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Impact of Fish Oil and Vitamin E Maternal Mice Dietary Supplementation on Offspring's Testis and Brain Development and Function

Ghasemi ZS[1,5], Akbarinejad V[2], Shahverdi A[1,3], Ramezani M[4], Kheymeh A[5], Hezavehei M[1], Esmaeili V[1], Taheri P[6], Numan Bucak M[7] and Alizadeh A[1]*

[1]Department of Embryology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran

[2]Department of Theriogenology, University of Tehran, Tehran, Iran

[3]Reproductive Epidemiology Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran

[4]Department of Biology, Central Tehran Branch, Islamic Azad University, Tehran, Iran

[5]Animal Core Facility, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran

[6]Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

[7]Department of Reproduction and Artificial Insemination, Selcuk University, Konya, Turkey

Abstract

According to developmental origins of health and disease hypothesis, we focused on maternal nutrition effects on testis alongside with brain. The aim was to evaluate feeding mice mothers with I) n-6 group, in which 300 mg Sunflower Oil (SO) and 50 IU vitamin E (standard levels of vitamin E (VE), II) n-3 group, in which 300 mg Fish Oil (FO) and 50 IU VE, III) n-6+VE group, in which 300 mg SO and 125 IU VE and IV) n-3+VE group, in which 300 mg FO and 125 IU VE were included in 1 kg of diet for 56 days on the testis and brain function. Seminiferous tubules diameter, seminiferous epithelium thickness and Leydig cell numbers were positively influenced by the main effect of VE (P<0.05). Number of seminiferous tubules and Sertoli cells were not influenced. Total and progressive motility were not influenced by the interaction effect of FO by VE and the main effect of FO; however, these motility parameters were positively impacted by the main effect of VE (P< 0.01). Number of neurons, glial cells and Y-maze findings were not affected by the interaction effect of FO by VE and the main effect of FO while it was adversely influenced by the main effect of vitamin E (P< 0.05). These results suggest that unique fatty acids in fish oil combined vitamin E supplementation is effective in both fetal and postnatal stages of brain and testes development which warrants further studies for male offspring reproduction.

Keywords: Embryonology; Reproductive development; Maternal nutrition; Fatty acids; Vitamin E

Introduction

It was show that consumption of n-6 fatty acids (FA) has dramatically increased over recent decades, particularly in developing countries [1]. n-6 FA are considered as essential FA and believed to be required for synthesis of a plethora of endogenous components playing pivotal role for normal function of several organs. However, excessive consumption of n-6 FA favors pro-thrombotic and pro-inflammatory mechanisms and could contribute to pathogenesis of various conditions including atherosclerosis, obesity, and diabetes [2]. These conditioned would be aggravated when over-consumption of n-6 FA is accompanied by under-consumption of n-3 FA since n-6: n-3 balance would be of utmost importance for proper regulation of various pathways, systems and organs [2,3], especially it has curial roles in maternal nutrition [4].

Among various categories of cells present in the body, three types of cells, including neurons, retina rod outer segments, and male gamete, have been recognized for possessing plasma membranes enriched in n-3 PUFAs [5]. The high levels Docosahexaenoic acid (DHA, C22:6n-3) of in the brain as well as retina suggests that this FA has important roles in retinal and neural function [6]. In

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*Correspondence:
Alizadeh Moghadam Masouleh AliReza, Department of Embryology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran.
Tel: 00982123562738
E-mail: alizadehmasouleh@royaninstitute.org; ar.alizadehmasouleh@ royan-rc.ac.ir

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this sense, n-3 PUFAs contribute to modulation in performance and structure of biological membranes such as ion permeability, membrane organization and elasticity may therefore facilitate brain function [7,8]. In primate models several investigations have shown that n-3 PUFAs deficiency in maternal and offspring diets result in altered brain and retinal FA composition and are associated with impaired brain and eye efficiency [9]. Besides, n-3 FA have pivotal roles in testis and male reproduction [10]. The testes and male gamete have a unique lipid component that is highly enriched in PUFAs, predominantly FA contains twenty-two carbons such as docosapentaenoic acid (DPA, 22:5 n-6) in mice and DHA in humans and ruminants. LA and Alphalinolenic acid (ALA; C18:3 n-3) together with their metabolites: Arachidonic acid (AA; C20:4 n-6), Eicosapentaenoic acid (EPA; C20:5 n-3) and DHA, are deposited in reproductive tissues and potentially influence the male reproductive function and fertility. Transformation of n-3 PUFAs to membrane of the respective cells and tissues is believed to occur over different stages of development through placental nutrition, mother's milk, and dietary sources [11]. Although numerous studies evaluated FA supplementation in maternal diet and confirmed the effects of several FA on offspring's brain and vision, little information exists regards effects maternal nutrition on male offspring reproductive organs.

