± 1.0 (45)	14.3 ± 3.1(36)	15.3 ± 1.5 (36)	14.0 ± 0.0 (36)
Maternal body weight gain (%)	44.1 ± 11.8	49.4 ± 8.4	48.9 ± 3.2	57.6 ± 7.9	60.8 ± 0.9
Maternal diet intake (g)					
GD1-7	19.6 ± 4.6	23.3 ± 8.9	16.8 ± 3.9	22.5 ± 3.6	18.6 ± 1.2
GD8-14	17.9 ± 4.8	23.5 ± 6.0	16.4 ± 2.6	20.5 ± 3.3	18.8 ± 1.9
GD15-20	19.8 ± 4.1	19.0 ± 1.7	17.9 ± 3.2	22.0 ± 7.4	19.2 ± 1.8

Mean ± SD (n=3-4 per group). Main effects were analyzed using two-way analysis of variance (ANOVA); The significant differences between EtOH and PF groups were analyzed by student's *t*-test, ($P<0.05$); No significant effects were identified. Cont, control; Cont+DHA, control+DHA; EtOH, ethanol; EtOH+DHA, ethanol+DHA; PF, pair-fed.

Table 3.3. The effect of dietary DHA on placental weight at GD20 in fetus exposed to EtOH

	Cont	Cont+DHA	EtOH	EtOH+DHA	PF	Significant effects (P<)		
						EtOH	Diet	EtOH* Diet
Female								
(g)	0.41 ± 0.05	0.44 ± 0.08	0.43 ± 0.087	0.43 ± 0.06	0.41 ± 0.06	NS	NS	NS
(%)	13.0 ± 1.4	12.13 ± 1.06	12.28 ± 1.626	15.46 ± 7.41	13.02 ± 1.78	NS	NS	NS
Male								
(g)	0.43 ± 0.069 ^{ab}	0.45 ± 0.10^{a}	0.41 ± 0.07^{b}	0.46 ± 0.11^{a}	0.43 ± 0.07	NS	0.02	0.0004
(%)	12.71 ± 2.03 ^{b}	12.50 ± 1.50^{b}	11.40 ± 2.11^{c}	13.88 ± 1.89^{a}	11.35 ± 2.17	NS	NS	0.0001

Mean ± SD (female, n=25-50 per group; male, n=16-29 per group). Main effects were analyzed using two-way analysis of variance (ANOVA); Values having different superscript letters are significantly different among the groups by Duncan's multiple range test; The significant differences between EtOH and PF groups were analyzed by student's *t*-test, ($P<0.05$). No difference was identified; NS, not significant;GD, gestational day; Cont, control; Cont+DHA, control+DHA; EtOH, ethanol; EtOH+DHA, ethanol+DHA; PF, pair-fed.

Table 3.4. The effect of dietary DHA on body weight from GD20 to PD90 in rats exposed to prenatal EtOH

Age (days)	Cont	Cont+DHA	EtOH	EtOH+DHA	PF	Significant effects (P<)		
						EtOH	Diet	EtOH*Diet
Female								
GD20 (n=25-50)	3.2 ± 0.3^{b}	3.4 ± 0.3^{a}	3.5 ± 0.3^{a}	3.2 ± 0.2^{b}	3.4 ± 0.3	NS	NS	0.0001
PD4 (n=7-20)	8.9 ± 1.6	9.2 ± 1.4	9.9 ± 1.6	9.4 ± 1.4	$8.0 \pm 0.8^{*}$	NS	NS	NS
PD21 (n=3-7)	59.8 ± 7.6	56.3 ± 7.0	70.9 ± 2.0	58.3 ± 11.4	60.0 ± 6.1	NS	NS	NS
PD49 (n=4-8)	234.6 ± 26.2	221.0 ± 21.8	255.8 ± 41.1	247.8 ± 23.5	224.3 ± 9.3	NS	NS	NS
PD90 (n=4-8)	359.9 ± 34.9^{b}	401.5 ± 82.6^{ab}	356.6 ± 47.1^{b}	441.0 ± 31.4^{a}	450.3 ± 68.0	NS	0.02	NS
Male								
GD20 (n=16-29)	3.4 ± 0.2^{b}	3.6 ± 0.4^{a}	3.6 ± 0.3^{a}	3.3 ± 0.3^{b}	$3.8 \pm 0.3^{*}$	NS	NS	0.0001
PD4 (n=7-15)	10.0 ± 0.6^{a}	9.8 ± 1.7^{a}	10.3 ± 0.7^{a}	8.5 ± 1.7^{b}	$8.3 \pm 1.3^{*}$	NS	0.04	NS
PD21 (n=2-9)	61.5 ± 7.5	57.3 ± 10.2	65.7 ± 5.6	64.2 ± 5.9	64.0 ± 2.4	NS	NS	NS
PD49 (n=3-9)	311.8 ± 12.1	301.8 ± 35.1	326.3 ± 21.8	316.3 ± 6.4	309.0 ± 21.3	NS	NS	NS
PD90 (n=6-10)	630.8 ± 59.2	631.0 ± 74.3	630.3 ± 92.1	632.3 ± 51.7	668.8 ± 58.5	NS	NS	NS

Mean ± SD. Main effects were analyzed using two-way analysis of variance (ANOVA); Main effects were analyzed using two-way analysis of variance (ANOVA); Values having different superscript letters are significantly different among the groups by Duncan's multiple range test; The significant differences between EtOH and PF groups were analyzed by student's *t*-test*;* * indicates a significant difference between EtOH and PF groups, ($P<0.05$). NS, not significant; GD, gestational day; PD, postnatal day; Cont, control; Cont+DHA, control+DHA; EtOH, ethanol; EtOH+DHA, ethanol+DHA; PF, pair-fed.

Table 3.5. The effect of dietary DHA on brain weight from GD20 to PD90 in rats exposed to prenatal EtOH

Age (days)	Cont	Cont+DHA	EtOH	EtOH+DHA	PF	Significant effects (P<)		
						EtOH	Diet	EtOH*Diet
Absolute								
Female								
GD20 (n=24-49)	0.16 ± 0.01^{ab}	0.17 ± 0.02^{a}	0.17 ± 0.01^{a}	0.16 ± 0.01^{b}	0.17 ± 0.01	NS	NS	0.01
PD4 (n=7-20)	0.37 ± 0.04^{bc}	0.39 ± 0.05^{b}	0.43 ± 0.04^{a}	0.35 ± 0.02^{c}	0.35 ± 0.02	NS	0.01	0.0003
PD21 (n=3-7)	1.54 ± 0.11	1.45 ± 0.09	1.57 ± 0.02	1.56 ± 0.24	1.46 ± 0.06	NS	NS	NS
PD49 (n=4-8)	2.09 ± 0.10^{a}	1.85 ± 0.15^{c}	2.03 ± 0.05^{ab}	1.94 ± 0.03^{b}	1.96 ± 0.10	NS	0.002	NS
PD90 (n=3-8)	2.10 ± 0.19	2.01 ± 0.10	2.04 ± 0.21	2.09 ± 0.06	2.15 ± 0.10	NS	NS	NS
Male								
GD20 (n=16-29)	0.16 ± 0.01^{ab}	0.18 ± 0.04^{a}	0.17 ± 0.02^{ab}	0.16 ± 0.02^{b}	0.17 ± 0.01	NS	NS	0.04
PD4 (n=7-15)	0.40 ± 0.02^{a}	0.35 ± 0.05^{b}	0.40 ± 0.02^{a}	0.32 ± 0.05^{b}	0.34 ± 0.03	NS	0.0001	NS
PD21 (n=3-9)	1.63 ± 0.08	1.46 ± 0.13	1.45 ± 0.23	1.49 ± 0.05	1.49 ± 0.08	NS	NS	NS
PD49 (n=3-9)	2.13 ± 0.12	2.00 ± 0.08	2.11 ± 0.02	2.09 ± 0.05	2.05 ± 0.08	NS	NS	NS
PD90 (n=6-10)	2.33 ± 0.06^{a}	2.20 ± 0.11^{b}	2.32 ± 0.14^{a}	2.27 ± 0.07^{ab}	2.32 ± 0.13	NS	0.02	NS
Relative								
Female								
GD20 (n=24-49)	5.07 ± 0.55^{a}	4.88 ± 0.40^{ab}	4.74 ± 0.33^{b}	4.91 ± 0.34^{ab}	4.85 ± 0.29	NS	NS	0.03
PD4 (n=7-20)	4.26 ± 0.50	4.19 ± 0.36	4.35 ± 0.51	3.75 ± 0.73	4.40 ± 0.43	NS	NS	NS
PD21 (n=3-7)	2.60 ± 0.32	2.61 ± 0.32	2.22 ± 0.04	2.74 ± 0.57	2.44 ± 0.16	NS	NS	NS
PD49 (n=4-8)	0.90 ± 0.11	0.84 ± 0.05	0.81 ± 0.10	0.79 ± 0.09	0.87 ± 0.06	NS	NS	NS
PD90 (n=3-8)	0.59 ± 0.07^{a}	0.51 ± 0.09^{ab}	0.58 ± 0.04^{a}	0.46 ± 0.03^{b}	0.48 ± 0.05	NS	0.01	NS
Male								
GD20 (n=16-29)	4.84 ± 0.36	4.95 ± 1.00	4.64 ± 0.41	4.87 ± 0.46	4.42 ± 0.33	NS	NS	NS
PD4 (n=7-15)	4.04 ± 0.27^{a}	3.54 ± 0.29^{b}	3.93 ± 0.17^{a}	3.85 ± 0.44^{a}	4.22 ± 0.50	NS	0.006	NS
PD21 (n=2-9)	2.68 ± 0.30	2.58 ± 0.27	2.42 ± 0.16	2.33 ± 0.24	2.33 ± 0.20	NS	NS	NS
PD49 (n=3-9)	0.68 ± 0.06	0.67 ± 0.09	0.65 ± 0.04	0.66 ± 0.01	0.67 ± 0.07	NS	NS	NS
PD90 (n=6-10)	0.37 ± 0.03	0.35 ± 0.03	0.37 ± 0.04	0.36 ± 0.03	0.35 ± 0.04	NS	NS	NS

Mean ± SD; Main effects were analyzed using two-way analysis of variance (ANOVA); Values having different superscript letters are significantly different among the groups by Duncan's multiple range test; The significant differences between EtOH and PF groups were analyzed by student's *t*-test, ($P<0.05$). NS, not significant; GD, gestational day; PD, postnatal day; Cont, control; Cont+DHA, control+DHA; EtOH, ethanol; EtOH+DHA, ethanol+DHA; PF, pair-fed.

Table 3.6. The effect of dietary DHA on liver weight from GD20 to PD90 in rats exposed to prenatal EtOH

Age (days)	Cont	Cont+DHA	EtOH	EtOH+DHA	PF	Significant effects (P<)		
						EtOH	Diet	EtOH* Diet
Absolute								
Female								
GD20 (n=25-49)	0.20 ± 0.04^{c}	0.22 ± 0.03^{b}	0.24 ± 0.04^{a}	0.24 ± 0.02^{a}	$0.23 \pm 0.04^{*}$	0.0001	NS	NS
PD4 (n=6-20)	0.34 ± 0.07	0.33 ± 0.05	0.33 ± 0.05	0.34 ± 0.08	0.30 ± 0.04	NS	NS	NS
PD21 (n=3-7)	2.06 ± 0.45	1.94 ± 0.27	2.62 ± 0.42	2.03 ± 0.47	2.09 ± 0.21	NS	NS	NS
PD49 (n=4-8)	8.69 ± 2.35	8.13 ± 0.85	8.56 ± 1.66	8.80 ± 1.31	7.32 ± 1.13	NS	NS	NS
PD90 (n=4-8)	8.88 ± 0.66^{b}	10.81 ± 2.35^{ab}	9.01 ± 1.15^{b}	11.98 ± 1.49^{a}	11.40 ± 2.83	NS	0.001	NS
Male								
GD20 (n=15-29)	0.22 ± 0.03	0.23 ± 0.04	0.25 ± 0.03	0.22 ± 0.04	0.26 ± 0.03	NS	NS	NS
PD4 (n=6-15)	0.36 ± 0.05	0.37 ± 0.12	0.39 ± 0.06	0.34 ± 0.07	$0.30 \pm 0.06^{*}$	NS	NS	NS
PD21 (n=3-9)	2.30 ± 0.47	2.09 ± 0.43	2.33 ± 0.42	2.27 ± 0.42	2.07 ± 0.10	NS	NS	NS
PD49 (n=3-9)	10.99 ± 1.96	11.06 ± 0.80	11.79 ± 1.02	10.47 ± 0.27	11.32 ± 0.97	NS	NS	NS
PD90 (n=6-10)	16.34 ± 1.5	18.21 ± 3.34	17.96 ± 3.67	20.28 ± 3.99	18.09 ± 2.43	NS	NS	NS
Relative								
Female								
GD20 (n=25-49)	6.30 ± 0.95^{c}	6.45 ± 0.88^{bc}	6.83 ± 0.80^{b}	7.40 ± 0.57^{a}	6.71 ± 0.69	0.0001	0.01	NS
PD4 (n=6-20)	3.81 ± 0.38	3.60 ± 0.46	3.51 ± 0.58	3.62 ± 0.54	3.77 ± 0.43	NS	NS	NS
PD21 (n=3-7)	3.42 ± 0.42	3.46 ± 0.27	3.97 ± 0.23	3.49 ± 0.37	3.48 ± 0.10	NS	NS	NS
PD49 (n=4-8)	3.68 ± 0.74	3.68 ± 0.18	3.33 ± 0.19	3.55 ± 0.33	3.25 ± 0.44	NS	NS	NS
PD90 (n=4-8)	2.47 ± 0.13^{b}	2.69 ± 0.19^{a}	2.53 ± 0.15^{b}	2.71 ± 0.25^{a}	2.51 ± 0.30	NS	0.01	NS
Male								
GD20 (n=15-29)	6.50 ± 0.88	6.31 ± 0.74	6.82 ± 0.92	6.70 ± 1.12	6.91 ± 0.58	NS	NS	NS
PD4 (n=6-15)	3.58 ± 0.48	3.38 ± 0.61	3.81 ± 0.56	3.79 ± 0.20	3.61 ± 0.40	NS	NS	NS
PD21 (n=2-9)	3.71 ± 0.32	3.64 ± 0.28	3.89 ± 0.05	3.53 ± 0.40	3.24 ± 0.13	NS	NS	NS
PD49 (n=3-9)	3.53 ± 0.63	3.70 ± 0.39	3.61 ± 0.10	3.31 ± 0.03	3.67 ± 0.29	NS	NS	NS
PD90 (n=6-10)	2.59 ± 0.16^{b}	2.88 ± 0.37^{ab}	2.83 ± 0.30^{ab}	3.22 ± 0.72^{a}	2.70 ± 0.18	NS	0.02	NS

Mean ± SD. Main effects were analyzed using two-way analysis of variance (ANOVA). Values having different superscript letters are significantly different among the groups by Duncan's multiple range test; The significant differences between EtOH and PF groups were analyzed by student's *t*-test, * indicates a significant difference between EtOH and PF groups, ($P<0.05$). NS, not significant; GD, gestational day; PD, postnatal day; Cont, control; Cont+DHA, control+DHA; EtOH, ethanol; EtOH+DHA, ethanol+DHA; PF, pair-fed.

Table 3.7. The effect of dietary DHA on selective serum fatty acids in dam and fetus exposed to EtOH

Fatty acids (%, w/w)	Cont	Cont+DHA	EtOH	EtOH+DHA	PF	Significant effects (P<)		
						EtOH	Diet	EtOH *Diet
Dam								
C18:2n-6	11.99 ± 2.33	11.78 ± 2.01	14.02 ± 0.51	13.04 ± 0.79	12.06 ± 1.02	NS	NS	NS
C18:3n-3	0.41 ± 0.23	0.41 ± 0.11	0.42 ± 0.07	0.46 ± 0.15	0.41 ± 0.11	NS	NS	NS
C20:4n-6	13.09 ± 2.51	12.19 ± 4.15	11.89 ± 5.33	11.88 ± 2.61	12.22 ± 1.54	NS	NS	NS
C22:5n-6	4.88 ± 1.81^{a}	2.27 ± 0.88^{bc}	4.48 ± 1.31^{ab}	1.63 ± 0.87^{c}	3.02 ± 1.17	NS	0.0003	NS
C22:6n-3	4.65 ± 0.91^{b}	9.40 ± 3.11^{a}	4.24 ± 1.18^{b}	7.92 ± 0.79^{a}	6.87 ± 2.17	NS	0.001	NS
n-3 PUFA	5.91 ± 1.08^{b}	10.60 ± 2.98^{a}	5.65 ± 1.74^{b}	9.16 ± 0.82^{a}	8.08 ± 2.02	NS	0.002	NS
n-6 PUFA	31.82 ± 1.88	27.98 ± 3.26	31.37 ± 4.31	28.35 ± 2.64	28.77 ± 2.72	NS	NS	NS
Fetus								
C18:2n-6	9.84 ± 1.72	8.04 ± 1.58	9.70 ± 0.60	11.07 ± 1.38	9.52 ± 1.40	NS	NS	NS
C18:3n-3	0.26 ± 0.10	0.19 ± 0.01	0.18 ± 0.08	0.16 ± 0.06	0.19 ± 0.01	NS	NS	NS
C20:4n-6	11.49 ± 1.29	8.12 ± 1.94	9.39 ± 0.93	9.62 ± 2.58	11.78 ± 0.92	NS	NS	NS
C22:5n-6	3.99 ± 0.98^{a}	1.77 ± 0.64^{b}	4.23 ± 0.54^{a}	1.46 ± 0.15^{b}	2.23 ± 1.44	NS	0.0001	NS
C22:6n-3	4.62 ± 0.77^{b}	8.49 ± 2.05^{a}	3.76 ± 0.44^{b}	7.61 ± 1.44^{a}	5.42 ± 1.37	NS	0.0001	NS
n-3 PUFA	3.18 ± 3.45^{a}	1.10 ± 0.17^{b}	1.26 ± 0.14^{b}	1.16 ± 0.21^{b}	1.48 ± 0.37	NS	0.02	NS
n-6 PUFA	21.82 ± 4.99^{a}	17.71 ± 3.77^{b}	20.97 ± 1.17^{ab}	22.54 ± 3.80^{a}	24.19 ± 1.27	NS	NS	0.02

Mean ± SD (dam, n=3-4 per group; fetus, n=4-5 per group). Main effects were analyzed using two-way analysis of variance (ANOVA); Values having different superscript letters are significantly different among the groups by Duncan's multiple range test; The significant differences between EtOH and PF groups were analyzed by student's *t*-test, (P<0.05). NS, not significant; Cont, control; Cont+DHA, control+DHA; EtOH, ethanol; EtOH+DHA, ethanol+DHA; PF, pair-fed.

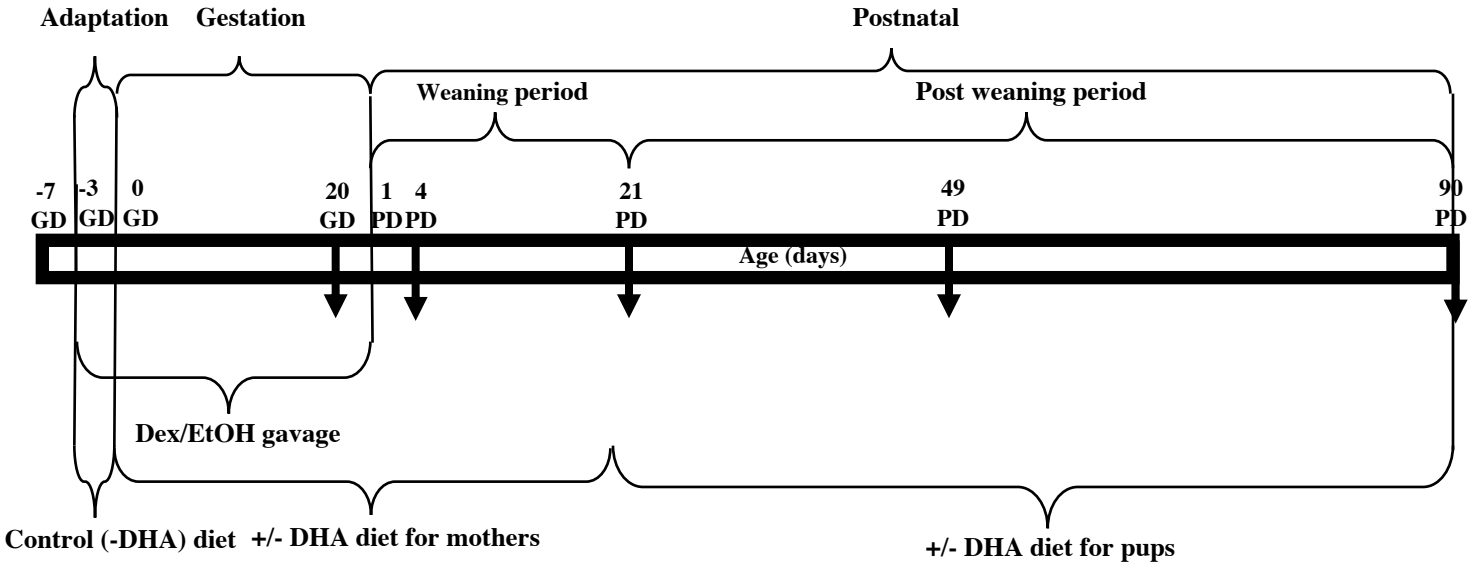


Figure 3.1. Overview of the experimental design
GD, gestational day; PD, postnatal day; EtOH, ethanol; Dex, dextrose; arrows indicate the termination days.

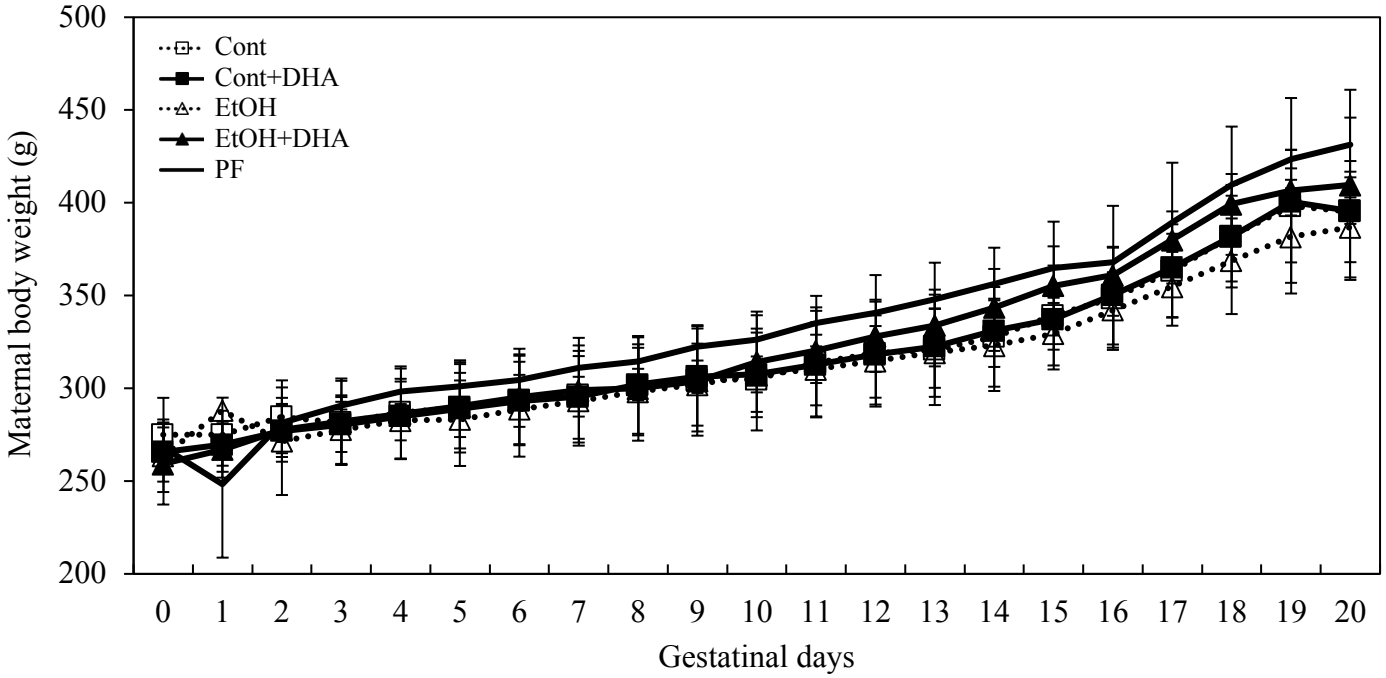


Figure 3.2. The effect of dietary DHA on maternal body weight that consumed EtOH during pregnancy
Mean ± SD (n=3-4 per group). Main effects were analyzed using two-way analysis of variance (ANOVA); The significant differences between EtOH and PF groups were analyzed by student's *t*-test, ($P<0.05$). Cont, control; Cont+DHA, control+DHA; EtOH, ethanol; EtOH+DHA, ethanol+DHA; PF, pair-fed.

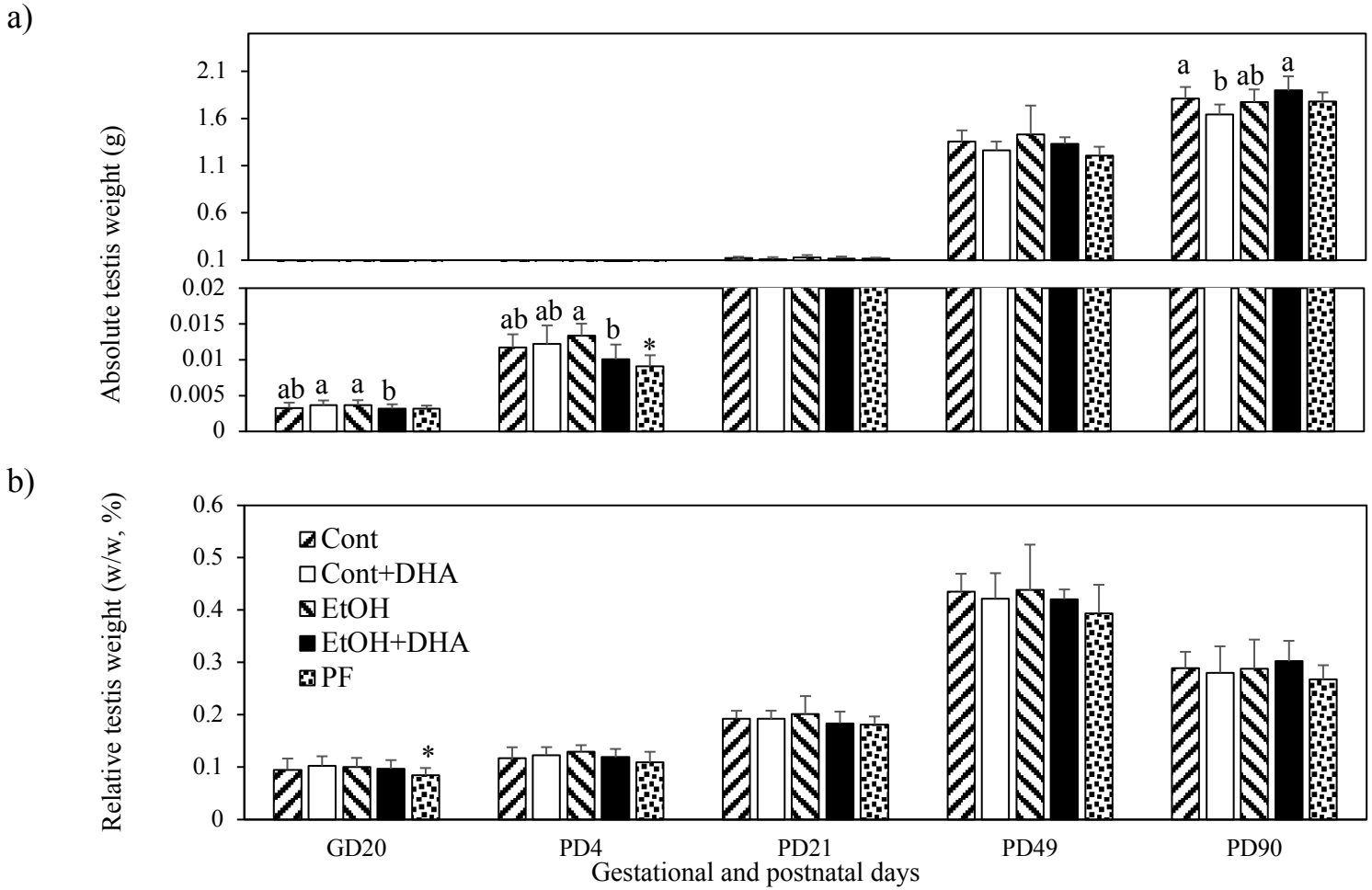


Figure 3.3. The effect of dietary DHA on a) absolute and b) relative testis weight from GD20 to PD90 in rats exposed to prenatal EtOH
Mean ± SD (Gestational day (GD) 20, n=16-28 per group; Postnatal day, (PD) 4, n=6-13 per group; PD21, n=2-8 per group; PD49, n=3-9 per group; PD90, n=5-10 per group). Main effects were analyzed using two-way analysis of variance (ANOVA); Values having different superscript letters are significantly different among the groups by Duncan's multiple range test; The significant differences between EtOH and PF groups were analyzed by student's t-test; * indicates a significant difference between EtOH and PF groups, ($P<0.05$); EtOH*Diet, GD20 ($P<0.004$); PD4, ($P<0.01$); PD90 ($P<0.006$). Cont, control; Cont+DHA, control+DHA; EtOH, ethanol; EtOH+DHA, ethanol+DHA; PF, pair-fed.

CHAPTER 4. THE EFFECT OF EARLY PROVISION OF DIETARY DOCOSAHEXAENOIC ACID ON TESTIS DEVELOPMENT, FUNCTION AND SPERM QUALITY IN RATS EXPOSED TO PRENATAL ETHANOL

"Birth Defects Research" (under review)

Abstract

Background and Objective: Although male fertility problems are diagnosed in adult life, evidence suggests that it might have a fetal origin. Prenatal ethanol (EtOH) exposure is associated with testicular hormonal imbalance and long-term adverse effects on male reproductive development and functions. Dietary docosahexaenoic acid (DHA, C22:6n-3) in adulthood is known to improve testicular functions and semen parameters, thereby affecting male fertility. This study investigated whether the early provision of dietary DHA influences testicular development and functions and sperm parameters in rats exposed to prenatal EtOH.

Methods: 9-10-week old female Sprague-Dawley rats were randomly selected to receive either EtOH (3g/kg, twice a day) or dextrose (isocaloric to EtOH) by oral gavage, throughout pregnancy. After becoming pregnant, they were fed either diet supplemented with (Cont+DHA; n=8, EtOH+DHA n=6) or without DHA (1.4%, w/w total fatty acids) (Cont, EtOH; n=8 dams each) and their pups were continued on their mothers' diet during experimental periods. Tissues were collected from the fetus (gestational day (GD) 20), neonates (postnatal day (PD) 4), weaning (PD21), peri-pubertal (PD49), and adult rats (PD90) analyzed for male hormone, testicular developmental markers, and sperm parameters.

Results: Dietary DHA significantly increased serum testosterone at GD20 ($P<0.02$) and sperm normal morphology at PD90 ($P<0.001$), regardless of EtOH exposure compared to the Cont group. In the non-EtOH-treated group, DHA also increased the height of germinal epithelium at PD49 ($P<0.03$). The EtOH-exposed group without DHA significantly increased the serum

testosterone at PD4 ($P<0.02$) and induced a marked decline in the testicular gene expression of StAR at PD49, ($P<0.02$) than those of Cont group. Sperm count and motility were not affected by either EtOH or DHA.

Conclusion: These findings indicate that regardless of exposure to prenatal EtOH, providing early dietary DHA may positively contribute to male fertility by impacting sperm morphology likely by increasing the testosterone in fetal life. Prenatal EtOH-induced alterations in circulating testosterone and the gene expression of protein involved in testosterone production during development that was normalized by dietary DHA.

Keywords: dietary docosahexaenoic acid, prenatal ethanol exposure, polyunsaturated fatty acids, reproductive functions, sperm parameters, testosterone

Introduction

Developmental delay in the central nervous system induced by prenatal ethanol (EtOH) exposure has been intensively studied. However, there is a wide range of other prenatal EtOH-related developmental deficits that have received little attention. The development and functions of the male reproductive system are known to be disrupted in men prenatally exposed to EtOH (Carter et al., 2014; Damgaard et al., 2007; Håkonsen et al., 2014; Ramlau-Hansen et al., 2010). Animal studies have also shown reproductive abnormalities in testicular hormonal balance and downstream impacts on pubertal development and spermatogenesis in adulthood due to prenatal EtOH exposure (Fakoya and Caxton-Martins, 2004; Handa et al., 1985; Lan et al., 2013; McGivern et al., 1992; Udani et al., 1985).

Testicular hormone production is active from the fetal period, with testosterone as a major regulator of testicular functions. Testosterone is a prerequisite for normal germ cell differentiation and spermatogenesis (Holdcraft and Braun, 2004; McLachlan et al., 2002). Since the shaping of various hormonally regulated reproduction process happens during the fetal period (Gore, 2008), any insult could interfere with normal reproductive function in adulthood. Maternal EtOH consumption impacts the fetal sexual organs and appears to suppress normal testosterone surges at both fetal (McGivern et al 1988) and neonatal age (McGivern et al., 1993). Exposure to EtOH prenatally also attenuates the plasma and brain level of testosterone (Kakihana et al 1980), impairs testicular steroidogenic enzyme activity (Kelce et al., 1989) and reduces the number of testosterone-producing cells in early life (McGivern et al 1988). In addition, blunting the normal pattern of Luteinizing hormone (LH) during development has been reported in prenatal EtOH-exposed rats (Handa et al 1985, Udani et al 1985, Wilson and Handa, 1997), implying the central dysregulation of testosterone production. Exposure to EtOH in early life also caused a delay in attaining the adult level of testosterone in rats (Udani et al 1985), a tendency towards a delayed pubertal development in men (Håkonsen et al 2014), and retardation in the onset of spermatogenesis in rats (Lan et al 2013). The data indicate a long-lasting effect of

prenatal EtOH exposure, showing changes in testosterone level resulting from early EtOH exposure. It is not known if appropriate nutrient supplementation can mitigate these effects when provided together with prenatal EtOH.

Testis and sperm have a unique lipid environment that is enriched in polyunsaturated fatty acids (PUFA) of 22 carbons, docosahexaenoic acid (DHA, C22:6n-3) for human and docosapentaenoic acid (DPA, C22:5n-6) for rodents (Retterstøl et al., 2001). These fatty acids are crucial in testicular structure, development and functions (Ayala et al., 1977; Bieri et al., 1969; Burr and Burr, 1930; Coniglio, 1994). Some evidence showed that dietary n-3 PUFA, particularly diet supplemented with DHA benefited male fertilizing capacity by altering the steroidogenic pathways (Gromadzka-Ostrowska et al., 2002; Hurtado de Catalfo et al., 2008; Nagata et al., 2000; Sebokova et al., 1990) and improving the sperm quality (Estienne et al., 2008; Mitre et al., 2004; Rooke et al., 2001). Furthermore, DHA improved the disruption in sperm parameters in both human (Conquer *et al* 2000, Safarinejad 2011) and animal models (Li et al., 2013; Roqueta-Rivera et al., 2011). However, there is a lack of evidence on the direct impact of DHA on testosterone production and testicular developmental markers during development and sperm parameters in adulthood.

This study investigated the impact of the early provision of dietary DHA on testicular functional changes in circulating testosterone and testicular developmental markers from GD20 to PD90 in rats prenatally exposed to EtOH. The sperm parameters in the adult stage were also examined.

Material and Method

Animals, diets, and treatments

All experimental procedures were approved by the University of Manitoba Animal Care Committee and were conducted according to the principles and guidelines of the Canadian Council on Animal Care (CCAC, 1993). Female Sprague-Dawley rats (9-10 weeks old, n= 30),

purchased from Charles Rivers Breeding Laboratories (Sherbrooke, Quebec, Canada) were acclimatized for a week in a temperature-controlled environment under a 12-h dark-light cycle. Rats were randomly assigned to receive either EtOH (3g/kg twice/day 50%, w/v, n=14), representing a moderate dose of EtOH exposure (Schambra et al., 2015) or dextrose (Cont, 80%, w/v, isocaloric to EtOH, n=16). This level of EtOH equals the amount reached by women having 1-2 drinks within an hour (Schambra et al., 2015). EtOH was introduced to animals gradually by increasing from 1/3 to 3/3 to reach the final dose for the first 3 days prior to mating (Chappell et al 2007). After EtOH adaptation, rats were impregnated by healthy male rats. During EtOH adaptation and mating period, animals were fed control diet. On the gestational day (GD) 0, defined as the first day of pregnancy, dams were housed individually and randomly assigned to receive either DHA-supplemented diet (1.4%, w/w, total fatty acids) or control diet. There were a total of 4 groups: i) control without EtOH (Cont, n=8); ii) control with DHA (Cont+DHA, n=8) iii) EtOH without DHA (EtOH, n=8); iv) EtOH with DHA (EtOH+DHA, n=6). Treatment with EtOH or dextrose continued for the entire pregnancy followed by abstinence after parturition. The experimental design is shown in **Figure 3.1** in Chapter 3. Since there is no difference in growth and dietary intake during pregnancy, the pair-fed group was not included in this Chapter.

Experimental diets were semi-purified, nutritionally balanced, and energy-dense, reflecting current dietary intake of the pregnant mother in North America (Denomme et al., 2005; Friesen and Innis, 2010; Hui et al., 2014). The only difference between the two experimental diets was the DHA level (0% vs. 1.4%, w/w fatty acids), which has been reported to be a sufficient amount for pregnant rats and growing pups (Wang et al., 2018; Weiser et al., 2015). Experimental diets were continued throughout the entire pregnancy and lactation period for dams. After being weaned at postnatal day (PD) 21, the pups were fed the same diet as their respective mothers, and continued until the end of the experimental period at the age 90 (PD90). Animals had *ad libitum* access to water and food. Diet intake and body weight of dams and pups were recorded and reported previously (Chapter 3).

For fetal samples, at GD20, almost half of dams in all four groups were anesthetized via isoflurane and cardiac puncture. The uterine horns were removed and male fetuses were identified by their anogenital distance using a dissecting microscope, weighed and then decapitated with sharp scissors. The remaining pregnant rats were allowed natural parturition. At PD4, litter was randomly culled to 8 pups/dam with an equal male to female sex ratio to allow equal access to mothers milk. Pups were kept with their natural mothers until weaning. Male pups in a litter were terminated at different time points that were chosen based on the rodent male sexual development; fetus at GD20, neonates at PD4, infants at PD21, adolescence at peri-puberty at PD49, and adult rats at PD90 (Clegg 1960, Ojeda et al 1980, Zanetti et al 2007). GD20 for the fetus was chosen for assessing the fetal testis development and function at which the fetal surge of testosterone has been completed (Baum et al., 1988). PD4 representing neonates in consideration of testis maturity that is coincident with re-initiation of gonocytes mitosis and their movement from center to the edge of seminiferous cord (McGuinness and Orth, 1992). PD21 represents weaning by which, Sertoli cells stop dividing (Steinberger and Steinberger, 1971), differentiate into non-proliferative cells (Wang et al., 1989), and form the blood-testis barrier (Vitale et al., 1973). While PD38-60 is considered as peri-pubertal period in the male rats (Schneider, 2013), PD49 was chosen at which the first spermatozoa can be detectable in seminiferous tubules (Clegg et al., 1960) and it is shortly before the initiation of the pubertal surge of testosterone at PD51 (Korenbrot et al., 1977). PD90 is defined as adulthood which is around the time that the concentrations of spermatozoa reach the highest in the rat (Sun and Flickinger, 1979). At each stage, pups were euthanized and blood samples were collected for measuring testosterone level and the male reproductive organ was removed for analyzing testis developmental markers and sperm parameters.

Histological analysis

The left testis at different developmental stages was fixed in Bouin's solution. After 4 hours, they were kept in 70% ethanol until embedding in paraffin, followed by sectioning in 5 µm and staining with hematoxylin and eosin (H&E). Histological markers for testis development are represented by the number and diameter of seminiferous cord at GD20 and seminiferous tubule diameter and height of germinal epithelium at PD49 and PD90, and the germ cells maturation at PD49 that were measured using an optical light microscope (Zeiss, Munich, Germany), coupled to a digital camera. Developmental markers at GD20 were measured for 8 testes per group at 200x magnification for the number and the diameter of the cords. At PD49 and PD90, the diameter was measured on 200 randomly selected seminiferous tubules per testis from three animals per group, by measuring across the minor axis at 100x magnification. The height of germinal epithelium in seminiferous tubules was measured from the boundary layer of the junction of the seminiferous tubule epithelium to tubular lumen at 100x magnification (Sprando et al., 1999). The germ cells maturation was determined by the detection of spermatozoa existed in the lumen per 200 tubules at PD49 (Knorr et al., 1970). Digital images were evaluated quantitatively for the testis developmental markers using ImageJ software (Image Processing and Analysis in Java).

Serum testosterone analysis

Blood samples were collected from the trunk at GD20, PD4 and from cardiac puncture at PD21, PD49 and PD90 for testosterone measurement. Serum testosterone was determined with a testosterone ELISA kit (Crystal Chem, USA), with coefficient of variation (CV) of <10%.

Determination of gene expression

Total RNA was extracted from the decapsulated testis at PD49 and PD90 according to the Trizol method (Chomczynski and Sacchi, 1987). As described by the manufacturer, 1 ml TRIzol

(Invitrogen, Canada) was added to 100 mg tissue homogenized using a PowerGen Model 125 Homogenizer. RNase-free DNAse I enzyme was used to remove contaminating genomic DNA (Promega, Madison, WI, USA). RNA concentration and purity of all samples were assessed using Nano-drop spectrophotometer 2000 (Thermo Scientific, Canada). 1.5 μg of total RNA was reverse-transcribed into cDNA with a High-Capacity cDNA Reverse Transcription kit, as described by the supplier's protocol (Applied Biosystems, Canada). Pairs of primers for each gene were designed using the National Centre for Biotechnology Information (NCBI) primer blast, from the mRNA sequence of the target gene and purchased from (University core and services, University of Calgary). The primer sequences are presented in **Table 4.1**. Real-time quantitative polymerase chain reaction (RT-qPCR) was conducted using a CFX Connect TM qRT-PCR Detection System (Bio-Rad, Canada) as described by the manufacturer. The reactions were in duplicate, including the forward and reverse primers of target gene, nuclease free water, samples cDNA and SYBR Green as a detector. Samples were run for 40 cycles (95°C for 15 secs for denaturation, 59°C for 20 secs for annealing/extension) using the Applied Biosystems StepOnePlus Real-Time PCR System. TATA box binding protein (Tbp) used as a housekeeping gene to normalize the relative expression of the target genes that is identified as stably expressed gene in testis (Svingen et al., 2009). Comparative ΔΔCt method was generated for data analysis (Livak and Schmittgen, 2001).

Sperm count, morphology and motility analysis

The cauda epididymis, from sexually matured rats at PD90, was removed, weighed and cut once with scissors, incubated in 2 ml 0.9% saline solution and maintained at 37°C for 5 min until the sperm suspension became slightly turbid. After incubation, the sperm suspension was analyzed as described below:

Sperm counts were measured on the warm Makler's chamber (New York microscope company Inc, Hicksville, NY, US), as described by the manufacturer. The sperm counts were analyzed

using guidelines set by the World Health Organization (WHO, 2010). The number of sperm counted in 10 squares of the grid in the same strip indicated the sperm counts in 10^6 per ml. *Sperm motility* was evaluated in a total of 100 sperm per animal by visual observation under a phase-contrast microscope (Zeiss, Munich, Germany) equipped with a video camera (Sprando et al., 2000). Sperm was classified as mobile or immobile with sperm motility was expressed in percentage.

Sperm morphology was analyzed according to *rat Sperm Morphological Assessment Guideline Document* (IRDG & CASA, 2000) as established in our laboratory (Merrells et al., 2009). An aliquot of epididymal sperm suspension was fixed in the formalin followed by staining with eosin Y and then smeared onto microscope slides. By using a phase-contrast microscope (Zeiss, Munich, Germany) at 400x magnification, a total of 200 random sperm per testis were classified into four categories in morphology, including normal morphology (hooked head and smooth tail) and abnormal head (missing head, flattened hook or pinhead), abnormal tail (missing tail, bent or coiled tail) and both abnormal head and tail (Merrells et al., 2009) and expressed as percentage.

Statistical Analysis

Statistical analysis was analyzed using two-way analysis of variance (ANOVA) for the main factors of EtOH and dietary DHA. Multiple comparisons were done using Duncan post-hoc test when an interaction exists between EtOH and DHA. When there was no interaction effect identified, the ANOVA was re-tested for the main factors, which enabled us to test against the correct degree of freedom of error. All data are expressed as mean ± standard error of mean (SEM). Statistical significance was set at $P< 0.05$.

Result

Testicular developmental markers

Effects of dietary DHA on testicular developmental markers were measured during development at GD20, PD21, PD49 and PD90 in rats with exposure to prenatal EtOH (**Table 4.2).** Testicular histological analysis at GD20 in all groups appeared normal. An ANOVA applied to each variable revealed that there were no significant changes in the number and the diameter of seminiferous cord among the groups **(Table 4.2; Figure 4.4-a)**. At PD21, the gonocytes have already moved from the center to the edge of the seminiferous tubules (**Figure 4.4 b**). The height of germinal epithelium and seminiferous tubule diameter were clearly different by adding DHA to the diet, but the number of animals per group was not sufficient to evaluate the statistical differences at this age due to losing samples during histological analysis procedure. At PD49, dietary DHA increased the height of germinal epithelium in rats fed DHA in Cont+DHA group (13%) and EtOH+DHA group (7.5%), compared to the Cont group, reaching a significant ($P<0.03$) difference only in the non-EtOH treated group **(Table 4.2; Figure 4.4-c)**. In adulthood (PD90), a significant ($P<0.01$) effect of EtOH was noted by increasing the heights in both EtOH-treated offspring compared to Cont+DHA (**Table 4.2; Figure 4.4-d**). Dietary DHA or prenatal EtOH exposure did not affect seminiferous tubule diameters in all ages tested and germ cells maturation at PD49. Indeed, the percentage of spermatozoa detected in the lumen of seminiferous tubules as a marker of germ cells maturation did not change significantly among all the groups (data not shown).

Serum testosterone level and testis StAR gene expression

Effects of dietary DHA on serum testosterone level was measured during development at GD20, PD4, PD21, PD49 to PD90 in rats with exposure to prenatal EtOH (**Figure 4.1)**. Overall, the level of serum testosterone was the highest in GD20 compared to all other age groups. Dietary DHA significantly increased ($P<0.02$) the testosterone level at GD20, regardless of the

EtOH exposure over those level in the Cont group. Although the DHA effect disappeared after birth, in early postnatal life, there was a significant ($P<0.02$) effect of prenatal EtOH on the testosterone level, increasing it from 0.82 to 1.78 ng/ml of serum in the Cont compared to the EtOH group. Although not significant, the level of testosterone was lower at PD49 and higher at PD90 in both EtOH-treated rats compared to their non-EtOH-treated counterparts.

Effects of dietary DHA on testicular mRNA expression of StAR were also measured at PD49 and PD90 in rats exposed to prenatal EtOH (**Figure 4.2**). The effect of dietary DHA was significant ($P<0.04$) on mRNA expression of StAR at PD90, where there was a reduction in the Cont+DHA group when compared to the EtOH group. However, the EtOH decreased the mRNA expression of StAR significantly ($P<0.02$) at PD49 only in EtOH group compared to the Cont group.

Sperm parameters

The effects of dietary DHA on sperm parameters in rats with exposure to prenatal EtOH were measured in sexually matured adult rats at PD90 (**Figure 4.3**). Dietary DHA significantly ($P<0.001$) increased the number of sperm with normal morphology, with no significant alterations in sperm counts and sperm motility in adult rats. Rats fed DHA diet in the Cont+DHA and EtOH+DHA had 74% and 71% normal sperm morphology, respectively compared to those fed the control diet in Cont group which had 55% normal sperm. The lone head and lone tail sperm were significantly ($P<0.05$) lower in rats fed the DHA diet compared to those consumed the Cont diet, regardless of EtOH treatment. No independent EtOH effect was identified in sperm parameters.

Discussion

The present study examined the effect of the early provision of DHA on testicular development and function by analyzing the testicular histology and testosterone level during

development in rats exposed to prenatal EtOH. This study also examined the sperm parameters in the adult stage. It was found that DHA provision through dam from pregnancy to lactation affected the circulating testosterone at GD20 and normalized serum testosterone, which was increased by the prenatal EtOH in early life. When the DHA diet was continued to adulthood, it improved the testis developmental markers at peri-puberty compared to those in the controls and was only significant in non-EtOH-treated animals. DHA also alleviated prenatal EtOH-reducing effect on gene expression of StAR, involved in testosterone production, at PD49. Sperm morphology increased in DHA-treated adult rats with and without exposure to prenatal EtOH. Prenatal EtOH exposure did not adversely affect the overall testis developmental markers during development and sperm parameters in adulthood. Overall, providing DHA in a nutrition-dense diet as designed in this study benefited sperm quality in rats with and without exposure to a moderate amount of prenatal EtOH.

Testicular developmental markers

From the present data, developmental markers were not changed by either EtOH or dietary DHA in the fetus. Similarly, no significant changes have been detected on the seminiferous diameter by any treatment in later life. These results also demonstrated that DHA did not significantly affect the height of germinal epithelium in those exposed to prenatal EtOH, but led to a 13% increase in none-EtOH treated rats at PD49. A previous study reported by Fakoya et al., (2004) showed that intubation of EtOH prenatally (5.8 g/kg) decreased clearly the height of germinal epithelium at PD42. In our results, although prenatal EtOH exposure did not modify germinal epithelium at PD49, it impeded the ability of DHA to increase the testicular histological marker. As reported in Chapter 3, testis weight in the Cont+DHA group was the lowest among all the groups at PD90. This was consistent with the results from the height of germinal epithelium and mRNA expression of StAR at the same stage, indicating that dietary

DHA might not benefit testis in adulthood in a rat model. This probably could be due to DHA which is not a critical fatty acid for testes at this age in a rat model.

Serum testosterone level and testicular gene expression of StAR

Since the majority of testosterone is produced in the testis, circulating testosterone was measured in this study. The level of testosterone at GD20 was higher than PD4 as expected. At this age, dietary DHA increased the level of testosterone, regardless of the EtOH exposure when compared to the Cont group. No other age groups were affected by the DHA diet. The studies conducted in adult rats found the increase of testosterone concentrations with dietary n-3 PUFA (Gromadzka-Ostrowska et al., 2002; Hurtado de Catalfo et al., 2008; Sebokova et al., 1990), but these were not follow-up studies. Sebokova et al., (1990) showed that an n-3 PUFA-rich diet modified the lipid composition of the Leydig cell membranes resulting in alterations in the number of LH receptors and thus the stimulation of the testosterone synthesis in a rat model. In parallel with the steroidogenic activity, the level of LH receptors increased in the fetal testis (Warren et al., 1984). Therefore, it might be possible that DHA quantitatively increased LH receptors in the fetal period to produce a greater level of testosterone. However, this study did not measure LH receptors to confirm our findings. A study on pregnant rats showed that DHA supplementation during pregnancy increased the level of high-density lipoprotein (HDL) cholesterol in offspring with no changes in the total cholesterol in the blood in rats (Gong et al., 2009). Based on our findings, another explanation for the higher level of testosterone in DHA-treated fetus might be the elevation in the amount of substrates for androgen production.

By GD20, the fetal surge of testosterone has been completed and the level of testosterone has been decreasing to the basic level in the plasma to reaching the lowest level after birth in rats (McGivern et al 1988). In our study, we did not find any independent effect of prenatal EtOH on the testosterone level at GD20. The data reported by Ward et al., (2003) showed no differences in testosterone level in EtOH-exposed animals over control level at the same age in rats.

However, a higher level of testosterone induced by prenatal EtOH in early life might be related to the inhibition of testosterone catabolism in the liver induced by EtOH (Sarkola and Eriksson, 2003). Therefore, it might be explained that the liver of pups at PD4 was not able to reduce the level of testosterone that had been recently surged after birth. Interestingly, DHA alleviated the effect of EtOH on this hormone.

Testicular gene expression of steroid acute regulatory protein (StAR) was also measured in this study, as it is the limiting factor in testosterone synthesis and responsible for transport of cholesterol into mitochondria to be used in testosterone production. At PD49, the rats exposed to prenatal EtOH significantly reduced gene expression of StAR in the EtOH group compared to the Cont group. Although failing to reach statistically significance, serum testosterone level was also lower at PD49 in the EtOH group than those without fetal EtOH exposure. Udani et al., (1985) also reported a lower level of testosterone at peri-puberty (PD55) but not in adulthood (PD110) in rats exposed to prenatal and postnatal ethanol exposure (Udani et al 1985), suggesting a delay in attaining the adult level of testosterone.

Prenatal EtOH exposure lowered the relative mRNA level of StAR at PD49 but not in DHA-treated group. Li et al., (2013) also revealed that dietary n-3 PUFA mitigated the lowering effect of non-alcoholic fatty liver disease on testosterone level by increasing the testicular StAR mRNA, and protein level leading to increased serum and testicular testosterone level in a rat model. A significant decline in the testicular responsiveness to LH, observed in an *in vitro* study of fetus exposed to prenatal EtOH, suggested the possibility of an EtOH-induced LH receptor deficiency (McGivern et al 1988). Moreover, a lower expression of StAR level has been shown in the LH receptor of knockout mice (Zhang et al., 2004). Thus, it is tempting to propose that the increasing impact of DHA on the number of LH receptor in Leydig cells might lead to mitigation in the testosterone EtOH-related reduction in mRNA expression of StAR in EtOH+DHA group at PD49. Further study is required to determine whether prenatal EtOH delays the surges of testosterone is a reliable finding and the possible underlying mechanism involved.