One of the concerns with n-3 PUFAs is the fact that these FA could easily be oxidated due to the susceptibility of their double bonds in the chain, culminating in rancidification and generation of unfavorable components [12-14]. As a result, antioxidants, including vitamin E, are used to protect n-3 PUFAs supplements from rancidity so as to maintain their beneficial impacts [15]. Yet there is little information available whether incorporation of vitamin E into supplements influence the positive effects of n-3 PUFAs, especially in maternal diet.

Prenatal period is considered as one the most important stages of development [16] and David Barker was one of the first scientists to describe a relation between events occurring in uterus and offspring's hypertension and type 2 diabetes which recognized as Developmental Origins of Health and Disease (DOHaD) hypothesis [17,18]. Indeed, adding some fatty acids during prenatal and lactation period have beneficial effects (longer gestation period, improve offspring's growth and diminished hazard of pregnancy complications) [19]. High levels of the n-3 PUFAs are concentrated in nervous system membrane phospholipids, particularly phosphatidylethanolamine (PE) and phosphatidylserine (PS), during fetal and neonatal development, paralleling membrane expansion in neurogenesis, dendritic aborization, and synaptogenesis [20]. Uniquely, Prior to birth, all of the DHA accumulated in the fetal brain must originate from n-3 FA in the maternal diet *via* placental transfer [21]. Many studies have shown that n-3 FA deprivation during development results in decreased DHA in brain membrane phospholipids, reduced performance in learning tasks, altered activity of membrane receptors and proteins, and altered metabolism of several neurotransmitters, including dopamine [20]; nevertheless, they did not investigate reproductive function alongside central nervous system function. Further, there are studies indicating alterations in reproductive function of the females as a consequence of maternal under-nutrition status [22-24] yet little information exists on male offspring [25]. In this regard, Bielli *et al*., 2001 supplemented ewe diet during pregnancy and they illustrated that number of Leydig and Sertoli cells were increased in lambs [26]. In other study, female rats fed high fat diet during pregnancy and lactation. In male's offspring, the median age of pubertal onset was lowest in high fat diet whereas control males were eldest at pubertal onset [27]. However, to our knowledge, the question of whether supplementing maternal diet with PUFA source with and without vitamin E affects male offspring semen quality alongside with brain function has not been addressed.

Accordingly, the current study was designed to investigate the effects of supplementation of maternal diet, basically containing n-6 FA, with n-3 FA and/or vitamin E during pre-conceptional, prenatal and postnatal periods on reproductive as well as brain development and function of male offspring in mice.

Materials and Methods

Study design, animals and experimental diets

This study was approved by the Ethics Committee of the Royan Institute (Project Code: 95000053). Forty mature female NMRI mice (age: 8 weeks) were housed at the animal center of Royan Institute (Tehran, Iran) under a standard 12-hour light/ 12-hour dark schedule with free access to food and water. The mice were fed four different experimental diet containing: I) n-6 group, in which 300 mg sunflower oil and 50 IU vitamin E (standard levels of vitamin E) were included in 1 kg of diet, II) n-3 group, in which 300 mg fish oil and 50 IU vitamin E were included in 1 kg of diet, III) n-6+VE group, in which 300 mg sunflower oil and 125 IU vitamin E (high levels of vitamin E) were included in 1 kg of diet and IV) n-3+VE group, in which 300 mg fish oil and 125 IU vitamin E were included in 1 kg of diet. Major FA of sunflower oil is 54.5% linolenic acid (C18:2 n-6). Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) levels in fish oil were 12 and 18% respectively. n-6: n-3 FA ratios were 97.2 and 46.6 for sunflower and fish oil, respectively. Female mice had ad libitum access to experimental diets during pre-conceptional (for 14 days before mating), pre-natal (over the course of pregnancy; 21 days), and post-conceptional (from parturition to weaning; 21 days). Therefore, mothers fed for 56 days by experimental diets. The sex ratio far mating was 2 females: 1 male. Pregnancy was determined based on the presence of vaginal plug, at which the male was removed from the cage and that day was designated as day 0.5 of gestation. After weaning, all offspring fed a similar standard diet until puberty.

At nine weeks of age, offspring body weight was recorded and male offspring were anesthetized with intra-peritoneal injection of 100 mg/kg ketamine 10% (Alfasan, Woerden, the Netherlands) and 10 mg/kg xylezine 2% (Alfasan, Woerden, the Netherlands).

Y-maze test

This is based on the mice behavior that natural tendency to prospect a new surroundings. The apparatus consists of three identical arms placed at 120° around an equilateral triangular platform in the center. At nine weeks of age (n=15/each group), to assess spatial learning of the animal, they were introduced to the Y-maze test, which described by Holter *et al*., 2015 [28].

Anogenital distance measurement

The anogenital distance (AGD) was measured (age: 9 weeks; n=15/each group) with digital caliper (Stoelting, USA) from the anus to genital papilla as described by Driesche *et al*., 2011 [29].

Sperm analysis

Spermatozoa were obtained by cutting the cauda epididymis from each mouse (n=10/each group). The cauda epididymis was dissected and placed in 1 ml of pre-warmed T6 culture medium that contained bovine serum albumin for epididymal sperm preparation.

Table 1: Food intake, water consumption, litter size, proportion of male pups, body weight of male pups and anogenital distance (AGD) of male pups in n-6, n-3, n-6+VE and n-3+VE groups.

	n-6	n-3	n-6+VE	n-3+VE
Food intake during gestation (kg)	0.714	0.927	0.705	1.197
Water consumption during gestation (ml)	1310	3030	1300	1717
Food intake during lactation (kg)	1.596	2.638	1.615	2.868
Water consumption during lactation (ml)	2320	3095	2300	3625
Litter size	8.17 ± 0.70^{a}	11.83 ± 0.70^{b}	10.67 ± 0.70^{ab}	9.62 ± 0.61^{ab}
Proportion of male pups (%)	46.51 (20/43)	41.54 (27/65)	48.98 (24/49)	43.48 (30/69)
Body weight at weaning (gr)	15.69 ± 0.43^{a}	12.42 ± 0.76^{b}	13.48 ± 0.48^{ab}	14.92 ± 0.63^{a}
Body weight at puberty (gr)	37.93 ± 0.87^{a}	38.13 ± 0.78^{a}	33.17 ± 0.73^{b}	37.48 ± 0.47^{a}
Weight gain from weaning to puberty (gr)	21.95 ± 0.85^{ab}	23.49 ± 0.97^{a}	19.59 ± 0.91^{b}	20.67 ± 0.95^{ab}
AGD (mm)	13.12 ± 0.20^{a}	14.16 ± 0.24^{b}	12.42 ± 0.25^{a}	14.49 ± 0.25^{b}

^{ab}Values with different superscripts within rows differ ($P<0.05$).

Gentle agitation along with tearing of the tissue was applied to make spermatozoa to swim-up into the medium. Spermatozoa were put in T6 medium tube and incubated (37°, CO_2) for 40 minutes. Sperm parameters were evaluated by CASA (computer-assisted sperm analysis), consisting of a phase contrast microscope (Eclipse E-200, Nikon Co., Japan) with a warm plate, which was equipped with Sperm Class Analyzer® software (SCA, full research version 5.1, Microptic Co., Spain). The Eosin staining protocol was recommended for viability assessment (mixing 5 μl of sperm sample with 5 μl of dye (0.5% w/v; Merck Chemical Co., Darmstadt, Germany). Viability was assessed by counting 200 cells under phase contrast at 1000×magnification. Sperm displaying partial or complete purple staining were considered dead while spermatozoa showing strict exclusion of stain were regarded as alive.

Testes and brain tissue preparation

Tissue samples (n=6/each group) of the testis from each mouse were excised and immersed in 4% paraformaldehyde and then embedded in paraffin wax using standard techniques. Serial sections (8 μm) were prepared in the coronal planes and stained with hematoxylin and eosin (H&E). The slides were photographed by use of an Olympus BX51 light microscope with Olympus DP12 camera. Total number of seminiferous tube, seminiferous epithelium and diameter, spermatogonia, spermatocyte, Leydig and Sertoli cells were count using image analysis software (ImageJ; National Institutes of Health, Bethesda, MD, USA) [25].

Mouse brains were fixed by transcardial perfusion of 4% paraformaldehyde (n=6/each group). After perfusion the brain specimens were removed from the skulls and further fixed in 10% formalin for several days. For histological examination, fixed brain specimens were embedded in paraffin and sectioned at 8 μm thickness. Brain sections were mounted on glass slides and stained with Gimsa. Olympus (BX51) light microscope with Olympus DP12 camera was used to capture images from hippocampal region. Cells with distinct nucleus, dark blue staining and relatively larger size were considered as neurons, and cells with amorphous shape, lighter teal blue staining and relatively smaller size were considered as glial cells. Neuron and glia cells were count with ImageJ software [20].

Statistical analysis

Continuous data were analyzed by linear regression using GLM procedure of SAS program and binary data were analyzed by logistic regression using GENMOD procedure including function link logit in the model. LSMEANS statement was used to perform multiple comparisons. All analyses were conducted in SAS (SAS Inc., NC, USA). Experimental groups differences were considered significant at $P\leq0.05$ and $P\leq0.1$ for trends.