Sperm parameters

While this study did not show the positive effects of dietary DHA on sperm motility and sperm counts, the DHA diet induced a significant increase in the number of sperm with normal morphology in both EtOH and non-EtOH treated rats, indicating it may be a good source for male reproductive health. No previous study has evaluated the relation between the early provision of dietary DHA on sperm parameters in adulthood. However, the improved sperm morphology and counts in adult testis following DHA consumption are not surprising, since the positive relationship between dietary DHA and sperm parameters has been shown previously in both human (Afeiche et al., 2014; Attaman et al., 2012; Eslamian et al., 2012; Vujkovic et al., 2009; Conquer et al., 2000; Robbins et al., 2012; Safarinejad, 2011) and animal studies (Rooke et al., 2001; Yeste et al., 2011). Consistent with our study, Attaman et al., (2012) reported a positive relationship between an n-3 PUFA diet and normal sperm morphology in which the sperm head defect was lowered. In addition to sperm morphology, a positive association was previously reported between sperm motility and sperm count and higher intake of n-3 PUFA, fish and seafood (Afeiche et al., 2014; Eslamian et al., 2012; Vujkovic et al., 2009); that was not found in our current study. However, our finding is consistent with the results of another clinical trial examining the effect of DHA on the semen analysis of asthenozoospermic men with abnormal sperm motility. In this study, compared to those with placebo, DHA supplementation failed to show an improvement in sperm concentrations and motility, but did not examine sperm morphology (Conquer et al 2000). Animal experiments also support the beneficial effect of n-3 PUFA supplementation on sperm morphology reported in boars (Yeste et al 2011). Similarly, feeding tuna oil reduced the proportion of sperm with abnormal morphology but with higher motility in boars (Rooke et al 2001). The discrepancy in these results from different studies might be related to the species-differences and the different amount and duration of n-3 PUFA supplementation. Whether an early provision of dietary DHA can improve the poor sperm

motility and sperm count in adulthood needs to be further investigated, considering the vital fatty acids in the rodent.

In this study, prenatal EtOH did not appear to affect sperm motility, sperm count and sperm morphology in adult rats. These findings were in agreement with the data reported by McGivern et al., (1992) who found no significant differences in sperm count in adult rats exposed to fetal EtOH, with no report on other parameters. The number of sperm with normal morphology was not significantly lower in EtOH with control diet as compared to DHA-treated groups. The results from a follow-up study of 347 men over two decades showed no alterations in sperm motility and morphology although around 32% decreased sperm concentration was recorded among boys whose mothers consumed ethanol $\geq$ 4.5 drinks/week compared to those consuming <1 drinks/week during gestation (Ramlau-Hansen et al 2010). More studies are required to confirm the adverse effect of prenatal EtOH on sperm parameters.

Conclusion

The effect of dietary DHA on testis function, developmental markers, and sperm parameters in adult rats in maternal EtOH-treated rats has, to our knowledge, not been investigated. The early provision of dietary DHA through dam during pregnancy markedly augmented the level of serum testosterone in the fetus and continuous addition of DHA to the diet of offspring increased testis sperm normal morphology in adult rats, regardless of prenatal EtOH exposure. Moreover, dietary DHA during development impeded the effect of EtOH on circulating testosterone and the gene expression of protein involved in testosterone production during development. Dietary DHA also increased testis developmental markers at peri-puberty compared to those in the controls but was only significant in non-EtOH treated animals. Overall, providing DHA in an energy-dense diet in our study increased sperm quality, likely by increasing testosterone levels in the fetal life. Prenatal EtOH exposure did not adversely affect the overall testis developmental markers during development and sperm parameters in adulthood. Due to the critical importance of testicular lipid composition in testicular functions, it will be

important in future studies to explore whether fetal EtOH exposure affects testicular lipid composition, and whether an early provision of dietary DHA influences the testicular lipid composition during development, particularly in those exposed to prenatal EtOH.

Table 4.1. Primers used for determination of gene expression

Gene	Primers (5'-3')	Reference Sequence	Amplicon size
StAR	CACACTTTGGGGAGATGCCT (S) GGCTGGCGAACTCTATCTGG (AS)	NM_031558.3	198 Bp
Tbp	CCCCGGTGGAAGACAGTTTTA (S) GCCCTGAGCATAAGGTGGAA (AS)	XM_008758748.1	198 Bp

All primers were designed using NCBI primer blast. S, sense primer; AS, anti-sense primer; StAR, steroidogenic acute regulatory; Tbp; TATA box binding protein.

Table 4.2. The effect of dietary DHA on testicular developmental markers from GD20 to PD90 in rats exposed to prenatal EtOH

Histological markers	Cont	Cont+DHA	EtOH	EtOH+DHA	Significant effects (P<)		
					EtOH	Diet	EtOH* Diet
GD20							
Seminiferous Diameter (μm)	45.2 ± 1.6	42.4 ± 2.0	41.5 ± 2.2	41.9 ± 1.7	NS	NS	NS
Number of seminiferous	81.6 ± 1.0	87.6 ± 3.0	82.0 ± 3.8	81.3 ± 5.9	NS	NS	NS
PD49							
Seminiferous Diameter (μm)	261.5 ± 11.0	265.6 ± 7.3	245.4 ± 8.5	243.2 ± 9.6	NS	NS	NS
Height of germinal epithelium (μm)	63.9 ± 2.0^{b}	72.2 ± 1.5^{a}	65.5 ± 0.3^{ab}	68.7 ± 3.3^{ab}	NS	0.03	NS
PD90							
Seminiferous Diameter (μm)	257.9 ± 8.6	259.9 ± 7.2	270.1 ± 4.7	249.5 ± 12.1	NS	NS	NS
Height of germinal epithelium (μm)	60.3 ± 2.6^{ab}	57.2 ± 2.6^{b}	66.3 ± 1.5^{a}	61.9 ± 0.6^{a}	0.01	NS	NS

Mean ± SEM (Gestational day (GD) 20, n=8 per group; Postnatal day (PD) 49, n=2-4 per group; PD90, n=3-4 per group. Main effects were analyzed using two-way analysis of variance (ANOVA); Values having different superscript letters are significantly different among the groups by Duncan's multiple range test (P<0.05). NS, not significant; Cont, control; Cont+DHA, control+DHA; EtOH, ethanol; EtOH+DHA, ethanol+DHA.

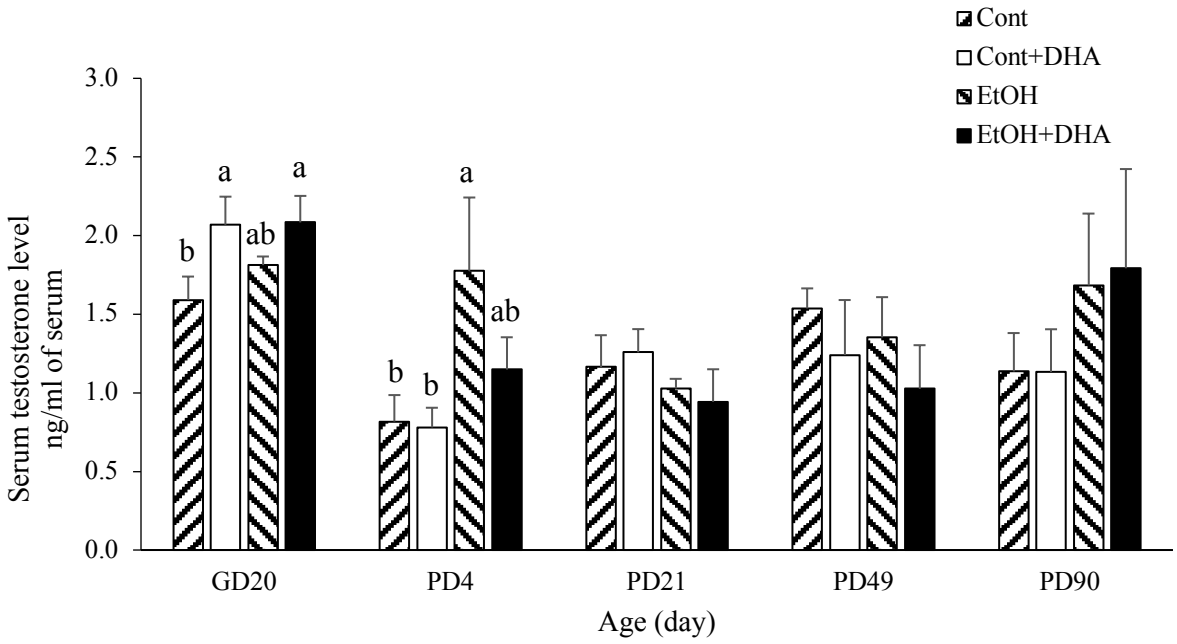


Figure 4.1. The effect of dietary DHA on serum testosterone level from GD20 to PD90 in rats exposed to prenatal EtOH

Mean ± SEM (Gestational day (GD) 20, n=8 per group; Postnatal day (PD) 4, n=5-7 per group; PD21, n=3-5 per group; PD49, n=3-7 per group; PD90, n=5-8 per group). Main effects were analyzed using two-way analysis of variance (ANOVA); Values having different superscript letters are significantly different among the groups by Duncan's multiple range test ($P<0.05$); The effect of DHA, GD20 ($P<0.02$); the effect of EtOH, PD4 ($P< 0.02$). Cont, control; EtOH, ethanol; Cont+DHA, control+DHA; EtOH+DHA, ethanol+DHA.

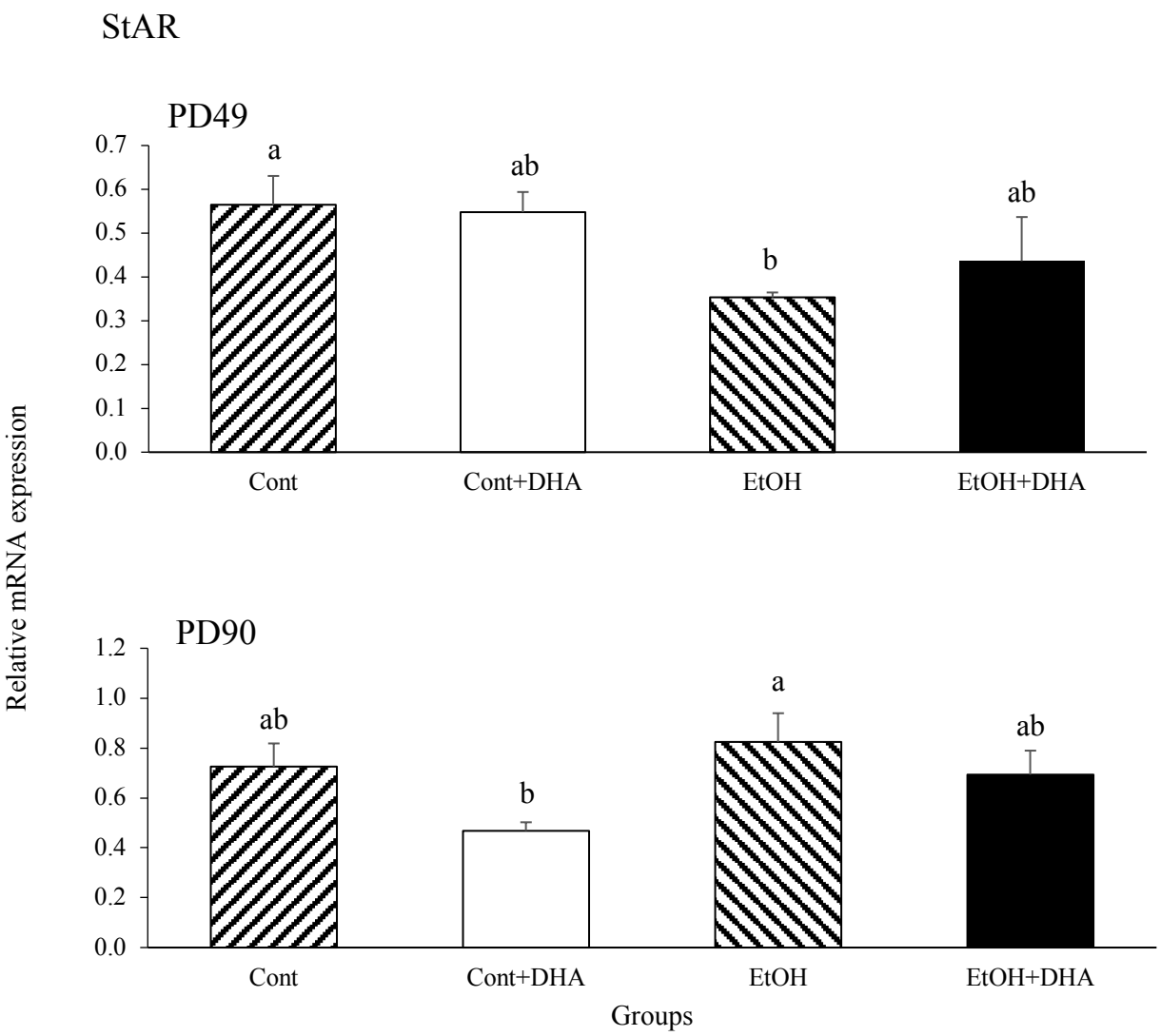


Figure 4.2. The effect of dietary DHA on mRNA expression of StAR at PD49 and PD90 in rats exposed to prenatal EtOH

Bars represent mean ± SEM (Postnatal day (PD) 49, n=3-5 per group; PD90, n=5-6 per group). Measured by $2^{\Delta\Delta Ct}$ using Tbp as the house keeping gene; Main effects were analyzed using two-way analysis of variance (ANOVA); Values having different superscript letters are significantly different among the groups by Duncan's multiple range test ($P<0.05$); The effect of DHA, PD90 ($P<0.04$); EtOH, PD49 ($P< 0.02$). StAR, steroidogenic acute regulatory; Cont, control; Cont+DHA, control+DHA; EtOH, ethanol; EtOH+DHA, ethanol+DHA.

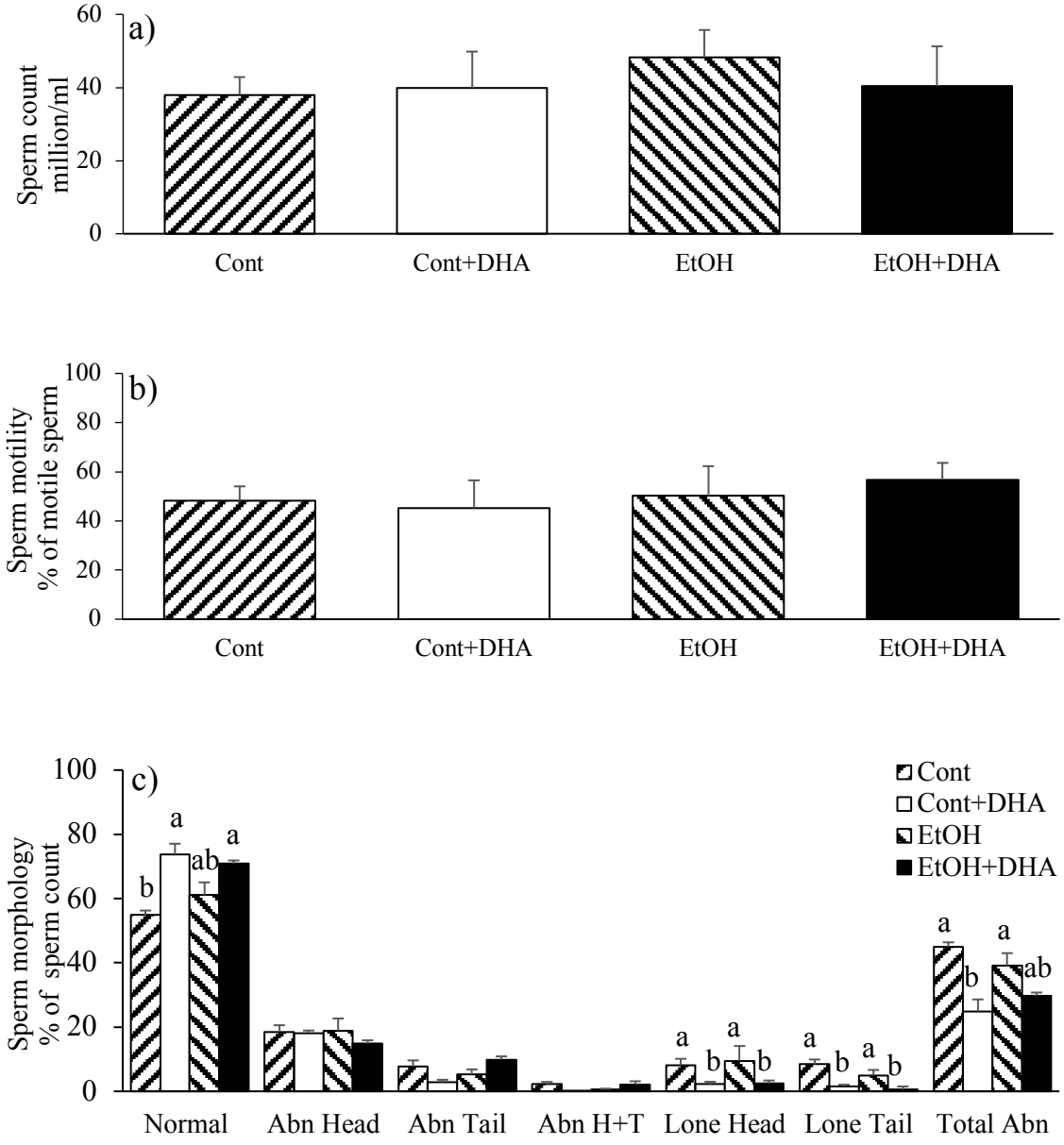


Figure 4.3. The effect of dietary DHA on sperm parameters, a) sperm count, b) sperm motility and c) sperm morphology in adult rats exposed to prenatal EtOH
Mean ± SEM (Sperm count, n=5-9 per group; Sperm motility, n=3-8 per group; Sperm morphology, n= 4-9 per group). Main effects were analyzed using two-way analysis of variance (ANOVA); Values having different superscript letters are significantly different among the groups by Duncan's multiple range test ($P<0.05$); The effect of DHA, Normal ($P<0.001$); Abn, abnormal ($P<0.04$); Lone head ($P<0.05$); Lone tail ($P<0.001$). Cont, control; Cont+DHA, control+DHA; EtOH, ethanol; EtOH+DHA, ethanol+DHA.

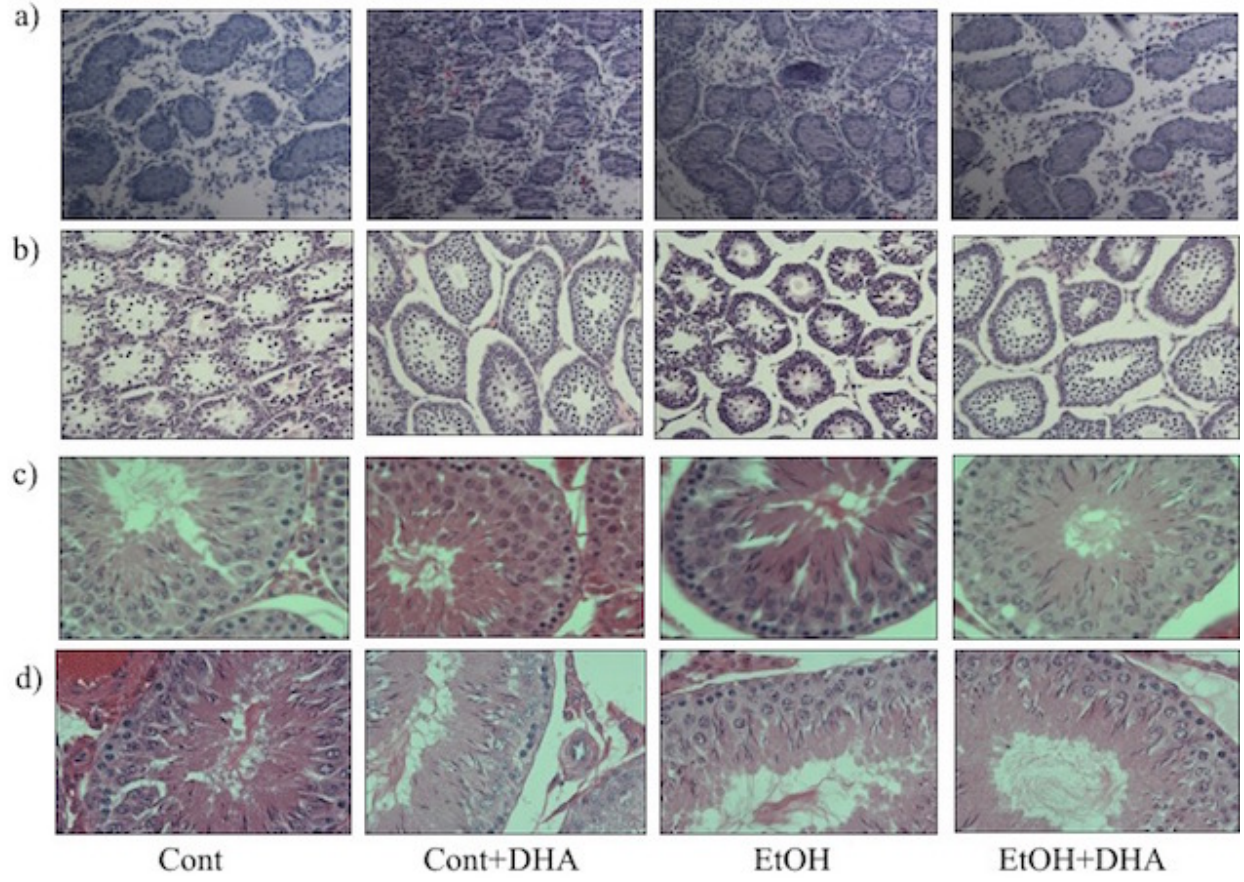


Figure 4.4. The effect of dietary DHA on testicular histology from GD20 to PD90 in rats exposed to prenatal EtOH

Hematoxylin and Eosin (H&E) staining of testis in four groups a) at Gestational day (GD) 20 magnification 200, b) at Postnatal day (PD) 21 magnification 200, c) at PD49 and d) at PD 90 magnification 400; Cont, control; Cont+DHA, control+ DHA; EtOH, ethanol; EtOH+DHA, ethanol+DHA.

CHAPTER 5. FATTY ACID COMPOSITION OF TESTIS DURING DEVELOPMENT ARE DIFFERENTLY RESPONSIVE TO DIETARY DOCOSAHEXAENOIC ACID IN OFFSPRING EXPOSED TO PRENATAL ETHANOL

Will be submitted to "Reproduction, Fertility, and Development" (In preparation)

Abstract

Background and Objective: Dietary docosahexaenoic acid (DHA, C22:6n-3) and ethanol (EtOH) consumption are known to be potent lipid modulators in testis. The present study investigated the effect of dietary DHA on developmental profiles of testis fatty acid composition in rats exposed to prenatal EtOH.

Methods: 9-10-week old female Sprague-Dawley rats were randomly selected to receive either EtOH (3g/kg, twice a day) or dextrose (isocaloric to EtOH) by oral gavage, for the entire pregnancy. Upon pregnancy, dams were fed either control or diet supplemented with (Cont+DHA; n=8, EtOH+DHA n=6) or without DHA (1.4%, w/w total fatty acids) (Cont, EtOH; n=8 dams each) and their pups were continued on their dam's diet after weaning until the termination points. Testes were collected at different stages from fetus at gestational day (GD) 20, neonates at postnatal day (PD) 4, weaning at PD21, peri-pubertal at PD49, and adult rats at PD90 for analyzing the testis fatty acid composition.

Results: Fatty acid composition of testis was undergone significant changes during development. The striking alterations were an elevation in n-6 polyunsaturated fatty acid (PUFA), predominantly n-6 docosapentaenoic acid (DPA), and n-6 very long chain fatty acids (VLCFA) mostly at PD21 and PD49, respectively, replacing n-3 PUFA, monounsaturated (MUFA) and saturated fatty acids (SFA). Dietary DHA induced a significant ($P<0.0002$) accumulation of DHA in testis at all ages regardless of prenatal EtOH exposure, with the highest at PD49. In contrast to immature testis, n-6 DPA and n-6 VLCFA level were not affected by diet, prenatal

EtOH exposure or their interaction in mature testis from PD49. Prenatal EtOH increased ($P<0.006$) both n-3 PUFA and DHA only in fetal testis.

Conclusion: n-6 DPA and n-6 VLCFA seem to be the major fatty acids in the rat testis, increasing during development. Testis at PD49 revealed a feature of the adult testis in response to the dietary DHA, with no changes in testis n-6 DPA and n-6 VLCFA level amongst the groups. Prenatal EtOH did not adversely affect the lipid composition in testis during development.

Keywords: docosahexaenoic acid, docosapentaenoic acid, prenatal ethanol, diet, testis, development

Introduction

The testis lipid environment is characterized by a relatively large amount of polyunsaturated fatty acids (PUFA) with a 22 carbon chain, increasing during development. Thus, it is believed that they play an important role in testis development and functions (Ayala et al., 1977; Bieri et al., 1969; Burr and Burr, 1930; Coniglio, 1994). Testicular lipid compositions are species specific (Suh et al., 2011). Rat testicular plasma membrane is richly endowed in docosapentaenoic acid (DPA, C22:5n-6) while docosahexaenoic acid (DHA, C22:6n-3) predominates in the human testes (Retterstøl et al., 2001). One of the earliest studies found that the huge rise in the level of n-6 DPA in rat testis during development paralleled their functional and structural development leading to germinal maturity (Davis et al., 1966). The details of the n-6 DPA developmental profiles, however, are still unclear.

The study of lipid metabolism in male fertility led to the discovery of the essential role of PUFA in fertility problems. Most studies agree that the distinctively high level of DHA in human spermatozoa is associated with normal sperm quality and quantity (Aksoy et al., 2006; Zalata et al., 1998), which are the markers of normal fertility. Animal studies revealed that testicular degeneration in rats, induced by environmental insults, caused a significant decline in testis n-6 DPA level (Davis and Coniglio, 1967). Underdeveloped testis, obtained from obese Zucker rats, also revealed significantly a lower level of n-6 DPA in rat testis (Suh et al., 2011; Zanetti et al., 2007). This evidence suggests that the level of n-6 DPA might be used as a marker of testis maturity in male rats.

It is well-known that testis membrane fatty acids are affected by dietary fat types, which are directly related to the maintenance of the cell membrane for its optimal functions. Dietary fat intake can thus affect the male reproductive function by modulating the fatty acid compositions of testis and spermatozoa (Bieri et al., 1969; Brinsko et al., 2005; Yan et al., 2013; Zaniboni and

Cerolini, 2009). A diet enriched in n-3 PUFA, DHA in particular, showed improved sperm parameters in humans (Comhaire et al., 2000; Robbins et al., 2012; Safarinejad, 2011) and increased testosterone production in rats (Sebokova et al., 1990). Supplementation of n-3 PUFA also improved sperm parameters in a rat model of non-alcoholic fatty liver disease (Li et al., 2013). Furthermore, the testis of obese rats responded more to n-3 PUFA dietary intervention, with 2-fold more DHA incorporation into the testis than those in the lean group (Suh et al., 2011). Studies on rats supplemented with dietary DHA during development showed that n-6 DPA could be functionally replaced by DHA in the testis (Ayala and Brenner, 1983; Ayala et al., 1977). These studies showed that the rat testis is well-responded to dietary lipids and n-3 PUFA dietary intervention can improve the reproductive function of species with either DHA or n-6 DPA as the predominant testicular fatty acid.