Results

Litter size and proportion of male offspring

Food intake and water consumption were recorded during experiment (Table1). Litter size was larger in n-3 group than n-6 group ($P=0.007$), but there was no significant difference among other groups ($P>0.05$; Table 1). However, the proportion of male offspring was not influenced by treatments ($P>0.05$; Table 1).

Body weight

At weaning, male pups were lighter in n-3 group compared with n-6 and n-3+VE groups ($P<0.05$), whereas at puberty, male pups in n-6+VE group had lesser body weight than male pups in n-6, n-3 and n-3+VE groups ($P<0.001$). Weight gain of male pups from weaning to puberty was not affected by the main effect of fish oil and the interaction effect of fish oil by vitamin E ($P>0.05$), yet it was negatively influenced by the main effect of vitamin E ($P=0.007$; Table 1).

Anogenital distance (AGD)

AGD was longer in n-3 and n-3+VE groups as compared with n-6 and n-6+VE groups ($P<0.05$), indicating that this measure was influenced by the main effect of maternal consumption of fish oil ($P<0.0001$; Table 1).

Testis tissue

Number of seminiferous tubules was not affected with experimental diets ($P>0.05$). Diameter of seminiferous tubules and thickness of seminiferous epithelium were not affected by the interaction effect of fish oil by vitamin E and the main effect of fish oil ($P>0.05$), yet it was positively influenced by the main effect of vitamin E ($P<0.05$; Figure 1). The interaction effect of fish oil by vitamin E did not influence number of Leydig cells ($P>0.05$); nonetheless, the main effect of vitamin E positively impacted number of Leydig cells ($P=0.026$) and the main effect of fish oil tended to negatively impact number of Leydig cells ($P=0.067$; Figure 1). Number of Sertoli cells was not influenced by treatments ($P>0.05$; Figure 1). Number of spermatogonia was greater in n-3 than n-6 group ($P=0.002$), but it was not significantly different among other groups ($P>0.05$; Figure 1). Number of spermatocytes was not affected by the interaction effect of fish oil by vitamin E and the main effect of vitamin E ($P>0.05$); however, it was positively impacted by the main effect of fish oil

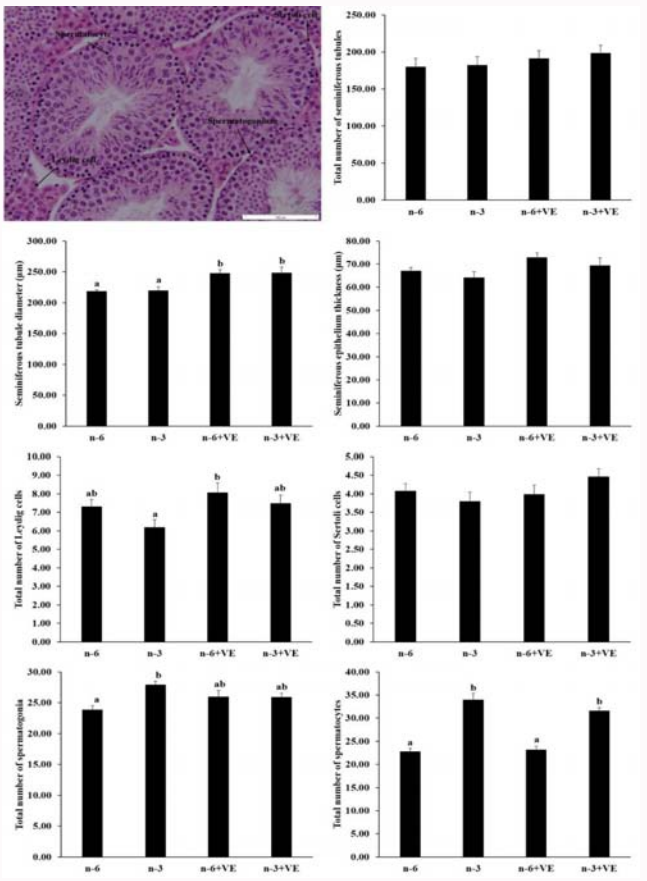

Figure 1: Microscopic image of the testis tissue of the control group [Hematoxyline-eosin (H&E) stain×10] and total number of seminiferous tubule number and diameters, thickness of seminiferous epithelium, number of Leydig cells, Number of Sertoli cells, Number of spermatogonia, and Number of spermatocytes in experimental groups. The mother mice were fed four different diets: **I)** n-6 group, in which 300 mg sunflower oil (SO) and 50 IU vitamin E (standard levels of vitamin E (VE), **II)** n-3 group, in which 300 mg fish oil (FO) and 50 IU VE, **III)** n-6+VE group, in which 300 mg SO and 125 IU VE (high levels of vitamin E) and **IV)** n-3+VE group, in which 300 mg FO and 125 IU VE were included in 1 kg of diet for 56 days. Data were presented as means±SEM (n=6/each group). a,b Values with different superscripts significant differences among the different experimental group (P<0.05).