The development and functions of the offspring male reproductive system are known to be negatively affected when exposed prenatally to ethanol (EtOH) (Carter et al., 2014; Damgaard et al., 2007; Håkonsen et al., 2014; Ramlau-Hansen et al., 2010). Fetal EtOH exposure can disrupt testicular hormonal balance with downstream impacts on pubertal development and sperm production later in life (Fakoya and Caxton-Martins, 2004; Handa et al., 1985; Lan et al., 2013; McGivern et al., 1992; Udani et al., 1985). EtOH exposure during gestation exerted lipid modulatory effects influencing the level of DHA in some tissues, such as brain in offspring with neurodevelopmental dysfunction (Burdge and Postle, 1995), but its impact on the testicular lipid composition has not been explored. Therefore, both dietary lipid and EtOH consumption are lipid modulators in tissues and can affect testis function.

This study determined the fatty acid composition of the testis of rats during development, from fetus to adulthood, and examined how dietary DHA might affect these fatty acid alterations in those exposed to prenatal EtOH.

Material and Method

Animal and treatments

The timeline of experiments, EtOH and dietary treatments, are illustrated schematically in **Figure 3.1** in Chapter 3. All experimental procedures were performed under a protocol approved by the University of Manitoba Animal Care Committee following the principles and guidelines of the Canadian Council on Animal Care (CCAC, 1993). Female Sprague-Dawley rats (9-10 weeks old; n= 30) were obtained from Charles Rivers Breeding Laboratories (Sherbrooke, Quebec, Canada). Animals were housed in an environmentally controlled room with a 12:12-hr light-dark cycle and allowed free access to standard rat chow and water. After three days of acclimation, EtOH was gradually introduced to animals by increasing the dose of EtOH from 1/3 to 3/3 to reach the final dose for the first three days (Chappell et al., 2007). Female rats were randomly assigned to receive by gavage either EtOH (3g/kg twice/day, 50%, w/v, n=14), representing a moderate dose of EtOH exposure (Schambra et al., 2015), or dextrose (Cont, 80% (w/v), isocaloric to EtOH, n=16). This level of EtOH equals the amount reached by women having 1-2 drinks within an hour (Schambra et al., 2015). After EtOH adaptation, two female rats were housed with one male for mating. The control diet was used during the time of EtOH adaptation and mating period. Treatment with EtOH or dextrose continued for the entire pregnancy and dams were abstained from EtOH after parturition.

Diets

Experimental diets were formulated in our laboratory from a nutritionally complete semi-

purified diet, as described in Chapter 3, **Table 3.1,** with the only difference being in the level of DHA (1.4% (w/w) of total fatty acids) between the two experimental diets, which has been reported to be a sufficient amount of DHA for pregnant rats and growing pups (Wang et al., 2018; Weiser et al., 2015). The total energy density was 4.17 Kcal/g diet composed of carbohydrate (50%), protein (17.5%) and fat (32.5%), reflecting nutrient intake of the pregnant mother in North America (Denomme et al., 2005; Friesen and Innis, 2010; Hui et al., 2014). Upon confirmation of pregnancy, dams were housed individually and in each group, randomly assigned to receive either a DHA-enriched diet or control diet on gestational day (GD) 0, defined as the first day of pregnancy. Therefore, a total of 4 groups were available: i) control without EtOH (Cont, n=8); ii) control with DHA (Cont+DHA, n=8) iii) EtOH without DHA (EtOH, n=8); iv) EtOH with DHA (EtOH+DHA, n=6). Dams were fed these diets for the entire pregnancy and throughout the lactation period. After weaning at postnatal day (PD) 21, the pups received the same diet as their mothers until PD90. Animals had access to water and food *ad libitum*. On GD20, half of the dams in all four groups were anesthetized via isoflurane and cardiac puncture. The ovarian horn was removed and using dissecting microscope male fetuses were identified by their anogenital distance, weighed, and then decapitated. The remaining dams were allowed to give birth naturally. At PD4, litter was randomly culled to 8 pups/dam with equal male to female sex ratio to allow equal access to dam's milk. Pups were remained with their mothers until weaning. Offspring were terminated at different time points at GD20 (fetus), PD4 (neonate), PD21(weanling), PD49 (peri-puberty), and PD90 (adult) for analysis of testis lipid composition. The termination days chosen are based on the rodent male sexual development (Clegg, 1960; Ojeda et al., 1980; Zanetti et al., 2007), as explained in Chapter 4.

Fatty acid analysis

Testis fatty acids were analyzed in a manner similar to that of the simplified method described by Kang and Wang (Kang and Wang, 2005). Saponification was carried out by adding 0.5 ml of a 0.5 M aqueous solution of methanolic potassium hydroxide (KOH) to ~7 mg decapsulated testis, collected from different developmental stages, followed by homogenization with a Dounce homogenizer. The methylation procedure was conducted using 1 ml of distilled hexane and 1 ml of 14% boron trifluoride in methanol (BF3) in methanol to the saponified samples and then they were heated for one hour at 110°C (Suh et al., 2009). The fatty acid methyl esters were analysed by gas chromatography. Separation of fatty acid methyl esters was carried out using a microcapillary column (15 m × 0.1 mm inner diameter and 0.2 m film thickness; BPX70; NC, USA), and a chromatography system (Vista 6010 GLC and Vista 402 data system; Varian Instruments, Mississauga, ON, Canada). Hydrogen was used as a carrier gas under a flow rate of 0.5 mL/min. The initial temperature was 130°C, then increased to 175°C at 20°C/min held for 1 min, then was increased to 200°C at 6°C/min, held for 0.5 min, raised again to 200°C at 6°C/min with no hold and finally increased to 280°C at 30°C/min. The injector/detector temperatures were 270°C and 290°C, respectively. Standard FAME 461 (Nucheck Prep Inc. Waterville, MN, USA) was used for peak identification.

Determination of gene expression

The decapsulated testis at PD49 and PD90 was homogenized and total RNA was isolated according to the Trizol method (Chomczynski and Sacchi, 1987). As described by the manufacturer, 1 ml Trizol was added to 100 mg tissues with the use of RNA prep Tissue Kit (Invitrogen, Canada). RNase-free DNAse I enzyme was used to remove contaminating genomic DNA (Promega, Madison, WI, USA). RNA quantity and purity of all samples were verified by

using Nano-drop spectrophotometer 2000 (Thermo Scientific, Canada). 1.5 μg of total RNA was reverse-transcribed into cDNA with a High-Capacity cDNA Reverse Transcription kit, as described by the supplier's protocol (Applied Biosystems, Canada). Pairs of primers for each gene, presented in (**Table 5.1)**, were designed using NCBI primer blast, from the mRNA sequence of the target gene and purchased from University core and services, University of Calgary). Real-time quantitative polymerase chain reaction (RT-qPCR) was conducted using a CFX Connect TM qRT-PCR Detection System (Bio-Rad, Canada) as described by the manufacturer. The reactions were in duplicate including the forward and reverse primers of target gene, nuclease free water, samples cDNA and SYBR Green as a detector. Samples were run for 40 cycles (95°C for 15 secs for denaturation, 59°C for 20 secs for annealing/extension) using the Applied Biosystems StepOnePlus Real-Time PCR System. TATA box binding protein (Tbp) used as a housekeeping gene to normalize the relative expression of the target genes that is identified as stably expressed gene in testis (Svingen et al., 2009). Comparative ΔΔCt method was generated for data analysis (Livak and Schmittgen, 2001).

Statistical Analysis

Statistical analysis was analyzed using two-way analysis of variance (ANOVA) for the main factors of prenatal EtOH exposure and dietary DHA. Multiple comparisons were tested using Duncan post-hoc test when an interaction between EtOH and diet was observed. When there was no interaction effect identified, the ANOVA was re-tested for the main factors, which enabled us to test against the correct degree of freedom of error. Student *t*-test was used to compare the data from GD20 and PD90. All data are expressed as mean ± standard deviation of mean (SD). Statistical significance was set at $P< 0.05$.

Results

Developmental profiles of fatty acid compositions in testis during development from fetus to adulthood

Developmental profiles of total and individual fatty acids in developing testis from fetus to adult rats are shown in **(Figure 5.1 and 5.2).** The level of total saturated fatty acid (SFA) decreased during the development, with a significant reduction ($P<0.05$) at PD90 than GD20. Overall, total SFA were the most abundant fatty acids from the fetal stage to PD21, comprising approximately 41% (w/w) of the total fatty acids in testis. Among SFA, palmitic acid (C16:0) was quantitatively the highest SFA at all ages, increasing considerably in the developing testis, from 21% to 31% (w/w of total fatty acids) during development, whereas the abundance of stearic acid (C18:0) diminished as the testis matured, from 16% to 7% (w/w of total fatty acids). The level of total monounsaturated fatty acid (MUFA) in rat testis was characterized by a significant constant decrease ($P<0.05$) from GD20 to PD90, dropping from around 28% to 12% (w/w of total fatty acids). Total n-3 PUFA also showed a similar significant ($P<0.05$) decrease as did total MUFA. However, total n-6 PUFA gradually increased from 26% at GD20 to 43% (w/w of total fatty acids) at PD90, becoming the dominant fatty acid class at PD49 and PD90. Among n-6 PUFA, linoleic acid (LA, C18: 2n-6) and arachidonic acid (AA, C20:4n-6) comprised approximately 3-4% (w/w) and 15-16% (w/w of total fatty acids), respectively. AA level remained relatively constant throughout development. AA was the major n-6 PUFA in early life until PD49, but n-6 DPA increased throughout development becoming the main n-6 PUFA in the mature testis. The level of DHA was slightly higher than n-6 DPA in early life at GD20 and PD4 but decreased significantly as testis develops from GD20 to PD90 ($P<0.05$). Similar to n-6 DPA, there was a marked increase in total n-6 very long chain fatty acids (VLCFA) at PD21, with the

highest level at PD90. No specific effects of EtOH on fatty acids were identified during testis maturation.

Dietary DHA and testis fatty acid composition in rat exposed to prenatal EtOH

Testis fatty acid composition at GD20

The major fatty acid detected in fetal rat testis was SFA followed by MUFA comprising approximately 40% and 27% (w/w) of total fatty acids, respectively. The fetal testis is comprised of 25-28% (w/w) of total n-6 PUFA, whereas n-3 PUFA accounts for about 5-8% (w/w). The level of n-6 VLCFA found to be minor, at around 1% (w/w of total fatty acids), in rat testis at GD20 (**Table 5.2**). There was a significant independent effect of dietary DHA for total PUFA, with an increase in n-3 PUFA and a reduction in n-6 PUFA in comparison to the control diet ($P<0.0001$). Of these fetal testis, DHA level was greater (~1.8 times) in DHA-treated groups than controls. Regardless of EtOH exposure, dietary DHA fed to dam significantly increased n-3 docosapentaenoic acid (DPA, C22:5n-3) in testis ($P<0.0001$), whereas AA decreased ($P<0.004$) by dietary DHA, which was more pronounced in those exposed to prenatal EtOH. Both total and individual n-6 VLCFA, C24:4n-6 and C24:5n-6 were reduced ($P<0.0001$) by adding DHA to the diet. Exposure to EtOH significantly increased the level of DHA and total n-3 PUFA compared to the non-EtOH-treated groups ($P<0.006$). There was an interaction between EtOH and DHA for n-6 DPA precursor, LA, and n-6 DPA levels, being the highest ($P<0.04$) and the lowest ($P<0.0007$) in EtOH+DHA group, respectively.

Testis fatty acid composition at PD4

Neonatal rat testis contained approximately 41% SFA, 24% MUFA, 5-6% n-3 PUFA, 29-30% n-6 PUFA and 1% n-6VLCFA (w/w of total fatty acids) at PD4 (**Table 5.3**). The response

of testis fatty acid composition to DHA diet at PD4 was almost similar to GD20. The animals fed dietary DHA had a trend of elevated total n-3 PUFA, with a 1.6 fold increase in testis DHA level when compared to those fed the control diet ($P<0.0001$). Dietary DHA intervention exerted a significant reducing effect on total and individual n-6 PUFA and n-6 VLCFA, n-6 DPA, C24:4n-6 and C24:5n-6 ($P<0.04$) at PD4. A significant ($P<0.0001$) reduction was also observed in n-6/n-3 PUFA ratio in DHA diet compared to the control diet. Prenatal EtOH exposure or its interaction with diet did not significantly affect total and major individual fatty acid composition at PD4.

Testis fatty acid composition at PD21

The testis composition of three-week-old pups was approximately 41% SFA, 21% MUFA, 3-6% n-3 PUFA, 28-32% n-6 PUFA and 3% n-6VLCFA (w/w of total fatty acids) (**Table 5.4**). There was a significant independent effect of dietary DHA at PD21. In keeping with the results observed for neonatal testis, total n-3 PUFA increased but n-6 PUFA decreased and were responsible for a lower ratio of n-6/n-3 PUFA in DHA treated groups than in the controls ($P<0.0001$). By adding DHA to the dam's diet, DHA level increased ($P<0.0001$) around 2.1 times relative to that of animals fed by control diet. Dietary DHA also increased ($P<0.002$) the level of LA in both DHA-treated groups, regardless of EtOH exposure. However, AA level remained significantly unchanged in all groups. Compared to non-EtOH-exposed animals, prenatal EtOH exposure independently did not affect testis lipid composition. The interaction between EtOH and diet was significant ($P<0.002$) for n-6 DPA and n-6 VLCFA, being the lowest in Cont+DHA group of all the groups.

Testis fatty acid composition at PD49

Rat testis at PD49 contained approximately 40% SFA, 14% MUFA, 2% n-3 PUFA, 40% n-6 PUFA and 4 % n-6VLCFA (w/w of total fatty acids) (**Table 5.5**). At the same trend as

immature testis, dietary DHA increased significantly total n-3 PUFA and DHA (2.2 times) at PD49, regardless of prenatal EtOH exposure to those fed the control diet ($P<0.0003$). The testis ratio of n-6/n-3 PUFA also decreased in DHA-treated groups ($P<0.0001$). Unlike the previous ages, DHA did not change testis n-6 DPA level and total n-6 PUFA in peri-puberty. Similarly, dietary DHA did not exert a reducing impact on total n-6 VLCFA but conversely, elevated some individual n-6 VLCFA, including C24:5n-6 relative to controls ($P<0.005$). However, a higher level of LA remained the highest ($P<0.0005$) in DHA treated groups than the controls. The independent effect of prenatal EtOH and its interaction with diet for testicular fatty acid composition at PD49 was not statistically significant.

Testis fatty acid composition at PD90

Rat testis at PD90 contained approximately 39% SFA, 13% MUFA, 1% n-3 PUFA, 43% n-6 PUFA and 5% n-6VLCFA (w/w of total fatty acids) (**Table 5.6**). The effect of dietary DHA was significant for both total and major individual SFA, palmitic acid (C16:0), and increased compared to those in control diet ($P<0.03$). However, dietary DHA reduced total MUFA in comparison to the controls ($P<0.002$). Similar to PD49, dietary DHA induced alterations only in total n-3 PUFA and DHA (~1.8 times) ($P<0.0001$) but not in total n-6 PUFA and n-6 VLCFA and n-6 DPA compared to non-DHA groups. The testis ratio of n-6/n-3 PUFA also decreased in DHA-treated groups ($P<0.0001$). Dietary DHA also increased ($P<0.02$) the level of LA at PD90, as shown in previous ages. The alterations induced by prenatal EtOH exposure were not statistically significant at PD90. The interaction between EtOH and diet was significant, with n-6 DPA level in EtOH+DHA being the lowest amongst the groups.

The effect of dietary DHA on gene expression of enzymes involved in testis lipid metabolism at PD49 and PD90 in rats exposed to prenatal ethanol

The results for gene expression of enzymes involved in testicular lipid metabolism of the peri-pubertal and adult rats at PD49 and 90 have been shown in (**Figure 5.3 and 5.4).** There was no significant independent effect of diet on the expression of genes involved in lipid metabolism in testis at PD49 or PD90. Prenatal EtOH exposure significantly increased mRNA expression of FADS1 ($P<0.007$) and FADS2 ($P<0.0004$) increased in both EtOH- treated animals, EtOH and EtOH+DHA groups, compared to Cont+DHA group at PD90. Furthermore, at the same age, FABP9 mRNA expression was greater ($P<0.002$) in EtOH-exposed groups compared to Cont group. The interaction between EtOH and diet was significant at PD49 ($P<0.03$), reducing FADS2 mRNA expression in offspring in EtOH+DHA group than those of pups in the Cont+DHA group. Prenatal EtOH exposure did not affect the ELOVL2 at each time point.

Discussion

This study examined the developmental profiles of fatty acid composition in testis, from fetus to adulthood, and how dietary DHA and prenatal EtOH exposure might affect the fatty acid composition during testis development. The major findings were the considerable increase in n-6 PUFA, mainly n-6 DPA, replacing n-3 PUFA and MUFA. Both n-6 DPA and n-6 VLCFA were increased drastically at PD21and PD49. By adding DHA to the diet of dams and offspring, testis DHA level increased at all age groups, with the greatest response occurring at PD49. Dietary DHA also reduced the level of n-6 DPA and n-6 VLCFA in testis at all ages except for PD49 and PD90. In this study, it is evident that prenatal EtOH exerted minimal effects on the testis fatty acid profiles. Overall, regardless of DHA provision in the diet, the characteristic increase of n-6 DPA and n-6 VLCFA coincides with age of sexual maturation. Testis specific fatty acids response to the dietary DHA were age-dependent.

Developmental profiles of fatty acid composition in testis during development from GD20 to PD90

Developmental profile of testis fatty acids from GD20 to PD90 rats was distinctive. During testis maturation, n-6 PUFA became the major fatty acids, while a concomitant decrease occurred in n-3 PUFA and MUFA. SFA levels maintained relatively constant throughout testis development except for adult rats relative to fetus. n-6 VLCFA also increased with testis maturation. A drastic enrichment in n-6 PUFA contributed predominantly by n-6 DPA, occurred at the peri-pubertal age (PD49), which is comparable to 11.5 years in human concerning sexual maturation (Quinn, 2005). The level of n-6 VLCFA began to increase at PD21, which is about equivalent to a 6-year-old human (Quinn, 2005). In agreement with our findings, a previous study found alterations of fatty acids in the developing testis had most modification taking place from PD28 to PD49 (Davis et al., 1966). This might suggest that PUFA accretion with DHA occurs concurrently with the sexual maturation in human as a predominant testis fatty acid. In contrast to n-6 DPA, AA a major n-6 PUFA in immature testis was maintained remarkably unchanged as testis matures. Similar findings in AA has been reported in the human testis during development (Coniglio et al., 1975). Moreover, the level of AA has not been changed in degenerated testis as well (Coniglio et al., 1975). Thus, elongation and desaturation from AA to n-6 DPA may work differently during development and disease states in testis, which needs further investigation.

The considerable increases in the level of n-6 PUFA during sexual maturation in rat testis indicate that the enzymatic system is in favor of n-6 PUFA, rather than n-3 PUFA. This is opposite from n-3 PUFA enrichment in brain and retina. It is clear that these testicular fatty acid changes coincide with the several phenomena that occur in testis leading to sexual maturity, including increased number and activity of Leydig cells, the maturity of Sertoli cells, the first

cycle of spermatogenesis, the production of primary spermatocyte and then appearance of full spermatogenesis with mature spermatozoa in seminiferous tubules (Clegg, 1960). Therefore, testis n-6 DPA and n-6 VLCFA content might be associated with sexual maturation in rat testis.

Fetal testis had a higher level of DHA compared to mature testis that is accompanied by some critical events in testis, including the fetal surge of testosterone and Sertoli cells proliferation at this age. Our results from the previous study showed an enhanced testosterone level at only GD20 in DHA-treated animals (Chapter 4). If DHA level at fetal rat testis exerts any vital role in testis function needs to be further investigated.

Dietary DHA on fatty acid composition of testis and expression of enzymes involved in lipid metabolism in rats exposed to prenatal EtOH

Dietary DHA induced a consistent change by increasing total n-3 PUFA and DHA level, regardless of fetal EtOH exposure or testis maturity, even though DHA is not a major fatty acid in rat testis. This study depicts that fetal and postnatal testis at PD4 and PD21 are responsive to maternal DHA diet either via placenta or breast milk. In our findings, the accumulation of DHA was highest at PD49, the period during which testis undergoes the most changes to become functionally mature, and was lowest at PD4 at which testis is functionally inactive.

It appears that the rat testis maintains a rather constant proportion of the n-6 DPA precursor, AA, with the dietary intervention of DHA. This might be related to the fatty acid composition in the formulated diet. In the diet used in this study, there was no EPA to compete with AA metabolism so that its level maintains the same in both DHA and control diet. The previous report also showed that the dietary intervention in the adult rat testis only changed the LA-derived fatty acids at the high dose of dietary LA. These changes were clearly pronounced in n-6 DPA than AA (Bieri and Prival, 1965). This might indicate that AA and n-6 DPA are

differently responsive to the dietary fatty acid content as observed in this study. Dietary DHA in our study induced a significant reduction in the level of n-6 DPA in testis during development in immature testis from GD20 to PD21. This might be directly related to higher level of DHA in those with DHA treatment that competes with n-6 DPA on incorporation into the testis lipid membrane. Moreover, n-6 DPA synthesis might be inhibited by dietary DHA by affecting the enzymatic system involved in PUFA synthesis. This might suggest that the lower level of n-6 DPA in the immature testis in DHA-treated animals is related to the inhibitory effect of dietary DHA on the activity of those enzymes involved, reducing the production of n-6 DPA only in immature testis, not in the mature one. Therefore, it is no surprise to observe no significant changes in the gene expression of enzymes involved in the production of n-6 DPA, FADS1 and FADS2, by dietary DHA in mature testis. The testis response to the dietary DHA in relation to total n-6 VLCFA paralleled that of n-6 DPA during development since n-6 PUFA are synthesized from n-6 PUFA precursors (Agbaga et al., 2010). This might indicate the ability of testis at PD49 and PD90 to control the level of testicular functionally vital fatty acids over the dietary lipid consumption likely via regulating the related enzymes. This ability of testis at PD49 is coinciding with an initiation of a full spermatogenic process (Clegg, 1960), suggesting that testis at PD49 shows a feature of the mature testis.

The independent effect of fetal EtOH exposure was demonstrated mostly at GD20, when EtOH crosses the placenta and reaches the fetus. EtOH exposure during pregnancy induced an increase in testis LA level with no changes in the level of AA in EtOH-treated animals, indicating the possibility of a higher uptake of this fatty acid by EtOH. Similarly, maternal EtOH increased testis n-6 DPA, DHA, and n-3 PUFA only if fetus was obtained from non-DHA consuming dams. This might be related to the effect of dietary DHA on down-regulation of testis

fatty acid transporters in DHA-treated group to blunt the effect of EtOH, as observed in the brain of pups treated by DHA-rich diet (Elsherbiny et al., 2015). In contrast to our results, the previous report showed reduced accumulation of DHA into the brain in the prenatal-EtOH exposed group (Burdge and Postle, 1995). This discrepancy might be related to the tissue-specific difference, or the difference in the dose or administration or duration of the EtOH exposure.

Conclusion

In conclusion, testis fatty acid composition changed significantly during development, with a substantial increase in n-6 PUFA, mainly n-6 DPA, replacing n-3 PUFA, predominantly DHA, and MUFA. Concurrently, the trend of total n-6 VLCFA resembled that of n-6 PUFA. Regardless of prenatal EtOH exposure and testis maturation, both maternal and post weanling dietary DHA significantly enhanced testis DHA level. Immature testis responded the dietary DHA to have lower level of n-6 PUFA, mainly n-6 DPA, and n-6 VLCFA. However, testis at PD49 revealed a feature of mature testis in adulthood, with no reduction in n-6 PUFA and VLCFA. Testis at PD49 also exerted the highest accumulation of n-6 DPA amongst ages and the most accretion of DHA in DHA-treated groups. These results support that the rat testis is specialized for n-6 PUFA especially n-6 DPA as well as n-6 VLCFA and likely their role in sexual maturity occur at the same age, regardless of dietary DHA. Prenatal EtOH exerted a minimal effect on these alterations. Further investigation on the sensitivity of these fatty acids to dietary DHA in testis lipid classes is also interesting as their incorporation are specifically varied among different lipid profiles.

Table 5.1. Primers used for determination of gene expression

Gene	Primers (5'-3')	Reference Sequence	Amplicon size
FADS1	CATCAGCCACTACGCTGGTC (S) AGCGCCTTATTCTTGGTGGG (AS)	NM_053445.2	150 Bp
FADS2	GGAGGAGTAGAAGACAAAAGCTG (S) GTTACCTCCCTTCCCCATGCT (AS)	NM_031344.2	154 Bp
FABP9	TGCAGACAACCGGAAAGTGA (S) GGCGTCTACCCATCTGCTAC (AS)	XM_017022088.1	191 Bp
ELOVL2	GCGGAATTCTTGGACAACATGTTTGGACCA (S) GACTGCGGCCGCCGCTTCACCTCATTGCACCTTCTTG (AS)	NM_001109118.1	231 Bp
Tbp	CCCCGGTGGAAGACAGTTTTA (S) GCCCTGAGCATAAGGTGGAA (AS)	XM_008758748.1	198 Bp

S, sense primer; AS, anti-sense primer; FADS, fatty acid desaturase enzyme, FABP, fatty acid binding protein; ELOVL, elongation of very long chain fatty acid protein; Tbp, TATA box binding protein.

Table 5.2. The effect of dietary DHA on testis fatty acids at GD20 in rats exposed prenatal EtOH

Fatty acids (%, w/w)	Cont (n = 4)	Cont+DHA (n = 4)	EtOH (n = 8)	EtOH+DHA (n = 3)	Significant effects (P<)		
					EtOH	Diet	EtOH* Diet
C14:0	0.85 ± 0.18	0.76 ± 0.07	0.63 ± 0.30	0.75 ± 0.14	NS	NS	NS
C16:0	22.08 ± 1.16	21.26 ± 0.67	21.45 ± 0.54	21.00 ± 0.32	NS	NS	NS
C18:0	16.15 ± 0.62	16.31 ± 0.65	16.07 ± 0.67	16.33 ± 0.37	NS	NS	NS
C18:1n-9	17.70 ± 0.92^{b}	18.53 ± 0.83^{ab}	17.90 ± 0.41^{ab}	19.04 ± 1.45^{a}	0.02	NS	NS
C18:1n-7	3.86 ± 0.39	3.92 ± 0.26	3.90 ± 0.29	2.91 ± 1.16	NS	NS	NS
C18:2n-6	3.64 ± 0.28^{c}	4.12 ± 0.09^{b}	3.93 ± 0.16^{b}	4.41 ± 0.11^{a}	0.001	0.0001	0.04
C18:3n-3	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	NS	NS	NS
C20:0	0.32 ± 0.04	0.28 ± 0.04	0.28 ± 0.06	0.33 ± 0.02	NS	NS	NS
C20:3n-6	0.70 ± 0.07^{c}	0.84 ± 0.07^{ab}	0.76 ± 0.07^{bc}	0.92 ± 0.03^{a}	0.03	0.0001	NS
C20:4n-6	15.20 ± 0.66^{a}	14.70 ± 0.36^{ab}	15.29 ± 0.31^{a}	14.52 ± 0.23^{b}	NS	0.004	NS
C20:5n-3	0.20 ± 0.01^{b}	0.20 ± 0.01^{b}	0.19 ± 0.02^{b}	0.23 ± 0.02^{a}	NS	NS	0.03
C22:4n-6	4.15 ± 0.20^{a}	3.17 ± 0.25^{b}	3.95 ± 0.14^{a}	3.25 ± 0.11^{b}	NS	NS	NS
C22:5n-6	2.88 ± 0.31^{b}	1.36 ± 0.12^{c}	3.50 ± 0.21^{a}	1.19 ± 0.04^{c}	NS	NS	0.0007
C22:5n-3	0.52 ± 0.04^{b}	0.74 ± 0.08^{a}	0.57 ± 0.05^{b}	0.78 ± 0.10^{a}	NS	0.0001	NS
C22:6n-3	3.39 ± 0.16^{c}	6.49 ± 0.18^{a}	4.00 ± 0.26^{b}	6.60 ± 0.36^{a}	0.006	0.0001	NS
C24:4n-6	0.21 ± 0.01^{a}	0.16 ± 0.02^{b}	0.20 ± 0.01^{a}	0.18 ± 0.01^{b}	NS	0.0001	NS
C24:5n-6	0.47 ± 0.03^{a}	0.32 ± 0.04^{b}	0.48 ± 0.02^{a}	0.31 ± 0.01^{b}	NS	0.0001	NS
SFA	41.32 ± 2.21	39.86 ± 1.05	39.83 ± 0.57	39.84 ± 0.34	NS	NS	NS
MUFA	26.48 ± 1.29	27.25 ± 1.01	26.51 ± 0.41	26.98 ± 0.51	NS	NS	NS
n-3 PUFA	4.21 ± 0.15^{c}	7.51 ± 0.10^{a}	4.85 ± 0.27^{b}	7.69 ± 0.44^{a}	0.002	0.0001	NS
n-6 PUFA	27.15 ± 1.28^{a}	24.95 ± 0.35^{b}	27.98 ± 0.59^{a}	24.92 ± 0.37^{b}	NS	0.0001	NS
n-6VLCFA	0.84 ± 0.07^{a}	0.59 ± 0.08^{b}	0.81 ± 0.07^{a}	0.58 ± 0.06^{b}	NS	0.0001	NS
n-6/n-3 PUFA	6.46 ± 0.41^{a}	3.31 ± 0.03^{c}	5.78 ± 0.29^{b}	3.25 ± 0.13^{c}	0.009	0.0001	0.03

Mean ± SD. Main effects were analyzed using two-way analysis of variance (ANOVA). Values having different superscript letters are significantly different among the groups by Duncan's multiple range test (P<0.05). NS, not significant; AA, arachidonic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; VLCFA, very long chain fatty acids; GD20, gestational day 20; Cont, control; Cont+DHA, control+DHA; EtOH, ethanol; EtOH+DHA, ethanol+DHA.

Table 5.3. The effect of dietary DHA on testis fatty acids at PD4 in rats exposed to prenatal EtOH

Fatty acids (%, w/w)	Cont (n = 3)	Cont+DHA (n = 4)	EtOH (n = 3)	EtOH+DHA (n = 4)	Significant effects (P<)		
					EtOH	Diet	EtOH* Diet
C14:0	0.58 ± 0.17	0.52 ± 0.15	0.62 ± 0.08	0.58 ± 0.04	NS	NS	NS
C16:0	23.01 ± 0.20	21.81 ± 0.42	22.28 ± 1.76	21.45 ± 0.79	NS	NS	NS
C18:0	15.62 ± 0.42	16.15 ± 0.76	15.67 ± 0.98	16.10 ± 0.56	NS	NS	NS
C18:1n-9	17.74 ± 1.34	17.44 ± 2.45	17.54 ± 0.61	16.65 ± 0.44	NS	NS	NS
C18:1n-7	3.08 ± 0.25	2.84 ± 1.74	1.96 ± 0.77	2.32 ± 0.07	NS	NS	NS
C18:2n-6	4.58 ± 0.30	4.37 ± 0.21	4.68 ± 0.42	5.15 ± 0.56	NS	NS	NS
C18:3n-3	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.02	0.03 ± 0.01	NS	NS	NS
C20:0	0.29 ± 0.03	0.35 ± 0.10	0.29 ± 0.03	0.32 ± 0.05	NS	NS	NS
C20:3n-6	0.82 ± 0.06	0.88 ± 0.07	0.83 ± 0.08	0.96 ± 0.07	NS	NS	NS
C20:4n-6	18.91 ± 0.25	18.41 ± 1.37	19.07 ± 0.68	18.17 ± 0.37	NS	NS	NS
C20:5n-3	0.21 ± 0.02	0.23 ± 0.04	0.22 ± 0.02	0.22 ± 0.03	NS	NS	NS
C22:4n-6	4.37 ± 0.14^{a}	3.67 ± 0.43^{b}	4.08 ± 0.43^{a}	3.47 ± 0.07^{b}	NS	0.0006	NS
C22:5n-6	2.01 ± 0.20^{a}	0.87 ± 0.14^{b}	2.05 ± 0.58^{a}	1.11 ± 0.19^{b}	NS	0.0001	NS
C22:5n-3	0.92 ± 0.12	1.00 ± 0.05	0.91 ± 0.06	0.95 ± 0.10	NS	NS	NS
C22:6n-3	3.31 ± 0.23^{b}	5.03 ± 0.29^{a}	3.64 ± 0.91^{b}	4.94 ± 0.15^{a}	NS	0.0001	NS
C24:4n-6	0.26 ± 0.01^{a}	0.22 ± 0.03^{ab}	0.26 ± 0.03^{a}	0.21 ± 0.01^{b}	NS	0.003	NS
C24:5n-6	0.32 ± 0.02^{a}	0.25 ± 0.05^{b}	0.32 ± 0.05^{a}	0.25 ± 0.01^{b}	NS	0.002	NS
SFA	40.36 ± 0.28	40.47 ± 0.31	41.03 ± 2.03	40.46 ± 0.97	NS	NS	NS
MUFA	23.35 ± 0.44	24.09 ± 2.14	23.94 ± 0.78	23.49 ± 0.77	NS	NS	NS
n-3 PUFA	4.52 ± 0.22^{b}	6.35 ± 0.34^{a}	4.85 ± 0.99^{b}	6.20 ± 0.26^{a}	NS	0.0001	NS
n-6 PUFA	31.10 ± 0.41^{a}	28.55 ± 1.97^{b}	29.51 ± 2.23^{ab}	29.29 ± 0.61^{ab}	NS	0.04	NS
n-6VLCFA	0.67 ± 0.05^{a}	0.53 ± 0.11^{b}	0.66 ± 0.1^{a}	0.55 ± 0.05^{ab}	NS	0.005	NS
n-6/n-3 PUFA	6.88 ± 0.26^{a}	4.50 ± 0.26^{b}	6.23 ± 1.07^{a}	4.73 ± 0.27^{b}	NS	0.0001	NS

Mean ± SD. Main effects were analyzed using two-way analysis of variance (ANOVA). Values having different superscript letters are significantly different among the groups by Duncan's multiple range test (P<0.05). NS, not significant; AA, arachidonic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; VLCFA, very long chain fatty acids; PD4, postnatal day 4; Cont, control; Cont+DHA, control+DHA; EtOH, ethanol; EtOH+DHA, ethanol+DHA.

Table 5.4. The effect of dietary DHA on testis fatty acids at PD21 in rats exposed to prenatal EtOH

Fatty acids (%, w/w)	Cont (n = 6)	Cont+DHA (n = 4)	EtOH (n = 3)	EtOH+DHA (n = 3)	Significant effects		
					EtOH	Diet	EtOH* Diet
C14:0	0.51 ± 0.09	0.68 ± 0.47	0.59 ± 0.17	0.65 ± 0.26	NS	NS	NS
C16:0	28.17 ± 0.63	28.88 ± 1.09	28.61 ± 1.24	28.05 ± 0.66	NS	NS	NS
C18:0	11.14 ± 0.46	11.65 ± 0.77	10.93 ± 0.29	10.80 ± 0.63	NS	NS	NS
C18:1n-9	15.60 ± 0.70	18.19 ± 1.62	16.89 ± 0.99	17.93 ± 1.95	0.01	NS	NS
C18:1n-7	2.01 ± 0.32	2.02 ± 0.00	2.11 ± 0.33	1.32 ± 1.16	NS	NS	NS
C18:2n-6	3.50 ± 0.33 [b]	4.86 ± 0.83 [a]	3.92 ± 0.39 [ab]	4.87 ± 0.99 [a]	NS	0.002	NS
C18:3n-3	0.00 ± 0.00	0.05 ± 0.06	0.02 ± 0.02	0.05 ± 0.05	NS	NS	NS
C20:0	0.17 ± 0.07	0.24 ± 0.06	0.16 ± 0.05	0.22 ± 0.03	NS	NS	NS
C20:3n-6	0.77 ± 0.07 [b]	0.91 ± 0.08 [ab]	0.80 ± 0.02 [b]	0.98 ± 0.14 [a]	NS	0.003	NS
C20:4n-6	15.86 ± 0.7	14.82 ± 1.69	16.03 ± 0.56	14.87 ± 1.97	NS	NS	NS
C20:5n-3	0.11 ± 0.04	0.14 ± 0.03	0.10 ± 0.05	0.15 ± 0.02	NS	0.04	NS
C22:4n-6	2.32 ± 0.17	2.15 ± 0.21	2.28 ± 0.13	2.37 ± 0.27	NS	NS	NS
C22:5n-6	9.94 ± 0.58 [a]	4.40 ± 0.49 [d]	9.05 ± 0.39 [b]	5.70 ± 0.58 [c]	NS	0.0001	0.002
C22:5n-3	0.17 ± 0.03	0.21 ± 0.03	0.15 ± 0.06	0.15 ± 0.05	NS	NS	NS
C22:6n-3	2.61 ± 0.15 [b]	6.02 ± 1.03 [a]	2.80 ± 0.42 [b]	5.51 ± 0.50 [a]	NS	0.0001	NS
C24:4n-6	1.13 ± 0.09 [a]	0.91 ± 0.05 [b]	1.06 ± 0.01 [a]	1.08 ± 0.07 [a]	NS	0.01	0.005
C24:5n-6	2.03 ± 0.11 [a]	1.20 ± 0.13 [c]	1.90 ± 0.20 [a]	1.58 ± 0.03 [b]	NS	0.001	0.002
SFA	41.24 ± 0.81 [b]	42.62 ± 0.86 [a]	41.04 ± 0.87 [b]	40.75 ± 0.84 [b]	NS	NS	NS
MUFA	19.64 ± 0.93	21.32 ± 2.43	20.35 ± 0.61	21.42 ± 3.15	NS	NS	NS
n-3 PUFA	2.94 ± 0.16 [b]	6.45 ± 1.03 [a]	3.12 ± 0.51 [b]	5.90 ± 0.44 [a]	NS	0.0001	NS
n-6 PUFA	32.66 ± 1.47 [a]	27.37 ± 0.95 [b]	32.26 ± 0.80 [a]	29.08 ± 1.97 [b]	NS	0.0001	NS
n-6VLCFA	3.52 ± 0.20 [a]	2.24 ± 0.09 [d]	3.23 ± 0.23 [b]	2.84 ± 0.04 [c]	NS	0.0001	0.0002
n-6/n-3 PUFA	11.15 ± 0.69 [a]	4.31 ± 0.58 [b]	10.53 ± 1.72 [a]	4.93 ± 0.24 [b]	NS	0.0001	NS

Mean ± SD. Main effects were analyzed using two-way analysis of variance (ANOVA). Values having different superscript letters are significantly different among the groups by Duncan's multiple range test ($P<0.05$). NS, not significant; AA, arachidonic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; VLCFA, very long chain fatty acids; PD21, postnatal day 21; Cont, control; Cont+DHA, control+DHA; EtOH, ethanol; EtOH+DHA, ethanol+DHA.

Table 5.5. The effect of dietary DHA on testis fatty acids at PD49 in rats exposed to prenatal EtOH

Fatty acids (%, w/w)	Cont (n = 3)	Cont+DHA (n = 4)	EtOH (n = 3)	EtOH+DHA (n = 3)	Significant effects (P<)		
					EtOH	Diet	EtOH* Diet
C14:0	0.42 ± 0.25	0.28 ± 0.04	0.32 ± 0.03	0.29 ± 0.03	0.005	NS	0.005
C16:0	31.41 ± 0.24	31.31 ± 1.35	30.92 ± 0.84	31.35 ± 0.22	NS	NS	NS
C18:0	7.77 ± 0.37	7.25 ± 0.26	7.65 ± 0.43	6.88 ± 0.05	NS	0.002	NS
C18:1n-9	11.78 ± 2.99	10.09 ± 0.49	10.32 ± 0.66	10.525 ± 0.25	NS	NS	NS
C18:1n-7	1.84 ± 0.15	1.74 ± 0.17	1.61 ± 0.19	1.66 ± 0.25	NS	NS	NS
C18:2n-6	3.86 ± 0.21 [b]	4.39 ± 0.12 [a]	3.81 ± 0.16 [b]	4.29 ± 0.15 [a]	NS	0.0005	NS
C18:3n-3	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	NS	NS	NS
C20:0	0.18 ± 0.14	0.08 ± 0.03	0.10 ± 0.03	0.10 ± 0.00	NS	NS	NS
C20:3n-6	0.70 ± 0.17 [b]	0.98 ± 0.10 [a]	0.78 ± 0.03 [b]	1.07 ± 0.05 [a]	NS	0.0002	NS
C20:4n-6	15.79 ± 1.87	16.24 ± 0.12	16.7 ± 1.03	15.97 ± 0.04	NS	NS	NS
C20:5n-3	0.07 ± 0.03	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.00	NS	NS	NS
C22:4n-6	2.34 ± 0.42	2.45 ± 0.20	2.67 ± 0.23	2.29 ± 0.14	NS	NS	NS
C22:5n-6	16.27 ± 1.43	15.73 ± 0.38	16.85 ± 0.57	16.07 ± 0.55	NS	NS	NS
C22:5n-3	0.08 ± 0.03	0.11 ± 0.05	0.09 ± 0.02	0.07 ± 0.02	NS	NS	NS
C22:6n-3	0.90 ± 0.01 [b]	2.35 ± 0.41 [a]	1.02 ± 0.05 [b]	1.98 ± 0.38 [a]	NS	0.0002	NS
C24:4n-6	1.52 ± 0.17	1.66 ± 0.12	1.55 ± 0.12	1.58 ± 0.09	NS	NS	NS
C24:5n-6	1.48 ± 0.19 [b]	1.66 ± 0.09 [ab]	1.56 ± 0.11 [b]	1.81 ± 0.02 [a]	NS	0.005	NS
SFA	40.97 ± 0.76	40.15 ± 0.83	40.37 ± 0.75	39.87 ± 0.16	NS	NS	NS
MUFA	14.84 ± 3.71	12.84 ± 0.42	13.19 ± 0.52 [b]	13.53 ± 0.05	NS	NS	NS
n-3 PUFA	1.10 ± 0.02 [b]	2.63 ± 0.38 [a]	1.31 ± 0.08	2.17 ± 0.40 [a]	NS	0.0003	NS
n-6 PUFA	39.20 ± 4.07	40.10 ± 0.5	41.05 ± 0.97	40.01 ± 0.64	NS	NS	NS
n-6VLCFA	3.89 ± 0.37	4.29 ± 0.29	4.08 ± 0.38	4.42 ± 0.19	NS	NS	NS
n-6/n-3 PUFA	35.50 ± 2.91 [a]	15.53 ± 2.54 [b]	31.37 ± 2.5 2 [a]	18.83 ± 3.37 [b]	NS	0.0001	NS

Mean ± SD. Main effects were analyzed using two-way analysis of variance (ANOVA). Values having different superscript letters are significantly different among the groups by Duncan's multiple range test (P<0.05). NS, not significant; AA, arachidonic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; VLCFA, very long chain fatty acids; PD49, postnatal day 49; Cont, control; Cont+DHA, control+DHA; EtOH, ethanol; EtOH+DHA, ethanol+DHA.

Table 5.6. The effect of dietary DHA on testis fatty acids at PD90 in rats exposed to prenatal EtOH

Fatty acids (%, w/w)	Cont (n = 6)	Cont+DHA (n = 6)	EtOH (n = 5)	EtOH+DHA (n = 4)	Significant effects (P<)		
					EtOH	Diet	EtOH* Diet
C14:0	0.20 ± 0.11	0.23 ± 0.11	0.29 ± 0.01	0.32 ± 0.03	0.03	NS	NS
C16:0	30.36 ± 0.35 [b]	31.13 ± 0.31 [a]	30.26 ± 0.68 [b]	30.47 ± 0.77 [ab]	NS	0.03	NS
C18:0	7.01 ± 0.28 [ab]	6.84 ± 0.18 [b]	6.92 ± 0.20 [ab]	7.12 ± 0.20 [a]	NS	NS	0.04
C18:1n-9	10.36 ± 0.43 [a]	9.60 ± 0.11 [b]	10.37 ± 0.37 [a]	10.39 ± 0.66 [a]	0.02	NS	0.03
C18:1n-7	1.49 ± 0.37 [a]	1.57 ± 0.18 [a]	1.72 ± 0.28 [a]	1.12 ± 0.62 [b]	NS	NS	0.02
C18:2n-6	3.40 ± 0.35 [b]	3.73 ± 0.31 [a]	3.45 ± 0.36 [b]	3.83 ± 0.36 [a]	NS	0.02	NS
C18:3n-3	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.04	NS	NS	NS
C20:0	0.08 ± 0.04	0.06 ± 0.02	0.07 ± 0.02	0.09 ± 0.03	NS	NS	NS
C20:3n-6	1.20 ± 0.09	1.50 ± 0.05	1.21 ± 0.09	1.12 ± 0.61	NS	NS	NS
C20:4n-6	16.07 ± 0.49	15.22 ± 0.33	15.84 ± 0.91	16.13 ± 0.37	NS	NS	NS
C20:5n-3	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.02	NS	NS	NS
C22:4n-6	2.61 ± 0.11	2.54 ± 0.05	2.72 ± 0.32	2.62 ± 0.14	NS	NS	NS
C22:5n-6	19.53 ± 1.06 [a]	19.74 ± 0.44 [a]	19.76 ± 0.84 [a]	18.56 ± 0.73 [b]	NS	NS	0.04
C22:5n-3	0.06 ± 0.03	0.04 ± 0.01	0.04 ± 0.02	0.07 ± 0.02	NS	NS	NS
C22:6n-3	0.67 ± 0.04 [b]	1.31 ± 0.11 [a]	0.67 ± 0.04 [b]	1.28 ± 0.20 [a]	NS	0.0001	NS
C24:4n-6	1.65 ± 0.10	1.66 ± 0.09	1.62 ± 0.05	1.67 ± 0.14	NS	NS	NS
C24:5n-6	1.74 ± 0.12	1.77 ± 0.13	1.75 ± 0.11	1.79 ± 0.08	NS	NS	NS
SFA	38.42 ± 0.77 [b]	39.08 ± 0.47 [a]	38.15 ± 0.83 [b]	38.93 ± 0.91 [a]	NS	0.02	NS
MUFA	12.85 ± 0.70 [a]	12.09 ± 0.22 [b]	13.18 ± 0.36 [a]	12.60 ± 0.17 [ab]	NS	0.002	NS
n-3 PUFA	0.86 ± 0.07 [b]	1.48 ± 0.11 [a]	0.84 ± 0.06 [b]	1.50 ± 0.20 [a]	NS	0.0001	NS
n-6 PUFA	43.01 ± 0.76	42.90 ± 0.51	43.22 ± 0.52	42.47 ± 0.66	NS	NS	NS
n-6VLCFA	4.87 ± 0.86	4.46 ± 0.29	4.62 ± 0.26	4.50 ± 0.22	NS	NS	NS
n-6/n-3 PUFA	50.27 ± 4.22 [a]	29.24 ± 2.45 [b]	51.93 ± 4.19 [a]	28.65 ± 3.69 [b]	NS	0.0001	NS

Mean ± SD. Main effects were analyzed using two-way analysis of variance (ANOVA). Values having different superscript letters are significantly different among the groups by Duncan's multiple range test (P<0.05). NS, not significant; AA, arachidonic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; VLCFA, very long chain fatty acids; PD90, postnatal day 90; Cont, control; Cont+DHA, control+DHA; EtOH, ethanol; EtOH+DHA, ethanol+DHA.

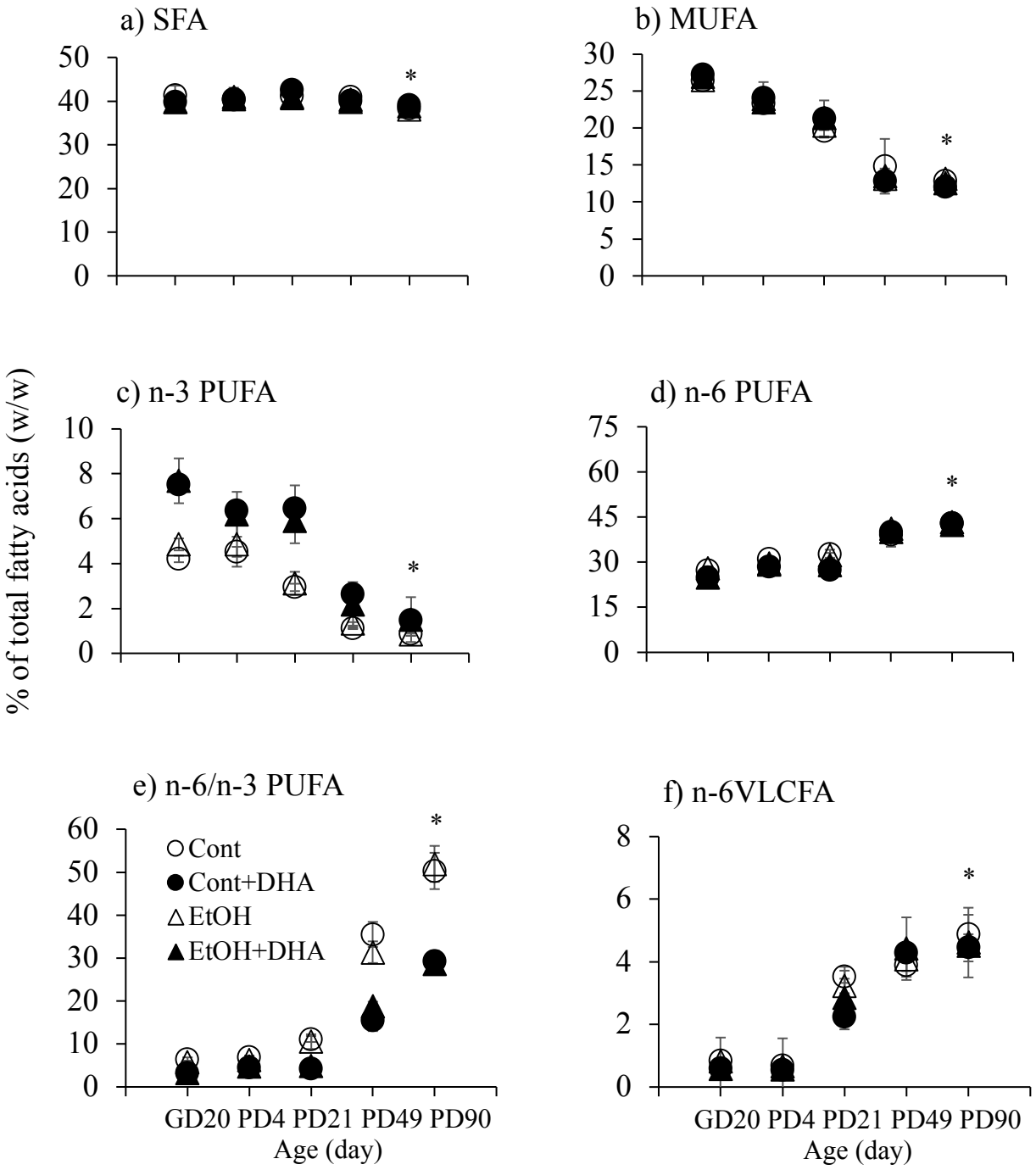


Figure 5.1. Developmental profiles of testis total fatty acid composition from GD20 to PD90

Mean ± SD (Gestational day (GD) 20, n=3-8 per group. Postnatal day (PD) 4, n=3-4 per group, PD21, n=3-6 per group, PD49, n=3-4 per group, PD90, n=4-6 per group). The difference between age was tested by student's *t*-test, ($P<0.05$). * indicates a significant difference between GD20 and PD90. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; VLCFA, very long chain fatty acids; Cont, control; Cont+DHA, control+DHA; EtOH, ethanol; EtOH+DHA, ethanol+DHA.