Table 2: Sperm parameters of male pups in n-6, n-3, n-6+VE and n-3+VE groups.

Parameter	n-6	n-3	n-6+VE	n-3+VE
Concentration(M/ml)	6.0±0.30^{a}	7.4±0.34^{b}	8±0.40^{b}	7.8±0.41^{b}
Total motility %	41.0±5.07^{a}	47.0±4.2^{ab}	52.0±6.04^{ab}	63±3.56^{b}
Progressive motility %	22±3.81^{a}	27±4.4^{ab}	33±5.44^{ab}	42±4.69^{b}
VCL (µm/s)	50.8±6.93^{a}	76.5±12.66^{ab}	76.6±11.89^{ab}	96.4±7.25^{b}
VSL (µm/s)	12±2.47^{a}	31.7±8.58^{ab}	26.8±7.15^{ab}	38.8±4.81^{b}
VAP (µm/s)	21.7±3.42^{a}	42.2±9.33^{ab}	40.1±8.57^{ab}	53.3±4.45^{b}

^{ab}Values with different superscripts within rows differ (P<0.05).

(P<0.0001; Figure 1).

Sperm parameters

Semen concentration was greater in n-3, n-6+VE and n-3+VE groups than n-6 group (P<0.05; Table 2). Total and progressive motility were not influenced by the interaction effect of fish oil by vitamin E and the main effect of fish oil (P>0.05); however, these motility parameters were positively impacted by the main effect of vitamin E (P<0.01; Table 2). VCL, VSL and VAP were not affected

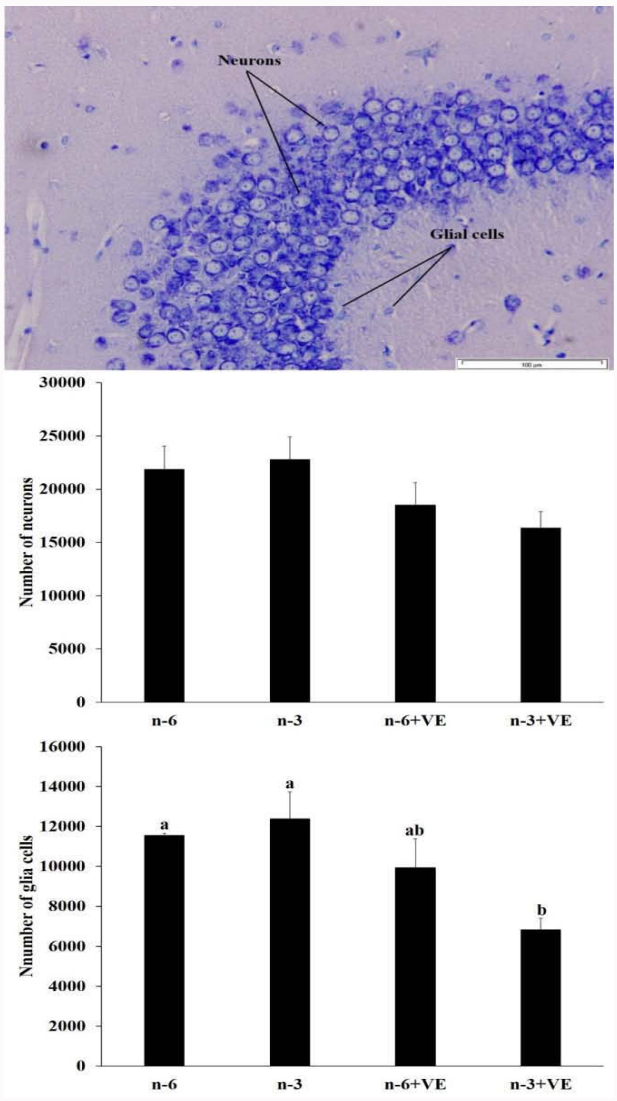

Figure 2: Microscopic image of neurons and glial cells of the control group [Giemsa stain×10] and differences between experimental groups. The mother mice were fed four different diets: **I)** n-6 group, in which 300 mg sunflower oil (SO) and 50 IU vitamin E (standard levels of vitamin E (VE), **II)** n-3 group, in which 300 mg fish oil (FO) and 50 IU VE, **III)** n-6+VE group, in which 300 mg SO and 125 IU VE (high levels of vitamin E) and **IV)** n-3+VE group, in which 300 mg FO and 125 IU VE were included in 1 kg of diet for 56 days. Data were presented as means±SEM (n=6/each group). a,b Values with different superscripts significant differences among the different experimental group (P<0.05).

by the interaction effect of fish oil by vitamin E (P>0.05), but these velocity variables were positively influenced by the main effects of fish oil and vitamin E (P<0.05; Table 2).