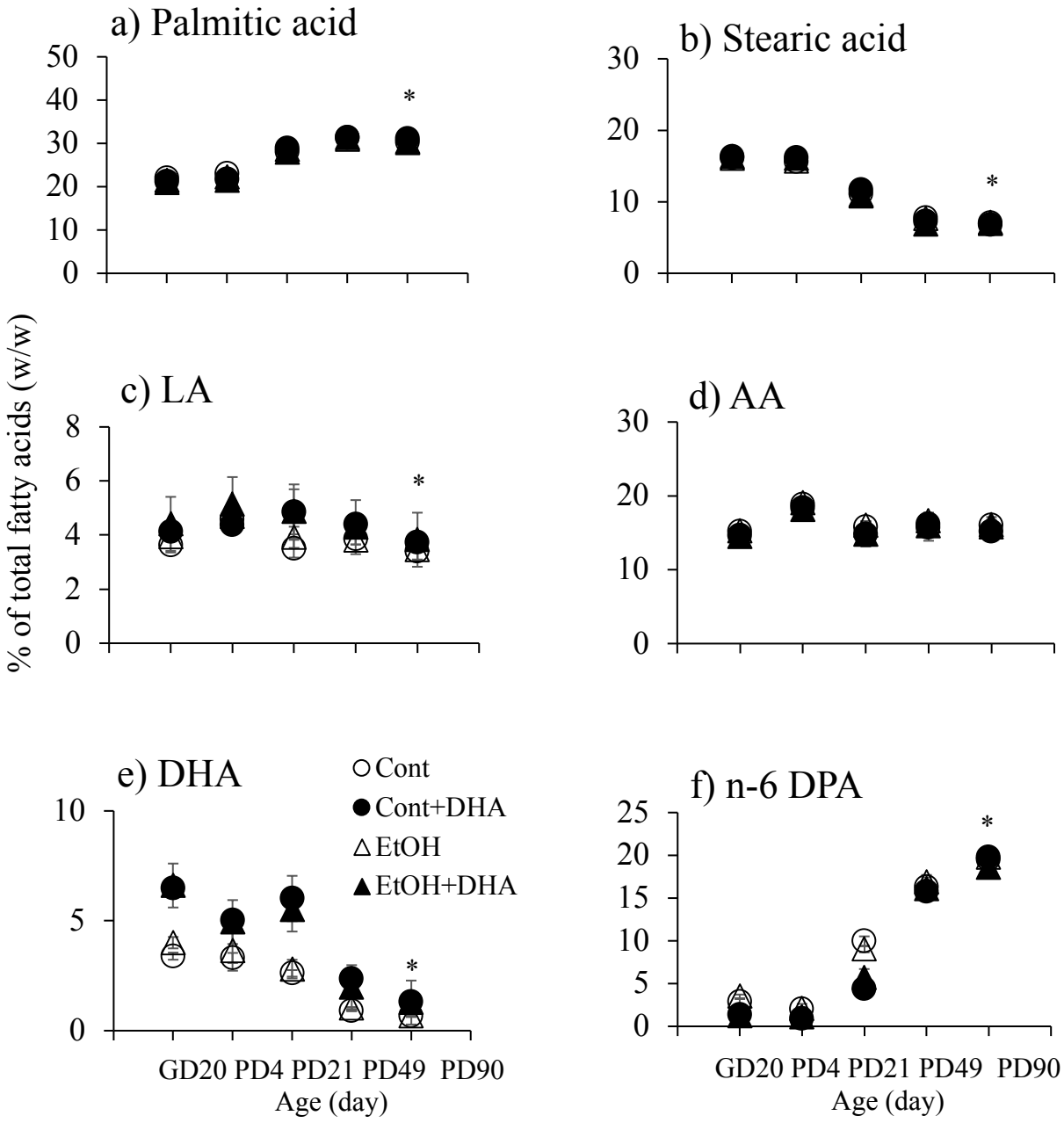
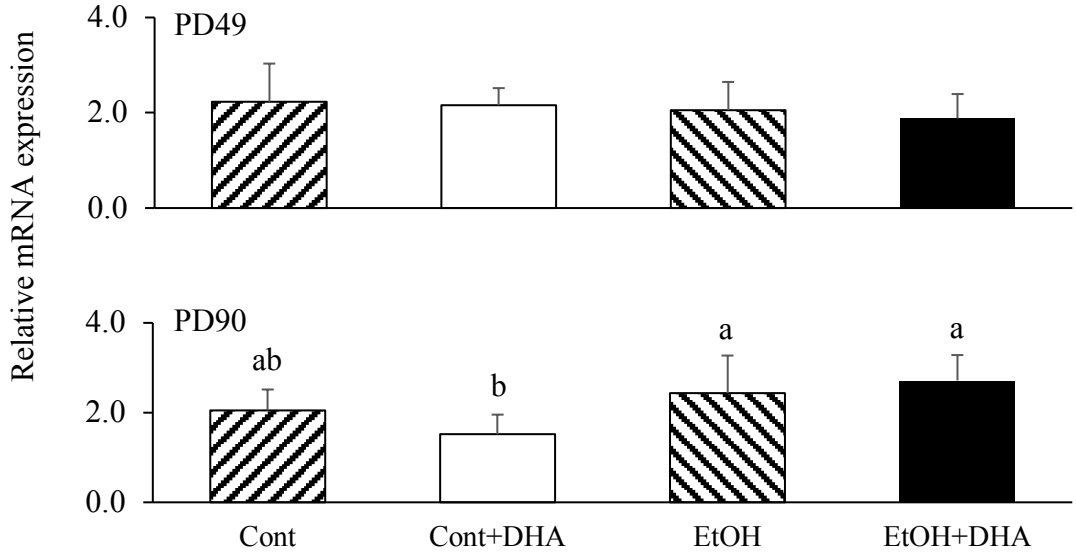


Figure 5.2. Developmental profiles of testis total fatty acid composition from GD20 to PD90

Mean ± SD (Gestational day (GD) 20, n=3-8 per group. Postnatal day (PD) 4, n=3-4 per group, PD21, n=3-6 per group, PD49, n=3-4 per group, PD90, n=4-6 per group). The difference between age was tested by student's *t*-test, ($P<0.05$). * indicates a significant difference between GD20 and PD90. AA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; Cont, control; Cont+DHA, control+DHA; EtOH, ethanol; EtOH+DHA, ethanol+DHA.

a) FADS1



b) FADS2

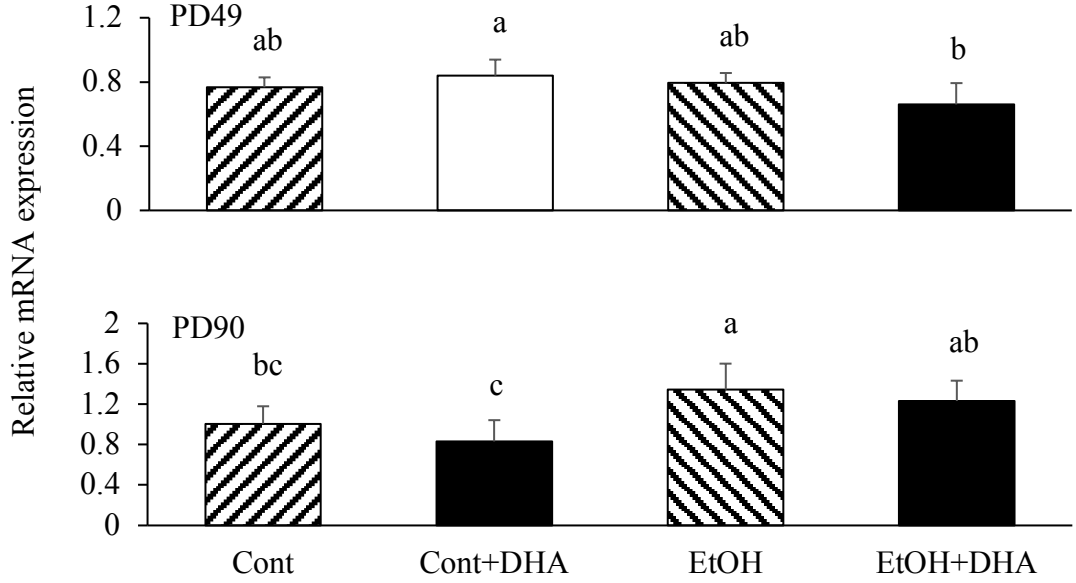


Figure 5.3. The effect of dietary DHA on mRNA expression of a) FADS1) and b) FADS2 at PD49 and PD90 in rats exposed to prenatal EtOH

Bars represent mean ± SD (Postnatal day (PD) 49, n=3-5 per group; PD90, n=5-6 per group). Analyzed via the $2^{\Delta\Delta Ct}$ method using Tbp as the house keeping gene. Main effects and interaction were analyzed using two-way analysis of variance (ANOVA); Values having different superscript letters are significantly different among the groups by Duncan's multiple range test ($P<0.05$). The effect of EtOH, FADS1, PD90 ($P<0.007$), FADS2, PD90 ($P<0.0004$); EtOH*DHA, FADS2, P49 ($P<0.03$). FADS, fatty acid desaturase; Cont, control; Cont+DHA, control+DHA; EtOH, ethanol; EtOH+DHA, ethanol+DHA.

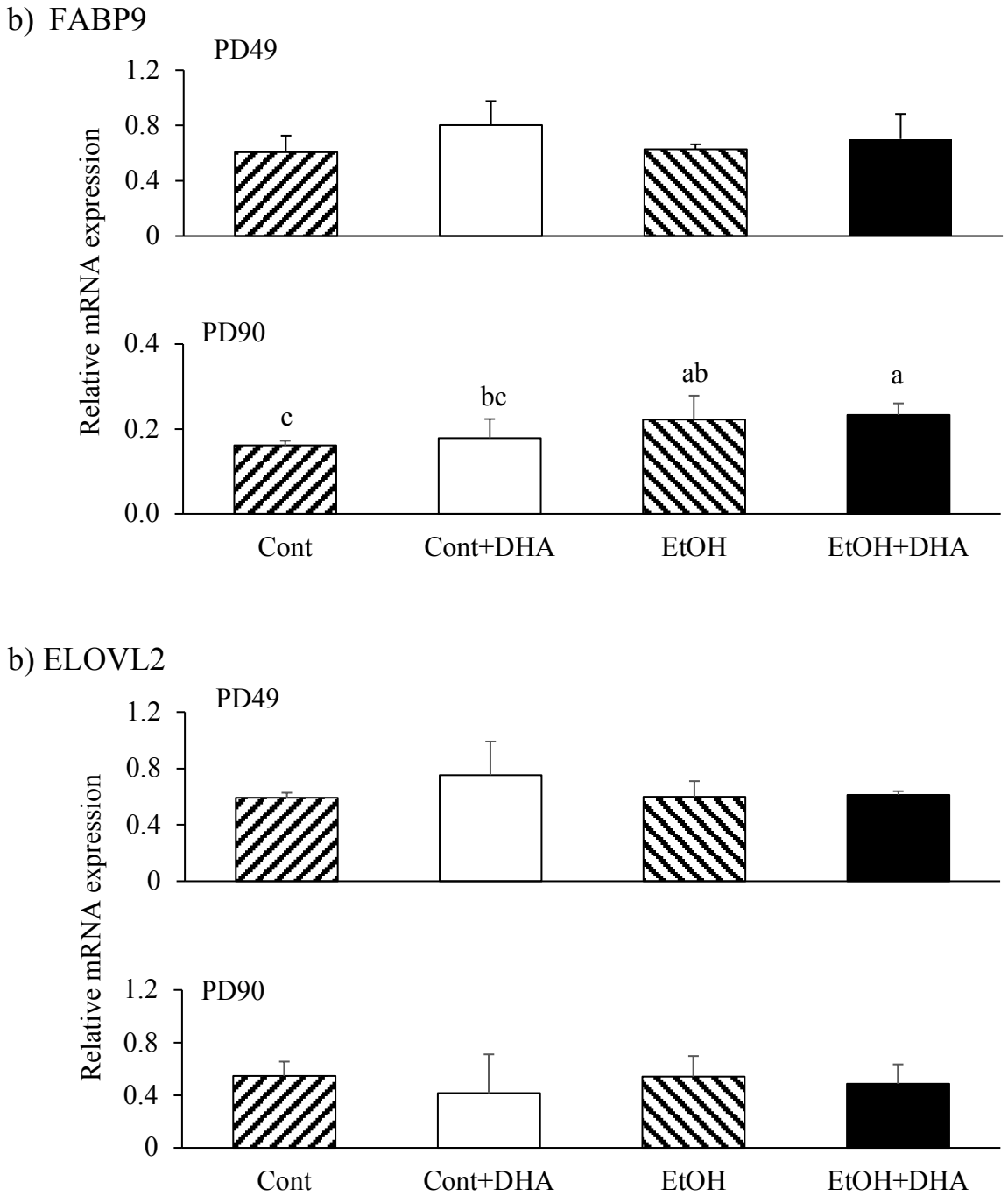


Figure 5.4. The effect of dietary DHA on mRNA expression of a) FABP9 and b) ELOVL2 at PD49 and PD90 in offspring exposed to prenatal EtOH

Bars represent mean ± SD (Postnatal day (PD) 49, n=3-5 per group; PD90, n=5-6 per group). Analyzed via the $2^{\Delta\Delta Ct}$ using Tbp as the house keeping gene. Main effects and interaction were analyzed using two-way analysis of variance (ANOVA). Values having different superscript letters are significantly different among the groups by Duncan's multiple range test ($P<0.05$). The effect of EtOH, FABP,PD90 ($P<0.002$). FABP, fatty acid binding protein; ELOVL, elongation of very long chain fatty acids protein; Cont, control; Cont+DHA, control+DHA; EtOH, ethanol; EtOH+DHA, ethanol+DHA.

CHAPTER 6. EARLY PROVISION OF DIETARY DOCOSAHEXAENOIC ACID INFLUENCED FATTY ACID PROFILES IN LIPID CLASSES IN DEVELOPMENTAL RAT TESTIS

Will be submitted to "Prostaglandins, Leukotrienes & Essential Fatty acids" (In preparation)

Abstract

Background and Objective: Previous studies have shown that the early provision of dietary docosahexaenoic acid (DHA) increased testis DHA concentration and sequentially improved sperm morphology in adult rats. This work further studied how dietary DHA impacts fatty acid distribution in lipid classes of the developing testis.

Methods: Thirty pregnant Sprague-Dawley rats randomly assigned to receive either control or diet supplemented by DHA (1.4%, w/w fatty acids) upon pregnancy. The same diet was provided for their offspring after weaning. The testis was collected from weaning at postnatal (PD) 21, peri-pubertal at PD49, and adult rats at PD90 for fatty acid analysis in the individual lipid classes, triacylglycerides (TAG), phospholipids (PL) and free fatty acids (FFA).

Results: The TAG fraction contained the greatest amount of n-6 DPA and n-6 very long chain fatty acids (n-6 VLCFA) regardless of DHA treatment, which increased throughout testis maturation. Compared to other testis lipid fractions, FFA contained the lowest amount of n-6 DPA in mature testis with a higher level of n-6 DPA precursors, arachidonic acid (AA). Dietary DHA augmented testis DHA level in all lipid fractions at all ages. Concurrently, dietary DHA declined the level of testis docosapentaenoic acid (DPA, C22:5n-6) at PD21 and only in PL at PD49.

Conclusion: Overall, testis main fatty acid distribution in lipid classes is age-dependent, and varies in responsiveness to dietary DHA. TAG, containing the greatest amount of rat testis specific fatty acids, might be a prime lipid class in testis development.

Keywords: dietary docosahexaenoic acid, testis lipid classes, docosapentaenoic acid, testis development, n-6 very long chain fatty acids

Introduction

The unique lipid environment of the mature testis plays a vital role in testis functions (Ayala et al., 1977; Bieri et al., 1969; Burr and Burr, 1930; Coniglio, 1994). This lipid characteristic is a marker of testis maturity as a major variation in lipid concentration is seen when comparing the mature and immature testis (Davis et al., 1966). In order to understand the normal development of the testis during sexual maturation, and sequentially the normal function of the testis, it is necessary to fully examine the fatty acid profile of major lipid classes. This is important as the relation between dietary fat and testis fatty acid composition and fertility potential is receiving increasing attention. However, there is little attention to how dietary fat can influence the testis fatty acid composition throughout development.

Dietary n-3 polyunsaturated fatty acid (PUFA), in particular docosahexaenoic acid (DHA, C22:6n-3), has been associated with improved testis maturity and function in a rat model (Ayala and Brenner, 1980; Ayala et al., 1977). As reported in Chapter 5, the early provision of dietary DHA (1.4% of total fatty acids) from the fetal period throughout development significantly increased the level of DHA in total lipids of immature and mature testis, with a substantial increase at weaning (postnatal day (PD) 21) and peri-puberty (PD49), associated with increased normal sperm morphology in adult male rats. The major fatty acid within rat testis is docosapentaenoic acid (DPA, C22:5n-6). Dietary DHA reduced n-6 DPA concentrations in the immature testis. However, as testis matured, n-6 DPA concentrations were independent of dietary DHA with no changes at PD49 in DHA-treated groups. Dietary DHA exerted a similar impact on the level of n-6 very long chain fatty acids (VLCFA, C>24) during development, which are uniquely enriched in the rodent testis. This suggests that the testis lipid concentration

has a fatty acid-specific response to dietary DHA majorly at PD49, which is a critical period of sexual maturation. Whether this phenomenon happens in major lipid classes is not known. Additionally, information about VLCFA distribution in developing testis lipid classes is limited. VLCFA cannot be obtained from the diet and must be synthesized *in situ* in the testis and spermatozoa from shorter chain fatty acids (Zadravec et al., 2011). Although the exact function of VLCFA is unknown, it has been reported that VLCFA play an important role in spermatogenesis (Kihara, 2012; Robinson et al., 1992) and the presence of VLCFA is detectable in testis upon the commencement of spermatogenesis (Furland et al., 2007).

Thus, this study investigated the effect of the early provision of dietary DHA on the fatty acid composition of major lipid classes in developmental testis during sexual maturity from PD21 to PD90.

Material and Method

Animal and diets

All animal experiments were approved by the University of Manitoba Animal Care Committee and followed the principles and guidelines of the Canadian Council on Animal Care (CCAC, 1993). Female Sprague-Dawley rats (9-10 weeks old; n= 30), obtained from Charles River Breeding Laboratories (Sherbrooke, Quebec, Canada), were used in this study. The animals were maintained in an environmentally controlled room and allowed free access to rat chow and water for the duration of the experiment. Following adaptation, female rats were mated in the proportion of two females to one male, during which animals were provided a control diet. The day in which sperm was detected by vaginal smear was considered day 0 of gestation. At this time, dams were housed separately and randomly assigned to consume one of two diets including a DHA-rich diet (DHA, n=14) or a control diet (Cont, n=16). Diets were formulated in

our laboratory from a nutritionally complete semi-purified diet as explained in Chapter 3, **Table 3.1**. The total energy-density was 4.17 Kcal/g diet composed of carbohydrate (50%), protein (17.5%) and fat (32.5%), reflecting nutrient intake of the pregnant mother in North America (Denomme et al., 2005; Friesen and Innis, 2010; Hui et al., 2014). The control and DHA diets were identical and differed only in their concentration of DHA (Cont: 0% vs. DHA: 1.4%, w/w fatty acids). It is reported that this level of DHA is sufficient for pregnant rats and growing pups (Wang et al., 1989; Weiser et al., 2015). The formulated diet was available to dams for the entire pregnancy and lactation period. Following parturition at PD4, male and female pups were randomly culled to 8 pups per litter in an equivalent sex ratio to allow for equal access to the dam's milk and were kept with their mothers until weaning. Pups consumed the same diet assigned to their mothers for the remainder of the experiment. Male pups were euthanized, and testis were collected at one of three time points for lipid analysis including: in weaning at PD21; peri-pubertal at PD49; and adulthood at PD90.

Lipid analysis

Total lipid was extracted from testis by the Folch method (Folch et al., 1957). A total of ~50 mg of decapsulated testes were homogenized using a Dounce homogenizer in appropriate proportions of chloroform and an aqueous solution of $CaCl_2$ (0.025 %, w/w) for extraction of total lipids. Ten ml of chloroform and methanol (2:1, v/v) were used for extraction. Separations into lipid classes including phospholipid (PL), triacylglyceride (TAG), 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 mobile phase was petroleum ether: diethyl ether: acetic acid (80:20:1 (v/v/v)). Aniline naphthalene sulfonic acid (0.1%, w/v) was used to visualize the lipid classes. TAG was saponified using 0.5 M of

methanolic potassium hydroxide (KOH) and heated in a sand bath at 110ºC for 1.5 hours. In order to prepare fatty acid methyl esters for all the lipid classes, boron trifluoride (BF3) in methanol was used by boiling at 110ºC for 1 hour (Suh et al., 2009).

Fatty acid analysis

Following lipid extraction, samples were sent directly for fatty acid analysis to separate fatty acid methyl esters using a microcapillary column (10 m × 0.10 mm inner diameter and 0.2 m film thickness; BPX70; NC, USA) and chromatography system (Vista 6010 GLC and Vista 402 data system; Varian Instruments, Mississauga, ON, Canada). Hydrogen was used as a carrier gas under a flow rate of 0.5 mL/min. The initial temperature was 130°C, then increased to 175°C at 20°C/min held for 1 min, then was increased to 200°C at 6°C/min, held for 0.5 min, raised again to 200°C at 6°C/min with no hold and finally increased to 280°C at 30°C/min. The injector/detector temperatures were 270°C and 290°C, respectively. Standard FAME 461 (Nucheck Prep Inc. Waterville, MN, USA) was used for peak.

Statistical analysis

Statistical analysis was analyzed using Student *t*-test to see the effect of diet at each age group between PD21 and PD90. All data is expressed as the mean ± standard deviation (SD). Statistical significance was set at $P< 0.05$.

Results

Fatty acid profiles of testis lipid classes during development from PD21 to PD90

The total testis lipids at PD21, PD49, and PD90 were separated into PL, monoacylglycerols, FFA, TAG, and CE and the separations were visualized as shown in (**Figure**

6.1). The total and individual fatty acid composition of developing testis from weaning to adulthood from different treatments is shown in **(Table 6.1 - 6.3).** Saturated fatty acids (SFA) were the predominant type of fatty acid from PD21 to PD90 with palmitic acid (C16:0) as the most abundant fatty acid in the testis. During development, a significant decline ($P<0.05$) in the total SFA was found in TAG and FFA, with a concomitant significant increase ($P<0.05$) in n-6 PUFA in all lipid classes, therefore becoming the main type of fatty acid in TAG and FFA in adulthood. However, in PL fractions, SFA remained as the major type of fatty acid. A noticeable feature regarding n-6 PUFA was the consistency in their distribution among lipid classes at all ages. n-6 DPA was quantitatively the main n-6 PUFA in most lipid classes, with TAG containing the highest proportion of n-6 DPA at all ages. The level of n-6 DPA notably elevated ($P<0.05$) in both PL and TAG classes as testis developed **(Figure 6.2)**. However, in testis FFA, the level of n-6 DPA precursors, linoleic acid (LA, C18:2n-6) and arachidonic acid (AA, C20:4n-6) increased ($P<0.05$) during development from PD21 to PD90 **(Figure 6.3)**. It appears that rat testis maintained a relatively constant proportion of AA throughout development in other lipid classes. In contrast to n-6 PUFA, the content of total n-3 PUFA significantly decreased ($P<0.05$) in all lipid classes from PD21 to PD90 **(Figure 6.3)**. DHA was a predominant n-3 PUFA and concentrations were highest in TAG and the lowest in PL at PD21. A clear decline ($P<0.05$) in DHA concentrations occurred during testis maturation from PD21 to PD90 **(Figure 6.2)**. The level of monounsaturated fatty acids (MUFA) in rat testis is characterized by a significant reduction ($P<0.05$) only in PL class during development. Total n-6 VLCFA was the highest in TAG and the lowest in PL at all ages. Although total n-6 VLCFA remained constant in FFA and PL during development, it was elevated ($P<0.05$) from PD21 to PD90 in TAG **(Figure 6.3)**.

The effect of dietary DHA on fatty acid profiles of testis major lipid classes from PD21 to PD90

Phospholipids

Fatty acid profiles of developing testis PL at PD21, PD49, and PD90 with and without DHA treatment are shown **(Table 6.1)**. At PD21, PL in rat testis contained approximately 44% SFA, 22% MUFA, 28% n-6 PUFA, 3% n-3 PUFA and 2% VLCFA **(Table 6.1)**. Dietary DHA significantly changed the fatty acid composition of PL. A particular change occurred in n-6 DPA and DHA concentrations including a considerable elevation of the DHA content (~138%) and a significant reduction in n-6 DPA in DHA-consuming pups ($P<0.001$). In contrast to n-6 DPA, LA was increased significantly ($P<0.01$) in DHA-fed animals compared to controls. Moreover, C26:5n-6 was the only n-6 VLCFA that was decreased ($P<0.05$) following DHA diet consumption in the PL class as compared to the control diet.

At PD49, PL in testis contained approximately 46% SFA, 14% MUFA, 36% n-6 PUFA, 2% n-3 PUFA and 2% VLCFA **(Table 6.1)**. The fatty acid composition of testis was significantly influenced by dietary DHA as observed at PD21. The concentration of n-6 DPA diminished ($P<0.05$) in the DHA-treated group, concomitant with an increase ($P<0.01$) in the concentration of LA. Incorporation of DHA continued to increase ($P<0.01$) by almost 118% in those receiving DHA in their diet compared to the control group at this age. Dietary DHA consumption significantly ($P<0.001$) enhanced the total n-3 PUFA content in testis, reducing ($P<0.01$) the ratio of n-6 to n-3 PUFA. C28:5n-6 was the only n-6 VLCFA that increased ($P<0.01$) in the PL class as compared to the control diet at PD49 in DHA-fed animals.

At PD90, SFA, MUFA, n-6 PUFA, n-3 PUFA and n-6 VLCFA contents in PL of rat testis were 44, 13, 39, 1 and 2%, respectively **(Table 6.1)**. In contrast to PD21 and PD49, n-6 DPA concentrations were independent of dietary intervention at PD90. However, dietary DHA

induced a significant increase ($P<0.001$) in the DHA level of testis by almost 88%. Similar to the previous age, the ratio of n-6 to n-3 PUFA in the testis continued to increase with maturation and was significantly affected by the DHA consumption due to a significant increase in the total n-3 PUFA ($P<0.0001$). At all ages, no profound changes were observed in total n-6 PUFA and n-6 VLCFA in DHA-fed animals.

Triacylglycerides

Fatty acid profiles of developing testis TAG at PD21, PD49, and PD90 with and without DHA treatment are shown **(Table 6.2)**. At PD21, testis TAG contained approximately 40% SFA, 15% MUFA, 20-30% n-6 PUFA, 5-13% n-3 PUFA and 8% VLCFA **(Table 6.2)**. Alterations in the fatty acid composition of TAG after dietary treatment were almost similar to those of the PL class. The fatty acid composition of PUFA altered considerably in the testis of DHA-fed animals, with a significant decrease ($P<0.001$) in n-6 DPA but an increase (~124%) ($P<0.01$) in DHA content. As a result, total n-3 and n-6 PUFA altered significantly ($P<0.001$), with a reduction ($P<0.05$) in the n-6 to n-3 PUFA ratio in those testis treated with dietary DHA. The testis AA content also increased significantly ($P<0.05$) in the DHA-treated group.

At PD49, TAG class in rat testis contained approximately 38% SFA, 15% MUFA, 33% n-6 PUFA, 2% n-3 PUFA and 11% n-6 VLCFA **(Table 6.2)**. In contrast to PL at the same age, the n-6 DPA level of the testis TAG class did not differ from the corresponding DHA group. However, similar to PL, DHA accretion ($P<0.01$) was detected in the TAG by almost 100%, regulating the increase ($P<0.01$) of total n-3 PUFA and thus reducing ($P<0.05$) the n-6 to n-3 PUFA ratio with DHA treatment. At PD49, dietary DHA exerted a lowering ($P<0.05$) effect on oleic acid with no overall changes in total MUFA content. Moreover, dietary DHA increased the proportion of C26:4n-6 ($P<0.05$) but did not alter total n-6 VLCFA.

At PD90, TAG in rat testis contained approximately 34% SFA, 13-18% MUFA, 39% n-6 PUFA, 1% n-3 PUFA and 10% n-6 VLCFA **(Table 6.2)**. DHA accumulated significantly ($P<0.001$) in adult testis by roughly 72% in those who received DHA compared to the corresponding controls. Moreover, oleic acid continued to decline at PD90, with a reduction in total MUFA content in DHA-treated animals ($P<0.05$).

Free fatty acids

Fatty acid profiles of developing testis FFA at PD21, PD49, and PD90 with and without DHA treatment are shown **(Table 6.3)**. At PD21, SFA, MUFA, n-3 PUFA, n-6 PUFA and n-6 VLCFA contents in FFA lipid class of rat testis were 44%, 14%, 4-11%, 43 and 5%, respectively **(Table 6.3)**. Similar to other lipid classes, DHA and n-6 DPA were altered significantly at PD21 in FFA. DHA accretion occurred with a significant increase in total n-3 PUFA ($P<0.001$), with a corresponding reduction in n-6 to n-3 PUFA ratio ($P<0.01$). n-6 DPA also decreased ($P<0.01$) but with no overall changes in total n-6 PUFA.

At PD49, SFA, MUFA, n-3 and n-6 PUFA and n-6 VLCFA contents in FFA of rat testis were 38%, 16%, 2%, 38% and 6%, respectively **(Table 6.3).** The only similar change observed in fatty acid composition at both PD49 and PD90 was DHA accumulation in the DHA-treated rats ($P<0.05$). However, total n-6 VLCFA increased ($P<0.05$) only in DHA-treated rats at PD90.

Discussion

Despite the vital role of dietary DHA on testis lipid composition on testis function and male fertility, limited studies have targeted the influence of dietary DHA on testis lipid composition throughout testis development. Considering the unique experimental model in our previous work focused on testis lipid composition from fetus to adult rats with or without DHA in offspring exposed to prenatal ethanol, the current study was an extension of that work. This

study further investigated the distribution of testis fatty acid concentrations in lipid classes from PD21 to PD90. Since no significant effect of prenatal ethanol was observed on testis total fatty acid during development, the present study focused only on the impact of dietary DHA. A complete characterization of fatty acid distribution in the different lipid classes was evaluated to provide a clear picture of any changes in fatty acid metabolism during development. In this study, similar to the results from testis total fatty acids, n-6 PUFA increased during development and replaced n-3 PUFA in rat testis in all lipid classes. Although testis fatty acid distribution in different lipid classes was age-dependent, TAG contained the highest level of n-6 DPA and n-6 VLCFA among lipid classes regardless of testis maturity, indicating their importance in rat testis. The present study also demonstrated that testis was responsive to dietary DHA intervention as DHA increased in all fractions at all ages. Among lipid classes, PL was more sensitive to dietary DHA as evidenced by a higher accumulation of DHA than n-6 DPA up to PD49. However, the n-6 DPA content in TAG was independent of dietary DHA at the same age. Overall, these results suggest that testis maturation is associated with a significant alteration in lipid composition with different distributions among lipid classes. Testis specific fatty acids of different lipid classes were responded differently to the dietary DHA during testis development.

Fatty acid profiles of testis lipid classes during development from PD21 to PD90

In this study, CE was visualized to be higher in PD21 than PD49 and PD90. A similar pattern was observed in our laboratory in underdeveloped testis from obese Zucker rats (Datar, 2015). This reduction of CE in immature testis might be due to a decrease in luteinizing hormone (LH) that occurs around PD21 (Ojeda et al., 1980). LH regulates the metabolism of CE in lipid droplets, increasing the level of free cholesterol in Leydig cells required for testosterone production (Weinbauer et al., 2010). Thus, a lower level of LH at PD21 might result in a higher

level of CE at the same age. Unfortunately, the fatty acids of CE were not measured due to the minute quantities present in this fraction.

In the present study, most lipid classes showed considerable changes in their constituent fatty acids during development from PD21 to PD90. These results support the previous data reported in Chapter 5 regarding testis total fatty acid concentrations. This includes a replacement of n-3 PUFA and MUFA content by n-6 PUFA in the total lipid content of the testis, which is comparable to that found in TAG and PL in the present study (Chapter 5). An earlier study also showed a similar pattern for SFA, MUFA and n-6 PUFA in testis lipid classes (Oshima and Carpenter, 1968). The proportion of n-6 PUFA was augmented during development at a similar rate in all lipid classes. n-6 DPA increased exclusively at all ages, with quantitatively different distributions in testis lipid classes primarily in TAG. Furthermore, n-6 VLCFA were predominantly present in TAG at all age groups, suggesting a great degree of importance of TAG for testis function to store the vital testis fatty acids regardless of testis maturity in a rat model. In the immature testis, DHA and n-3 PUFA were also found mostly in the TAG fraction. This might support our previous hypothesis regarding the essential role of DHA in immature testis development (height of germinal epithelium) and function (testosterone level) that was improved by dietary DHA, as reported in Chapter 4. Further lipid analysis on immature testis during the fetal period might provide further clarification. Although total SFA and MUFA decreased significantly during development, less is known regarding the role of these fatty acids in testis function.

Our findings showed that PL and TAG classes contained a relatively constant level of AA during development, although there was a considerable increase in AA level in FFA fraction was

demonstrated. This elevation may be related to the rise of FFA visualized following lipid separation.

The effect of dietary DHA on fatty acid profiles of testis major lipid classes from PD21 to PD90

Commencement of dietary DHA from the fetal period exerted profound alterations in the proportion of fatty acids in most lipid classes from the fetal period, throughout development from PD21 to PD90. Substantial changes were found mainly in the PUFA concentration in almost all lipid classes in the developmental testis. Although DHA is a minor component in the rat testis, it might have a critical role in male fertility, with the developmental testis responded to dietary DHA by increasing the levels of DHA in all lipid classes regardless of age. However, coinciding with testicular DHA reduction during development, the accretion rate of DHA associated with dietary DHA also decreased in the testis in all lipid classes. This suggests that the mature testis was less sensitive to dietary DHA in all testis lipid classes.

The level of n-6 DPA became independent of dietary DHA at PD49 in TAG and FFA classes, which was similar to that of testis total fatty acids. However, the level of n-6 DPA in PL was still influenced by dietary DHA at the same age. In addition, a reduced level of n-6 DPA with a higher amount of n-6 DPA precursors, mainly LA, indicates the lower synthesis of n-6 DPA in PL at PD21 and PD49. In contrast to our results, a previous study reported that DHA was quantitatively and functionally replaced by n-6 DPA in developmental testis obtained from pups consuming fish oils (17% DHA of total fatty acid) initiated from weaning (Ayala and Brenner, 1980; Ayala et al., 1977). The reason for the discrepancy might be related to the level of DHA provided in the diet (1.4 vs. 17% (w/w) of fatty acids). It is interesting to see how the early

provision of highly enriched dietary DHA might affect the rat testis response during development.

Dietary DHA only influenced total and individual level of MUFA in mature testis in TAG. It appears that MUFA, mainly oleic acid, was replaced by DHA and n-3 PUFA in DHA-treated groups at PD90. In this study, we did not find large differences in n-6 VLCFA throughout testis maturation. This might be related to the concentrations of VLCFA that were mostly found in sphingomyelin (SM) and ceramide (Cer) (Poulos et al., 1987). In the peri-pubertal rat, SM and Cer in testis are devoid of VLCFAs but increase on the commencement of spermatogenesis reaching 15% and 40% of total fatty acids, respectively (Furland et al., 2007). Further study is required to investigate the effect of DHA consumption during development on specific PL classes such as SM and Cer n-6 VLCFA content.

Conclusion

In conclusion, in the present study and in support of the results regarding testis total fatty acids n-6 PUFA, mainly n-6 DPA, increasingly replaced n-3 PUFA, predominately DHA during development, leading to an n-6 PUFA enriched environment for testis in all lipid classes. Additionally, testis specific fatty acids including n-6 DPA and n-6 VLCFA were found to be highest in TAG regardless of testis maturation suggesting a critical role of TAG throughout development. The present study also demonstrated that although DHA is not a primary fatty acid in rat testis, dietary DHA still induced a significant increase in DHA, regardless of lipid class and level of testis maturation. Among lipid classes, the PL fraction was more responsive to dietary DHA than TAG, as evidenced by a higher replacement of n-6 DPA by DHA even at PD49. FFA contained more n-6 DPA precursor than n-6 DPA compared to other lipid classes. Overall, the testis specific fatty acid distribution in lipid classes were age-dependent and responded

differently to dietary DHA. Further studies are needed to determine whether the early provision of dietary DHA can alter the fatty acid distribution of testis, and how this might impact the loss of fertility potential throughout aging.

Table 6.1. The effect of dietary DHA on fatty acid profiles of testis phospholipids from PD21 to PD90

Fatty acids (%, w/w)	PD21		PD49		PD90	
	Cont (n = 5)	DHA (n = 7)	Cont (n = 6)	DHA (n = 6)	Cont (n = 7)	DHA (n = 8)
C14:0	0.38 ± 0.12	0.50 ± 0.16	0.24 ± 0.13	0.17 ± 0.08	0.16 ± 0.03	0.28 ± 0.08
C16:0	32.41 ± 3.18	30.52 ± 0.88	35.62 ± 2.98	36.95 ± 3.44	34.99 ± 1.63	35.39 ± 1.48
C18:0	11.94 ± 1.59	11.05 ± 0.66	8.25 ± 0.64	8.34 ± 0.78	8.24 ± 0.54	8.12 ± 0.53
C18:1n-9	19.21 ± 2.13	20.25 ± 0.75	12.81 ± 0.82	13.38 ± 0.98	12.15 ± 0.74	12.34 ± 0.64
C18:1n-7	0.40 ± 0.22	0.49 ± 0.58	0.21 ± 0.19	0.39 ± 0.76	0.35 ± 0.65	0.08 ± 0.10
C18:2n-6	3.50 ± 0.56	4.50 ± 0.51**	3.32 ± 0.14	4.16 ± 0.19**	3.04 ± 0.17	3.27 ± 0.27
C18:3n-3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C20:0	0.17 ± 0.02	0.15 ± 0.06	0.11 ± 0.03	0.09 ± 0.04	0.10 ± 0.05	0.10 ± 0.03
C20:3n-6	0.68 ± 0.09	0.98 ± 0.13	0.92 ± 0.10	1.05 ± 0.18	1.03 ± 0.15	1.27 ± 0.18*
C20:4n-6	17.11 ± 1.73	16.95 ± 0.77	17.48 ± 1.82	17.30 ± 1.67	15.96 ± 0.49	15.74 ± 1.01
C20:3n-3	0.06 ± 0.07	0.06 ± 0.07	0.10 ± 0.08	0.10 ± 0.09	0.06 ± 0.02	0.06 ± 0.06
C20:5n-3	0.16 ± 0.02	0.17 ± 0.04	0.14 ± 0.03	0.14 ± 0.03	0.10 ± 0.05	0.11 ± 0.04
C22:4n-6	2.04 ± 0.25	2.20 ± 0.21	2.00 ± 0.24	2.02 ± 0.31	1.86 ± 0.09	1.68 ± 0.10**
C22:5n-6	7.07 ± 0.80	4.21 ± 0.69***	16.39 ± 0.46	14.24 ± 1.68*	17.80 ± 1.73	17.00 ± 1.28
C22:5n-3	0.09 ± 0.02	0.13 ± 0.04	0.02 ± 0.01	0.04 ± 0.02	0.02 ± 0.00	0.03 ± 0.01
C22:6n-3	1.74 ± 0.22	4.15 ± 0.57***	1.00 ± 0.16	2.18 ± 0.43**	0.61 ± 0.07	1.15 ± 0.13***
C24:4n-6	0.88 ± 0.12	0.87 ± 0.09	1.12 ± 0.13	1.18 ± 0.16	1.00 ± 0.06	0.99 ± 0.04
C24:5n-6	1.07 ± 0.16	0.91 ± 0.17	0.92 ± 0.15	0.97 ± 0.18	0.80 ± 0.07	0.81 ± 0.05
C26:4n-6	0.03 ± 0.02	0.02 ± 0.01	0.13 ± 0.02	0.13 ± 0.03	0.08 ± 0.04	0.11 ± 0.02
C26:5n-6	0.06 ± 0.01	0.04 ± 0.01*	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
C28:5n-6	0.03 ± 0.01	0.02 ± 0.01	0.07 ± 0.01	0.09 ± 0.01**	0.06 ± 0.02	0.07 ± 0.01
C30:5n-6	0.01 ± 0.01	0.01 ± 0.01	0.11 ± 0.02	0.11 ± 0.02	0.09 ± 0.02	0.07 ± 0.02
SFA	45.57 ± 4.61	42.94 ± 0.84	44.94 ± 3.75	46.30 ± 4.32	44.22 ± 1.93	44.45 ± 1.97
MUFA	21.20 ± 2.71	22.31 ± 1.05	13.84 ± 0.94	14.47 ± 1.19	13.09 ± 0.62	13.02 ± 0.51
n-3 PUFA	2.05 ± 0.29	3.51 ± 1.56	1.26 ± 0.18	2.46 ± 0.47***	0.79 ± 0.06	1.34 ± 0.15***
n-6 PUFA	29.10 ± 2.24	27.91 ± 1.73	37.54 ± 4.92	34.20 ± 5.81	39.81 ± 2.04	39.08 ± 2.22
n-6VLCFA	2.08 ± 0.27	1.85 ± 0.26	2.42 ± 0.30	2.56 ± 0.38	2.09 ± 0.14	2.10 ± 0.07
n-6/n-3 PUFA	14.46 ± 2.42	10.15 ± 6.02	30.62 ± 6.70	14.57 ± 4.55**	50.74 ± 4.13	29.35 ± 3.21***

Mean ± SD. The significant of differences between dietary treatments at each age were analyzed by student's *t*-test; *P<0.05, **P<0.01, ***P<0.001. PD, postnatal day; AA, arachidonic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; VLCFA, very long chain fatty acids; Cont, control; DHA, DHA diet.

Table 6.2. The effect of dietary DHA on fatty acid profiles of testistriacylglycerides from PD21 to PD90

Fatty acids (%, w/w)	PD21		PD49		PD90	
	Cont (n =7)	DHA (n = 8)	Cont (n = 8)	DHA (n = 7)	Cont (n = 9)	DHA (n = 6)
C14:0	1.67 ± 0.36	1.18 ± 0.81	0.79 ± 0.20	1.24 ± 1.24	0.89 ± 0.30	0.86 ± 0.10
C16:0	30.30 ± 3.45	21.66 ± 7.37	30.25 ± 4.38	29.68 ± 3.17	27.68 ± 2.79	28.70 ± 3.28
C18:0	7.06 ± 0.91	15.17 ± 5.43	5.89 ± 1.25	5.32 ± 0.86	4.37 ± 0.57	4.25 ± 0.46
C18:1n-9	12.47 ± 3.55	11.94 ± 2.32	13.93 ± 2.38	11.87 ± 0.64*	15.67 ± 4.19	11.32 ± 1.05*
C18:1n-7	0.85 ± 1.45	0.35 ± 0.16	1.42 ± 1.00	1.93 ± 0.95	1.27 ± 1.19	1.33 ± 1.85
C18:2n-6	3.45 ± 1.55	4.26 ± 1.88	3.56 ± 0.53	3.47 ± 0.62	3.87 ± 1.10	2.79 ± 0.44
C18:3n-3	0.56 ± 0.26	1.19 ± 1.87	0.22 ± 0.22	0.15 ± 0.20	0.38 ± 0.24	0.34 ± 0.32
C20:0	0.15 ± 0.11	0.14 ± 0.09	0.10 ± 0.11	0.12 ± 0.08	0.03 ± 0.03	0.03 ± 0.01
C20:3n-6	0.92 ± 0.18	0.80 ± 0.41	0.77 ± 0.25	0.82 ± 0.24	1.43 ± 0.13	1.75 ± 0.24**
C20:4n-6	2.62 ± 0.46	4.60 ± 1.69*	4.44 ± 0.86	3.95 ± 0.35	4.54 ± 0.67	4.98 ± 0.75
C20:3n-3	0.06 ± 0.07	0.04 ± 0.02	0.04 ± 0.02	0.08 ± 0.06	0.06 ± 0.07	0.02 ± 0.01
C20:5n-3	0.45 ± 0.17	2.37 ± 3.41	0.41 ± 0.30	0.53 ± 0.50	0.33 ± 0.23	0.30 ± 0.27
C22:4n-6	3.48 ± 0.48	4.67 ± 4.54***	5.86 ± 0.67	5.63 ± 0.91	5.20 ± 0.72	5.84 ± 0.41
C22:5n-6	19.27 ± 3.64	5.77 ± 3.67***	20.77 ± 1.70	18.89 ± 3.37	22 .43 ± 3.41	24.22 ± 3.76
C22:5n-3	0.11 ± 0.08	0.20 ± 0.28	0.04 ± 0.05	0.09 ± 0.06	0.02 ± 0.02	0.02 ± 0.03
C22:6n-3	4.25 ± 1.50	9.54 ± 4.97**	1.02 ± 0.18	2.05 ± 0.59**	0.54 ± 0.18	0.93 ± 0.10***
C24:4n-6	2.19 ± 0.33	2.65 ± 1.29	3.40 ± 0.41	3.49 ± 0.55	2.73 ± 1.08	3.39 ± 0.28
C24:5n-6	5.62 ± 1.36	3.45 ± 1.17*	5.23 ± 0.68	5.48 ± 0.93	4.45 ± 0.68	5.02 ± 0.47
C26:4n-6	0.21 ± 0.19	0.33 ± 0.23	0.20 ± 0.11	0.33 ± 0.08*	0.50 ± 0.08	0.52 ± 0.08
C26:5n-6	0.89 ± 0.29	0.99 ± 0.84	0.59 ± 0.14	0.66 ± 0.07	0.64 ± 0.09	0.69 ± 0.08
C28:5n-6	0.40 ± 0.30	0.40 ± 0.41	1.15 ± 0.19	1.29 ± 0.09	0.78 ± 0.35	0.93 ± 0.11
C30:5n-6	0.08 ± 0.02	0.04 ± 0.00	0.50 ± 0.09	0.59 ± 0.06	0.40 ± 0.06	0.40 ± 0.06
SFA	40.04 ± 4.00	37.91 ± 10.82	38.14 ± 4.67	37.59 ± 4.87	33.81 ± 3.04	34.66 ± 3.41
MUFA	14.91 ± 3.52	14.99 ± 3.38	15.95 ± 2.08	14.57 ± 1.14	17.62 ± 3.67	12.76 ± 1.73*
n-3 PUFA	5.17 ± 1.75	13.03 ± 1.98***	1.73 ± 0.41	2.88 ± 0.75**	1.32 ± 0.42	1.61 ± 0.54
n-6 PUFA	29.73 ± 3.28	19.76 ± 2.24***	33.10 ± 5.79	33.11 ± 3.98	37.83 ± 3.42	40.02 ± 4.28
n-6VLCFA	8.21 ± 3.11	7.66 ± 3.02	11.08 ± 1.22	11.84 ± 1.58	9.41 ± 1.93	10.95 ± 0.93
n-6/n-3 PUFA	4.45 ± 1.05	1.56 ± 0.33*	19.75 ± 4.56	12.33 ± 4.12*	31.58 ± 10.72	26.74 ± 7.13

Mean ± SD. The significant of differences between dietary treatments at each age were analyzed by student's *t*-test; *P<0.05, **P<0.01, ***P<0.001. PD, postnatal day; AA, arachidonic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; VLCFA, very long chain fatty acids; Cont, control; DHA, DHA diet.

Table 6.3. The effect of dietary DHA on fatty acid profiles of free fatty acids from PD21 to PD90

Fatty acids (%, w/w)	PD21		PD49		PD90	
	Cont (n = 8)	DHA (n = 7)	Cont (n = 6)	DHA (n = 4)	Cont (n = 12)	DHA (n = 11)
C14:0	1.28 ± 0.64	1.15 ± 0.27	1.05 ± 0.97	0.35 ± 0.06	0.59 ± 0.31	0.88 ± 1.41
C16:0	32.83 ± 4.80	30.79 ± 3.65	28.71 ± 2.71	27.25 ± 1.92	26.69 ± 2.06	26.15 ± 1.99
C18:0	10.53 ± 0.53	10.63 ± 1.79	9.00 ± 1.84	7.47 ± 0.66	7.44 ± 1.11	6.93 ± 0.81
C18:1n-9	11.93 ± 1.91	11.82 ± 2.23	15.13 ± 1.67	14.88 ± 0.92	14.40 ± 1.76	14.01 ± 0.97
C18:1n-7	1.42 ± 0.86	1.50 ± 1.01	0.80 ± 1.26	0.91 ± 1.57	1.36 ± 1.16	0.46 ± 0.77
C18:2n-6	3.74 ± 0.69	4.60 ± 0.88	7.74 ± 0.93	8.56 ± 0.19	6.54 ± 0.55	7.00 ± 0.71
C18:3n-3	0.29 ± 0.21	0.47 ± 0.34	0.46 ± 0.67	0.10 ± 0.09	0.24 ± 0.24	0.21 ± 0.18
C20:0	0.06 ± 0.02	0.07 ± 0.04	0.22 ± 0.25	0.07 ± 0.05	0.02 ± 0.01	0.23 ± 0.55
C20:3n-6	0.94 ± 0.19	1.15 ± 0.39	1.11 ± 0.15	1.07 ± 0.28	1.59 ± 0.17	1.61 ± 0.31
C20:4n-6	9.73 ± 2.53	8.86 ± 1.19	15.71 ± 2.63	18.14 ± 2.08	18.22 ± 1.55	17.61 ± 2.06
C20:3n-3	0.09 ± 0.03	0.08 ± 0.03	0.19 ± 0.20	0.09 ± 0.05	0.11 ± 0.07	0.11 ± 0.07
C20:5n-3	0.35 ± 0.25	0.35 ± 0.36	0.38 ± 0.38	0.06 ± 0.08	0.32 ± 0.26	0.32 ± 0.20
C22:4n-6	2.71 ± 0.29	2.81 ± 0.67	2.58 ± 0.67	2.87 ± 0.42	2.71 ± 0.32	2.71 ± 0.26
C22:5n-6	12.78 ± 2.02	7.21 ± 2.60**	9.15 ± 3.29	9.38 ± 1.84	13.16 ± 1.94	13.14 ± 2.00
C22:5n-3	0.15 ± 0.07	0.09 ± 0.06	0.04 ± 0.08	0.07 ± 0.04	0.03 ± 0.02	0.03 ± 0.02
C22:6n-3	3.59 ± 0.67	9.82 ± 2.98***	0.92 ± 0.43	1.37 ± 0.45*	0.57 ± 0.11	0.96 ± 0.23**
C24:4n-6	1.65 ± 0.27	1.61 ± 0.40	2.40 ± 0.48	2.56 ± 0.48	1.89 ± 0.55	2.19 ± 0.15
C24:5n-6	2.63 ± 0.22	2.26 ± 0.84	1.44 ± 0.41	1.57 ± 0.43	1.63 ± 0.35	1.72 ± 0.18
C26:4n-6	0.45 ± 0.04	1.11 ± 0.36	0.81 ± 0.84	0.54 ± 0.16	0.36 ± 0.08	0.43 ± 0.12
C26:5n-6	0.22 ± 0.08	0.25 ± 0.03	0.50 ± 0.26	0.43 ± 0.07	0.39 ± 0.07	0.38 ± 0.08
C28:5n-6	0.39 ± 0.07	0.21 ± 0.05	0.58 ± 0.17	0.62 ± 0.22	0.54 ± 0.09	0.50 ± 0.21
C30:5n-6	0.03 ± 0.01	0.02 ± 0.00	0.28 ± 0.10	0.34 ± 0.13	0.23 ± 0.06	0.27 ± 0.08
SFA	45.33 ± 5.38	43.55 ± 5.63	39.80 ± 4.31	35.49 ± 2.30	35.35 ± 3.23	35.01 ± 3.71
MUFA	14.32 ± 1.11	14.62 ± 1.58	16.89 ± 1.45	16.44 ± 0.78	15.83 ± 1.16	15.33 ± 0.87
n-3 PUFA	4.47 ± 0.73	10.81 ± 2.46***	1.99 ± 0.98	1.69 ± 0.57	1.23 ± 0.48	1.64 ± 0.50
n-6 PUFA	30.36 ± 4.68	25.58 ± 3.46	35.30 ± 5.23	40.32 ± 2.60	42.54 ± 3.70	42.53 ± 3.51
n-6VLCFA	5.37 ± 0.61	5.44 ± 1.61	6.02 ± 1.76	6.06 ± 1.46	5.04 ± 0.53	5.50 ± 0.42*
n-6/n-3 PUFA	6.81 ± 0.50	2.45 ± 0.57**	21.98 ± 10.37	27.93 ± 15.76	39.08 ± 14.09	29.45 ± 13.30

Mean ± SD. The significant of differences between dietary treatment at each age were analyzed by student's *t*-test*;* *P<0.05, **P<0.01, ***P<0.001. PD, postnatal day, AA, arachidonic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; VLCFA, very long chain fatty acids; Cont, control; DHA, DHA diet.