Brain tissue

Number of neurons and glial cells was not affected by the interaction effect of fish oil by vitamin E and the main effect of fish oil (P>0.05), but it was adversely influenced by the main effect of vitamin E (P<0.05; Figure 2).

Y-maze and total entrance

Treatments did not affect Y-maze findings (P>0.05). Although the interaction effect of fish oil by vitamin E and the main effect of fish oil did not influence total entrance numbers (P>0.05), the main effect

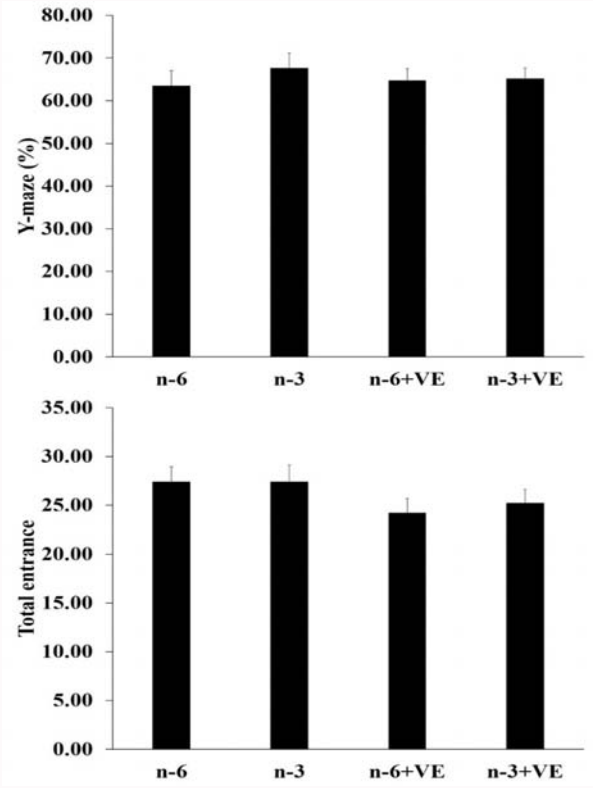

Figure 3: Y-maze test and total entrance in experimental groups. The mother mice were fed four different diets: **I)** n-6 group, in which 300 mg sunflower oil (SO) and 50 IU vitamin E (standard levels of vitamin E (VE), **II)** n-3 group, in which 300 mg fish oil (FO) and 50 IU VE, **III)** n-6+VE group, in which 300 mg SO and 125 IU VE (high levels of vitamin E) and **IV)** n-3+VE group, in which 300 mg FO and 125 IU VE were included in 1 kg of diet for 56 days. Data were presented as means±SEM (n=15/each group). Maternal dietary treatments did not affect Y-maze findings and total entrance.

of vitamin E tended to negatively impact total entrance numbers (P=0.090; Figure 3).

Discussion

This study provides compelling evidence on impressive effects of fish oil within favorable selects of antioxidant source in maternal mice diet on offspring's brain and testis. For the first time, we focused on maternal nutrition effects on testes alongside with brain. Consumption of diet enriched in n-3 PUFAs led to increment in the number of pups in comparison to consumption of n-6 PUFAs in the present study. By contrast, the study conducted by Yi *et al.*, indicated diet rich in n-3 PUFAs decreased ovulation rate, and in turn, litter size through disruption of prostaglandin synthesis at the time of ovulation in mice [30]. It is worth noting that litter size could be influenced by various factors including number follicles developing to pre-ovulatory stage, well-organized ovulatory properties and survival of embryos [31-33]. Hence, it is possible that the positive impact of n-3 PUFAs on litter size was mediated through ovulatory-independent mechanisms. In this context, Smits et al. reported that feeding sows with a diet containing fish oil before farrowing and during lactation could increase litter size [34]. Stimulatory effects of n-3 FA on oocyte quality and ovarian function were reported in previous studies [35,36]. These positive effects of n-3 PUFAs on bovine reproduction could be attributed to stimulated proliferation and steroidogenesis in bovine granulosa cells of ovarian follicles, greater oocyte developmental competence and reduction of PGF2α synthesis by uterine tissue. Similarly, Nateghi *et al.*, confirmed that fish oil could enhance ovarian function in laying hens [36]. Besides, increase in the number of fetuses in mice supplemented with fish oil has been attributed to mitigated generation of placental inflammatory cytokines and resultant greater fetal sustainability [37].

The lesser body weight of pups in n-3 group could to some extent be attributed to larger litter size in n-3 group as in the respective group higher number pups had access to limited nourishment supplied by their dam during suckling and this phenomenon disappeared following weaning by puberty, during which the pups had ad libitum access to food because it is well-established that the weight gain of the young decreases if the mother could not afford adequate milk production for extra pups [38,39]. Similarly, Fountain *et al.*, in mice and Reyes-Hernandez *et al.*, in rat reported that omega-3 FA consumption in mother's diet decreased offspring's body weight compared with control [40,41]. Further, the present study revealed consumption of vitamin E during preconsptional, prenatal and postnatal period by mothers culminated in inferior weight gain from weaning to puberty in offspring, for which the precise underlying mechanism is unknown. Although consumption of an appropriate amount of various vitamins including vitamin E have been suggested during gestation [42], it is possible that excessive consumption of these components bring about unfavorable outcomes which warrants further research.

Longer AGD in the offspring of mothers fed fish oil indicates a higher level androgen exposure during fetal period due to maternal consumption of n-3 PUFAs [29,43]. The higher androgen level might have originated from the effect of fish oil on the mother and maternal circulation since fish oil, particularly eicosapentaenoic acid, have been reported to promote testosterone production in murine [44]. Alternatively, FA components of fish oil could have passed through placenta and upregulated synthesis of androgens in the fetus. Irrespective of the origin of higher androgen exposure, it is noteworthy that androgens play critical roles in the development of male genital organs and the effect of prenatal consumption of fish oil on testicular development might have mediated through modulation of fetal androgen level [45]. Interestingly, previous studies on AGD and reproductive parameters in male illustrated significant positive associations between AGD and sperm concentration and total sperm motility [46]. Zhou *et al.*, showed a positive association among AGD and reproductive hormones which they suggested that AGD could prudently be considered in predicting reproductive outcomes in adult males [47]. Uniquely, Driesche *et al.*, showed that measurement of AGD may provide lifelong fetal androgen exposure during the masculinization programming window [29].

It was illustrated that mammalian sperm contains high levels of omega-3 fatty acids, mainly in form of 22 carbons FA [10]. Both total motility and progressive motility in offspring's who they mother consumed fish oil and vitamin E were higher than n-6 group. Although maternal diet effects on offspring semen quality was not reported, our results were consistent with results in humans [48,49], duck [50], dogs [51], and rams [52] which they confirmed that fish oil and vitamin E could improve semen quality. PUFA provide large amounts of FA for potential conversion to type-2 prostaglandins and typ-4 leukotriense, although prostaglandins in seminal plasma regulate various aspects of sperm function. Other possible mechanisms whereby dietary FA could promote spermatogenesis before puberty, was suggested as regulation of gene expression, especially peroxisome proliferator-activated receptor gamma (PPARγ) [53]. An important consideration

is the potential interaction of PUFA derived eicosanoids with the hypothalamus-pituitary-gonadal axis and the hormonal control of spermatogenesis. Thus, the effects of dietary PUFA on the secretion of GnRH, LH, FSH and testosterone, and on the responsiveness of the relevant types of cells to these hormone maybe worthy investigation [50]. Irrespective of the underlying mechanism, the present study indicates maternal dietary PUFA supplementation by n-3 series can improve sperm concentration, progressive motility and also emphasizes the importance of adequate dietary vitamin E in per oxidative reactions in semen.

In our study, dietary FA during pregnancy and weaning was clearly associated with increase of spermatogonia cells in offsprings. In a basic study, Ahluwalia *et al*., has reported that fat-free pelleted diet in 3-month-old male rabbits could extensive degenerative changes in the seminiferous tubules; no stage beyond secondary spermatocyte was evident [54]. Change in the level of nutrition can induce a reversible, non-pathological process that leads to change in testis mass, sperm output and the efficiency of spermatogenesis [55]. In this regards, feeding mature rats with high fat diet rich in saturated FA for 4 months result in reduction in seminiferous tubules diameter [56]. Some nutrients alter the seminiferous tubules diameter, the relative proportion of testis occupied by the seminiferous tubules, the proportion of the seminiferous tubule engaged by the epithelium of seminiferous, the relative proportion of interstitial tissue and the leydig cells total volume of [55]. Previously, it was suggested that change in dietary fat intake alters the responsiveness of leydig cells by changing LH-stimulated adenylate cyclase activity and testosterone synthesis. Therefore, it seems that modification of the lipid composition of the testicular plasma membrane by dietary treatment affects accessibility of LH/hCG receptors [57]. LH stimulate the interstitial cells located in the testes to produce testosterone, and FSH plays a role in spermatogenesis [58]. On the other hand, antioxidants increase the level of testosterone by affecting spermatogenic and sertoli cells and maintain the health of male reproductive system.