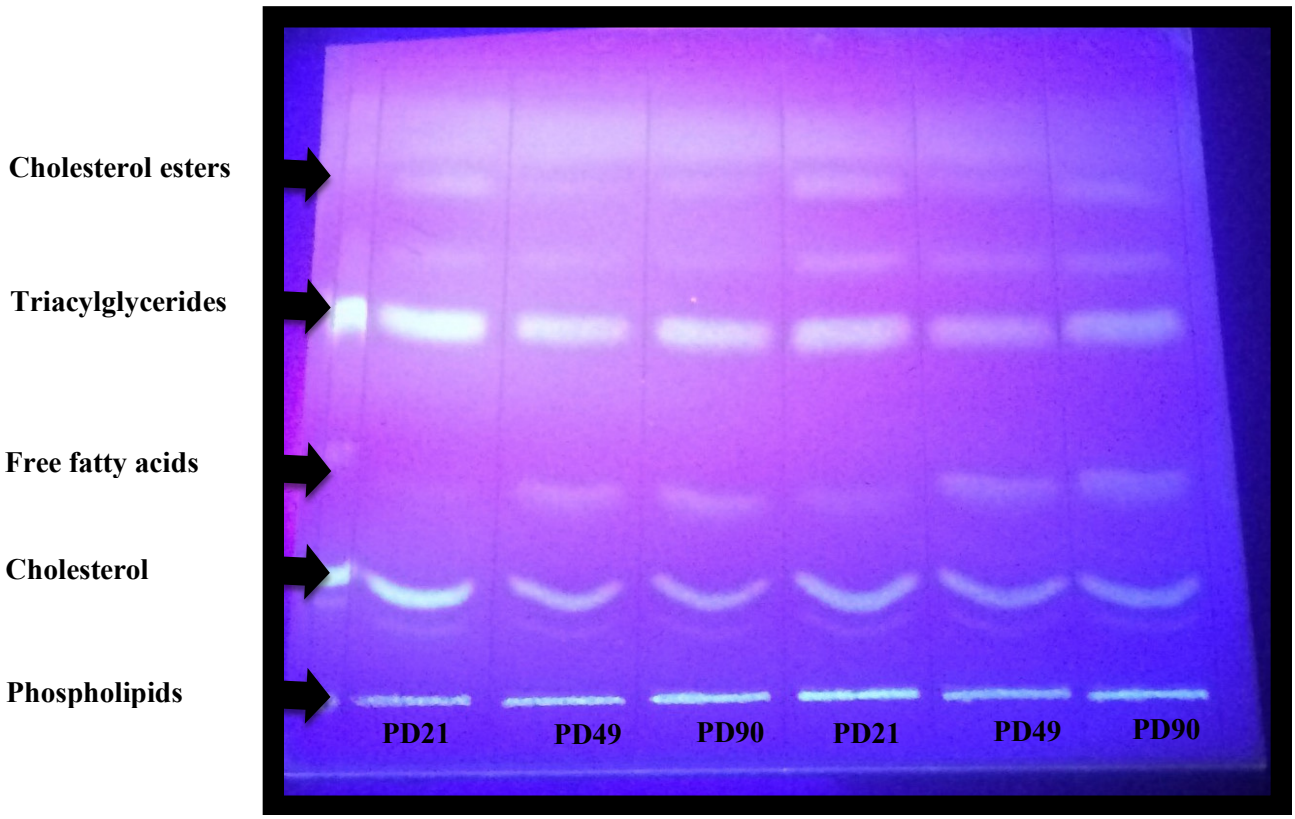
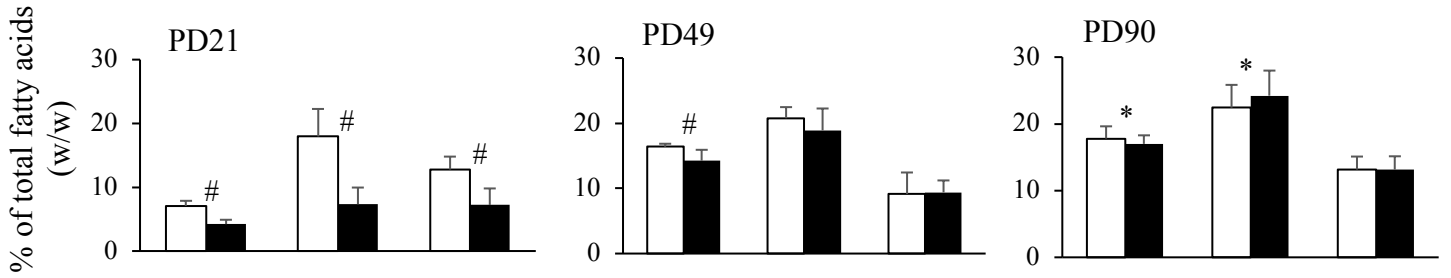


Figure 6.1. Typical testis lipid classes during development at PD21, PD49 and PD90
Illustration of the band separation observed in testis of different ages at postnatal day (PD) 21, 49 and 90; Approximately total lipid from 50 mg of tissue separated using a mobile phase of petroleum ether, diethyl ether and acetic acid in the ratio 80:20:1 (by volume), and band were visualized under UV with 0.1% aniline naphthalene sulfonic acid (w/v).

a) n-6 DPA



b) DHA

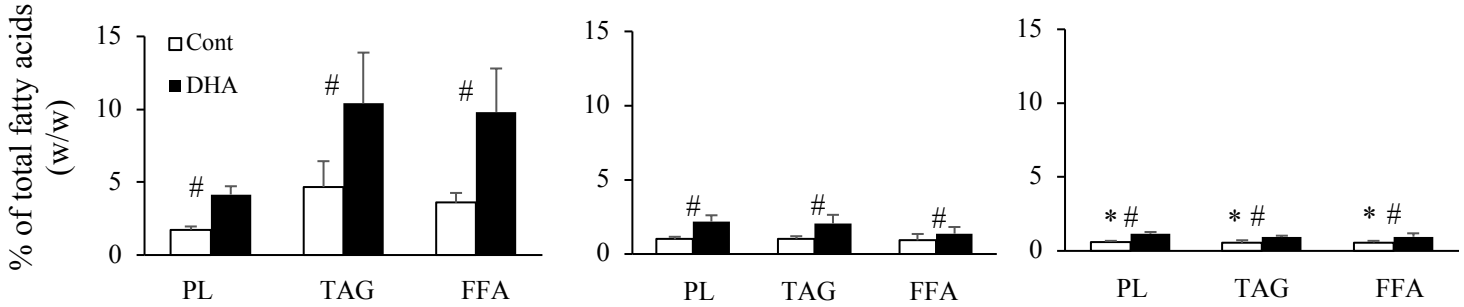


Figure 6.2. The fatty acid profiles of testis lipid classes during development from PD21 to PD90: a) n-6 DPA and b) DHA

Mean ± SD (Postnatal day (PD) 21, n=5-8 per group; PD49, n=4-8 per group; PD90, n=6-12 per group). The difference between diet or age was tested by student's t-test, ($P<0.05$). # indicates a significant difference between control and DHA treated groups in each lipid class, * indicates a significant difference between PD21 and PD90. DPA, docosapentaenoic acid; DHA, docosapentaenoic acid; PL, phospholipids; TAG, triacylglycerides; FFA, free fatty acids; Cont, control; DHA, DHA diet.

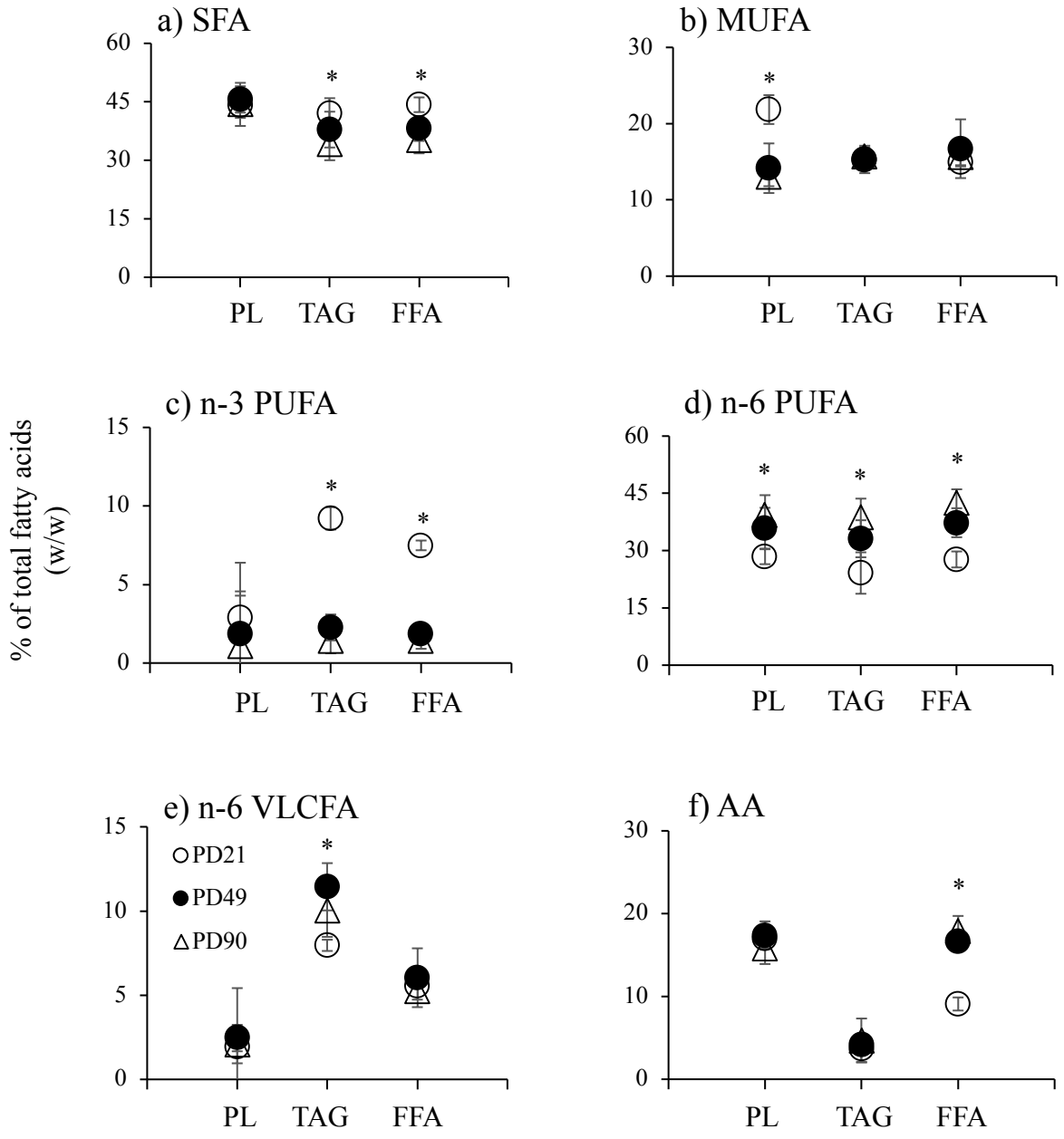


Figure 6.3. The fatty acid profiles of testis lipid classes during development from PD21 to PD90: a) SFA, b) MUFA, c) n-3 PUFA, d) n-6 PUFA, e) n-6 VLCFA ratio, f) AA

Mean ± SD (Postnatal day (PD) 21, n=5-8 per group; PD49, n=4-8 per group; PD90, n=6-12 per group). The difference between age was tested by student's *t*-test, ($P<0.05$). * indicates a significant difference between PD21 and PD90. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; VLCFA, very long chain fatty acid; AA, arachidonic acid; PL, phospholipids; TAG, triacylglycerides; FFA; free fatty acids.

CHAPTER 7. GENERAL DISCUSSION

There is limited research in relation to the effect of prenatal ethanol (EtOH) and dietary docosahexaenoic acid (DHA) on male reproductive health during development from fetus to adulthood. This study has provided an overview of the influence of dietary DHA on testis function in relation to testis lipid composition in offspring exposed to EtOH. How testicular lipid environment responds to the early provision of DHA during development was also investigated. To achieve this, the present study used the *Sprague-Dawley* rat, commonly used in the nutrition field and testis toxicology test for testicular development. The nutritionally balanced and energy-dense semi-purified diet was formulated in our laboratory to have a consistency in the nutrients provided in all groups to test our hypotheses. Fatty acids in total lipids (Chapter 5) and in each lipid class in testis (Chapter 6) were from the same animals.

Hypothesis I. Dietary DHA will improve the overall offspring development in relation to the fetal DHA status in those exposed to prenatal EtOH

Hypothesis I has been covered in Chapter 3. In order to test this hypothesis, the body weight, placental and testis weight of offspring exposed to prenatal EtOH during development, at gestational day 20 (GD20), postnatal day (PD) 4, PD21, PD49 and PD90, as well as the DHA level of fetal serum were measured. Prenatal EtOH exposure did not adversely influence on fetal DHA serum level and the offspring development. Body weight, placental and testis weight were not significantly affected by EtOH administration. As predicted, supplementing the diet with DHA increased fetal serum DHA level regardless of EtOH exposure, without any decline in that of fetus obtained from non-DHA-consuming dams. Although this study found that dietary DHA increased the body weight of the fetus, there was a significant reduction in the body weight of fetus obtained from DHA-consuming dams when they exposed to EtOH during gestational period. Therefore, the hypothesis I was not supported by the results in this study.

Hypothesis II. Dietary DHA will improve testis developmental markers in offspring exposed to prenatal EtOH

Hypothesis II has been covered in Chapter 4. To test this hypothesis, testis histological analysis, including the number and diameter of seminiferous cord at GD20, the height of germinal epithelium and diameter of seminiferous tubule at PD49 and PD90 and the germ cells maturation at PD49 were measured. Prenatal EtOH exposure did not adversely affect the testis developmental markers during development. Dietary DHA increased the height of germinal epithelium only at PD49, but not in offspring exposed to prenatal EtOH. Therefore, the hypothesis II was not supported by the results in this study.

Hypothesis III. Dietary DHA will increase the level of testosterone during development in offspring exposed to prenatal EtOH

Hypothesis III has been covered in Chapter 4. To test this hypothesis, the serum level of testosterone during development was measured and the mRNA expression of StAR, involved in testosterone production, at peri-puberty and adult rats was determined. According to our hypothesis, dietary DHA increased the level of testosterone but only in the fetus, in those with and without exposure to prenatal EtOH. As it was expected, dietary DHA normalized the prenatal EtOH-related changes in circulating testosterone and StAR mRNA expression during development. Therefore, hypothesis III was supported by the results of this study.

Hypothesis IV. Dietary DHA will increase sperm parameters in adult rats exposed to prenatal EtOH

Hypothesis IV has been covered in Chapter 4. Sperm parameters were analyzed in only adult rats exposed to prenatal EtOH with and without dietary DHA to test this hypothesis. The prenatal EtOH did not exert any significant adverse influence on sperm parameters in adult rats.

As predicted, dietary DHA increased the number of sperm with normal morphology regardless of the effect of prenatal EtOH exposure, with no effect on sperm counts and sperm motility Therefore, hypothesis IV was partially supported by the results in this study.

Hypothesis V. Total testis fatty acid level will be altered by dietary DHA as testis develops in offspring exposed to prenatal EtOH

Hypothesis V has been covered in Chapter 5. To examine this hypothesis, testicular lipid compositions were measured from fetus to adult rats at different time points as the testis matures. Prenatal EtOH did not exert a significant effect on testis fatty acid compositions, mainly on the fetus. In agreement with our hypothesis, testis fatty acid compositions differed in DHA groups from those of controls, regardless of the prenatal EtOH exposure. Dietary DHA induced a significant accumulation in testis DHA level, with the highest occurring at PD49. Therefore, hypothesis V was partially supported by the results in this study.

Hypothesis VI. Fatty acid level of major lipid class of testis will be altered by dietary DHA by increasing DHA level during development from PD21 to PD90

Hypothesis VI has been covered in Chapter 6. To test this hypothesis, we measured the fatty acid compositions in testis lipid classes, phospholipids, triacylglycerides and free fatty acids from PD21 and PD90. As expected, the distribution of major fatty acids in testis lipid classes was age-dependent, with being differently responsive to the dietary DHA. Dietary DHA increased testis DHA level in all lipid classes, regardless of testis maturation. In this study, hypothesis VI was supported by the results.

Overall, the adverse effect of prenatal EtOH exposure was not observed, particularly on the DHA content of maternal and fetal serum, as well as the offspring and testis development from fetus to adult rats. Prenatal EtOH exposure did not exert a significant impact on testis

developmental markers and lipid compositions during development. Therefore, it is not surprising that no significant changes have been revealed on sperm parameters in adult rats exposed to prenatal EtOH. Maternal EtOH exposure induced significant alterations in circulating testosterone and the gene expression of the protein involved in testosterone production during development, and were normalized by dietary DHA. It was concluded that in our study, the energy-dense semi-purified diet could have affected by maintaining optimal physiological state that might have mitigated the effects of prenatal EtOH exposure. Maternal dietary DHA at the dose used in this study significantly increased fetal DHA level in serum and testis, fetal testosterone level and improved fetal development with no impact on testis developmental markers. This indicates that DHA in maternal diet during gestation can affect offspring development and testis function. Maternal dietary DHA during lactation did not significantly change offspring development, testis size and serum testosterone level. However, it increased significantly the level of DHA in testis at PD4 and PD21 replacing by the n-6 PUFA (mainly n-6 DPA) and n-6 VLCFA. This shows that maternal dietary DHA during lactation can influence testis lipid composition. Dietary DHA after weaning increased testis developmental markers at PD49. Testis at this age also revealed the highest accumulation of DHA due to dietary DHA with no significant changes in the level of n-6 DPA. These results show that testis at PD49 was significantly responsive to dietary DHA, leading to improved testis developmental marker. This might be related to the vital changes that happen in peri-puberty from 35 to PD55-60, leading to sexual maturation. This is also supported by the highest relative weight of testis at PD49 amongst ages. All testis lipid classes significantly responded to the dietary DHA at all ages to contain higher levels of DHA in DHA-treated group. In adult rats, sperm normal morphology increased in DHA-treated rats in those with and without exposure to prenatal EtOH compared to controls. This might be related to the effect of dietary DHA on testosterone level at fetus and an overall increased DHA level in testis. As testis developed, the level of n-6 PUFA, mainly n-6 DPA significantly increased replacing n-3 PUFA, majorly DHA, MUFA and SFA. The distribution of

n-6 DPA and n-6 VLCFA in testis lipid classes is age-dependent, accumulating mainly in TAG. These results support the potential role of n-6 PUFA, mainly n-6 DPA and n-6 VLCFA, as well as TAG in rat testis development. Whether the early provision of dietary DHA improves testis function and sperm parameters requires further investigation.

Strengths and limitations

This research has provided some basic knowledge related to less understood and not as considerably investigated subject in male infertility. This study offered us a multifaceted approach in studying testis development and function under an EtOH-exposed condition coupled with a DHA supplemented diet. Utilizing a DHA enriched diet, relative to control diet devoid of DHA, but with identical macro- and micronutrients, total SFA, MUFA, and n-6/n-3 PUFA ratio made it possible to understand the stepwise metabolism of testis fatty acids evoked by only dietary DHA. Dietary DHA changed PUFA content, with no influence on the SFA and MUFA level, also offered us with an ideal platform to study PUFA metabolic changes in the testes. Providing this special diet from gestational period to adulthood permitted us to compare testicular responses to dietary DHA in immature and mature testis in relation to testis development, function and lipid composition.

The prenatal EtOH-exposed condition was not associated with adverse impact on testis maturation due to a complete and energy-dense diet utilized in our study rather than standard rat chow. Therefore, the determination of EtOH dose to evoke the tissue damage under the condition of this study might be helpful. Another limitation of this study is perhaps the ability to extrapolate these results to human studies as testis predominant fatty acid is species-specific. The number of testes used for analysis were low and varied due to 5 time points used in this study and lower percentage of males born compared to females. A larger number of tissues should be used to confirm the findings.

Recommendations for future research

Due to the critical importance of the lipid compositions in reproductive performance, the future investigation might be aimed at looking at the testis response to the early provision of dietary DHA in older adult rats to understand how the accumulation of DHA changes, along with aging, in relation to the testicular functional ability. A close look at the alterations of n-6 DPA and n-6 VLCFA in association with testis function might provide some approach to improved fertility at this period of time. Moreover, conducting observational studies to investigate the sperm fatty acid alterations in the EtOH-associated male fertility problems will cast light upon the underlying mechanism, fundamental for therapeutic approaches. Finally, although the recommendation for DHA intake required for normal growth and neural development has been determined for DHA, more studies are required to pursue if it is sufficient for males with reproductive health issues.

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Appendices

Appendix I. The effect of dietary DHA on serum fatty acid composition at GD20 in rats exposed to prenatal EtOH

Fatty acids (%, w/w)	Cont (n = 4)	Cont+DHA (n = 9)	EtOH (n = 5)	EtOH+DHA (n = 5)	PF (n = 5)	Significant effect		
						EtOH	Diet	EtOH* Diet
C14:0	1.25 ± 0.55[a]	1.15 ± 0.07[b]	1.39 ± 0.13[b]	1.30 ± 0.29[b]	1.20 ± 0.17	0.01	NS	0.006
C16:0	23.55 ± 1.79	26.42 ± 3.00	25.37 ± 0.77	24.27 ± 1.87	23.95 ± 0.69	NS	NS	NS
C18:0	11.72 ± 0.57	11.89 ± 0.53	12.00 ± 0.73	12.48 ± 0.50	12.78 ± 0.49	NS	NS	NS
C18:1n-9	21.01 ± 2.31	22.78 ± 2.46	22.46 ± 1.49	20.60 ± 1.71	19.78 ± 1.79	NS	NS	NS
C18:1n-7	2.76 ± 1.35	2.15 ± 1.27	2.00 ± 0.72	2.53 ± 0.49	2.75 ± 0.74	NS	NS	NS
C18:2n-6	9.84 ± 1.72[ab]	8.04 ± 1.58[b]	9.70 ± 0.60[ab]	11.07 ± 1.38[a]	9.52 ± 1.40	0.04	NS	NS
C18:3n-6	0.58 ± 0.20[a]	0.39 ± 0.14[b]	0.51 ± 0.04[ab]	0.41 ± 0.04[ab]	0.41 ± 0.03	NS	0.03	NS
C18:3n-3	0.26 ± 0.10	0.19 ± 0.01	0.18 ± 0.08	0.16 ± 0.06	0.19 ± 0.01	NS	NS	NS
C20:0	0.25 ± 0.15	0.29 ± 0.17	0.31 ± 0.07	0.25 ± 0.06	0.26 ± 0.08	NS	NS	NS
C20:3n-6	0.42 ± 0.24	0.51 ± 0.13	0.54 ± 0.09	0.59 ± 0.08	0.61 ± 0.04	NS	NS	NS
C20:4n-6	9.88 ± 3.73	8.12 ± 1.94	9.39 ± 0.93	9.62 ± 2.58	11.78 ± 0.92	NS	NS	NS
C20:3n-3	1.87 ± 3.46	0.02 ± 0.01	0.02 ± 0.01	0.04 ± 0.03	0.04 ± 0.01	NS	NS	NS
C20:5n-6	0.28 ± 0.16	0.27 ± 0.06	0.35 ± 0.02	0.33 ± 0.08	0.43 ± 0.25	NS	NS	NS
C22:2n-6	0.03 ± 0.05	0.00 ± 0.00	0.03 ± 0.06	0.08 ± 0.05	0.10 ± 0.06	NS	NS	NS
C22:4n-6	0.79 ± 0.11[a]	0.48 ± 0.10[b]	0.59 ± 0.11[b]	0.52 ± 0.07[b]	0.67 ± 0.08	NS	0.001	0.02
C22:5n-6	3.99 ± 0.98[a]	1.77 ± 0.64[b]	4.23 ± 0.54[a]	1.46 ± 0.15[b]	2.23 ± 1.44	NS	0.0001	NS
C22:5n-3	0.40 ± 0.12	0.28 ± 0.08	0.30 ± 0.06	0.35 ± 0.05	0.38 ± 0.14	NS	NS	NS
C22:6n-3	4.62 ± 0.77[b]	8.49 ± 2.05[a]	3.76 ± 0.44[b]	7.61 ± 1.44[a]	5.42 ± 1.37	NS	0.0001	NS
SFA	37.85 ± 3.19	41.02 ± 3.35	40.23 ± 1.20	39.46 ± 1.68	39.31 ± 0.63	NS	NS	NS
MUFA	32.51 ± 0.64[a]	31.65 ± 3.16[ab]	33.76 ± 0.76[a]	29.21 ± 1.77[b]	29.59 ± 1.58	NS	0.009	NS
n-3 PUFA	3.18 ± 3.45[a]	1.10 ± 0.17[b]	1.26 ± 0.14[b]	1.16 ± 0.21[b]	1.48 ± 0.37	NS	0.02	NS
n-6 PUFA	21.82 ± 4.99[a]	17.71 ± 3.77[b]	20.97 ± 1.17[ab]	22.54 ± 3.80[a]	24.19 ± 1.27	NS	NS	0.02

Mean ± SD; Main effects and interaction were analyzed using two-way analysis of variance (ANOVA); Values having different superscript letters are significantly different among the groups by Duncan's multiple range test ($P<0.05$). NS, not significant; SFA, saturated fatty acids; monounsaturated fatty acids, PUFA, polyunsaturated fatty acids; Cont, control; Cont+DHA, control+ DHA; EtOH, ethanol; EtOH + DHA, ethanol + DHA; PF, paired fed.

Appendix II. The effect of dietary DHA on serum fatty acid composition in dams consuming EtOH during entire pregnancy

Fatty acids (%, w/w)	Cont (n = 4)	Cont+DHA (n = 9)	EtOH (n = 5)	EtOH+DHA (n = 5)	PF (n = 5)	Significant effect		
						EtOH	Diet	EtOH* Diet
C14:0	0.51 ± 0.06	0.47 ± 0.03	0.49 ± 0.18	0.53 ± 0.15	0.58 ± 0.06	NS	NS	NS
C16:0	21.67 ± 1.80	20.46 ± 1.26	19.36 ± 1.98	21.1 ± 0.78	21.94 ± 0.93	NS	NS	NS
C18:0	11.09 ± 2.13	11.00 ± 2.86	10.59 ± 3.89	9.66 ± 1.54	10.65 ± 1.85	NS	NS	NS
C18:1n-9	23.41 ± 3.42	23.61 ± 7.19	27.05 ± 7.31	25.53 ± 3.16	24.39 ± 4.65	NS	NS	NS
C18:1n-7	1.36 ± 0.71	1.35 ± 0.61	0.86 ± 1.01	0.99 ± 0.98	1.35 ± 1.17	NS	NS	NS
C18:2n-6	11.99 ± 2.33	11.78 ± 2.01	14.02 ± 0.50	13.04 ± 0.79	12.06 ± 1.02	NS	NS	NS
C18:3n-6	0.72 ± 0.23	0.83 ± 0.12	0.74 ± 0.26	0.99 ± 0.22	0.78 ± 0.14	NS	NS	NS
C18:3n-3	0.41 ± 0.23	0.41 ± 0.11	0.42 ± 0.07	0.46 ± 0.15	0.41 ± 0.11	NS	NS	NS
C20:0	0.03 ± 0.01	0.04 ± 0.01	0.06 ± 0.06	0.03 ± 0.00	0.03 ± 0.01	NS	NS	NS
C20:3n-6	0.14 ± 0.07	0.18 ± 0.05	0.24 ± 0.23	0.13 ± 0.02	0.17 ± 0.12	NS	NS	NS
C20:4n-6	13.09 ± 2.51	12.19 ± 4.15	11.89 ± 5.00	11.88 ± 2.60	12.00 ± 1.54	NS	NS	NS
C20:3n-3	0.17 ± 0.13	0.14 ± 0.08	0.14 ± 0.12	0.07 ± 0.02	0.16 ± 0.17	NS	NS	NS
C20:5n-3	0.04 ± 0.03	0.04 ± 0.01	0.08 ± 0.10	0.03 ± 0.01	0.03 ± 0.01	NS	NS	NS
C22:2n-6	0.04 ± 0.00	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.03	0.05 ± 0.00	NS	NS	NS
C22:4n-6	0.92 ± 0.24	0.71 ± 0.14	0.89 ± 0.44	0.54 ± 0.13	0.64 ± 0.15	NS	NS	NS
C22:5n-6	4.88 ± 1.81 [a]	2.27 ± 0.88 [bc]	4.48 ± 1.31 [ab]	1.63 ± 0.87 [c]	3.02 ± 1.17	NS	0.0002	NS
C22:5n-3	0.63 ± 0.19	0.61 ± 0.05	0.77 ± 0.46	0.67 ± 0.11	0.60 ± 0.08	NS	NS	NS
C22:6n-3	4.65 ± 0.91 [b]	9.40 ± 3.10 [a]	4.24 ± 1.18 [b]	7.92 ± 0.79 [a]	6.87 ± 2.17	NS	0.006	NS
SFA	34.09 ± 3.17	32.68 ± 1.81	31.29 ± 2.85	31.98 ± 1.80	33.95 ± 2.41	NS	NS	NS
MUFA	28.17 ± 4.29	28.75 ± 7.81	31.55 ± 7.36	30.51 ± 3.59	29.2 ± 4.43	NS	NS	NS
n-3 PUFA	5.91 ± 1.08 [b]	10.59 ± 2.98 [a]	5.65 ± 1.74 [b]	9.16 ± 0.82 [a]	8.08 ± 2.02	NS	0.001	NS
n-6 PUFA	31.82 ± 1.88	27.98 ± 3.26	31.37 ± 4.31	28.35 ± 2.64	28.77 ± 2.72	NS	NS	NS

Mean ± SD; Main effects and interaction were analyzed using two-way analysis of variance (ANOVA); Values having different superscript letters are significantly different among the groups by Duncan's multiple range test ($P<0.05$). NS, not significant; SFA, saturated fatty acids; monounsaturated fatty acids, PUFA, polyunsaturated fatty acids; Cont, control; Cont+DHA, control+ DHA; EtOH, ethanol; EtOH + DHA, ethanol + DHA; PF, paired fed.