In the present study, the positive effects of fish oil on most germ cells were observed; but in the case of Leydig cells, sole fish oil reduced these cells and vitamin E as an antioxidant has prevented this effects. The putative role of Sertoli cells is nutritional support of germ cells and these cells has a constant capacity for the number of germ cells it can support [59]. In our study, sertoli cells were not affected by maternal nutrition. Beilli *et al*., showed under nutrition during pregnancy in sheep reduce testicular development in newborn, reduce sertoli cells and the future capacity for sperm production and fertility [60]. Finally, according to the present study, the effects of maternal nutrition during pregnancy and lactation on offsprings' sperm parameters indicated importance of fetal period. According to previous studies on omega-3 FA in human maternal diet, it is an exciting and emerging area for research and clinical studies.

For the first time, we focused on maternal nutrition effects on testes alongside with brain. In our study, total number of neurons in the hippocampus tended to decrease by vitamin E supplementation and both fish oil and vitamin E decreased the number of glial cells. Brain contains high neurons and glial cells, both of which arise from embryonic neural stem cells. Innis and coworkers' studies have suggested that n-3 FA shortage drops the size of neurons in brain, and decline the complexity of dendritic arborizations on cortical neurons [61]. Uniquely, Brenna in a vast literature review done on animal studies, reported that high n-6: low n-3 seed oils fed as the exclusive source of fat to pregnant and lactating animals leads to behavioral and neural abnormalities in the offspring [4]. In this regard, Sakayori *et al*., showed that inducing a nutritional imbalance of n-6/n-3 ratios in critical developmental periods has long-term functional consequences, revealing as advancement anxiety-related behavior in the adult mice offspring [62].

Although we could improve offspring sperm quality by maternal omega-3 supplementation, our findings in brain were unexpected. Niculescu *et al*., reported that postnatal n-3 supplementation enhances neurogenesis in the dentate gyrus of the offspring at postnatal day 19, but its beneficial effects are offset by n-3 deficiency in prenatal diet [63]. In Elsherbiny *et al*., study mother rats were fed DHA during lactation and up to 3 weeks' post-lactation [9]. In the brains of 3-week-old offspring a decrease of n-6 to 10%, an increase of n-3 to 15%, a decrease in the n-6/n-3 ratio of 22% and a decrease in the ratio of arachidonic acid to DHA of 20% were reported. But Helland *et al*., showed that supplemented pregnant women with EPA and DHA in gestation period, has no significant impact on electroencephalogram test results of offsprings neural development or novelty preference than control group [64]. On the other hand, the effects of vitamins on brain development is dependent on the area of the brain, the timing, dose and duration of vitamin exposure [65].

Brain is particularly susceptible to oxidative damage and has the lowest antioxidant enzyme activities among all tissues [15] and Vitamin E is essential for normal neurological function [66]. Although various studies have examined the effects of dietary vitamin E on brain function, little information exists on the effective use of antioxidants in maternal diet. For example, in 2008, Tsai *et al*., focused on the antioxidant status of chicks' brain by feeding their mothers with different doses of vitamin E. Daily consumption of 40 mg of vitamin E in the mother's diet had no effect on the level of vitamin E in the chicks' brain, but at 160 and 140 mg dosages resulted in an increase the level of vitamin E in the chicks' brain. Moreover, the level of ROS in the chicks' brain was reduced by increasing vitamin E in the mother's diet [15]. On the other hand, increased levels of superoxide dismutase activity in chick's brain were positively associated with high doses of vitamin E in the mother's diet. Vitamin E recycle alpha lipoic acid which is metal chelator leading to the decrease in level of brain iron by chelating Fe^{+2}. Their results showed an insignificantly increasing the activity of superoxide dismutase of the chick brain with increasing maternal supplementation.

In conclusion, fish oil and vitamin E decreased the number of glial cells. Although we could improve offspring sperm quality by maternal omega-3 supplementation, our findings in brain were unexpected. The essentiality of some nutrients to neuronal development, alongside the little information of requirements for these nutrients during prenatal and lactation period warrants deeper investigations on their impact on several tissue. These results suggest that unique FA in fish oil combined vitamin E supplementation is effective in both fetal and postnatal stages of brain and testes development which warrants further studies for male offspring reproduction.

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