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ORIGINAL ARTICLE

Sperm Biology

Dietary n-6:n-3 ratio and Vitamin E improve motility characteristics in association with membrane properties of boar spermatozoa

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This study was aimed to evaluate the effects of dietary n-6:n-3 ratio and Vitamin E on the membrane properties and motility characteristics of spermatozoa in boars. Forty Duroc boars were randomly distributed in a 2 × 2 factorial design with two n-6:n-3 ratios (14.4 and 6.6) and two Vitamin E levels (200 and 400 mg kg^{-1}). During 16 weeks of treatment, fresh semen was collected at weeks 0, 8, 12, and 16 for measurements of motility characteristics, contents of fatty acids, membrane properties (membrane fluidity and membrane integrity), and lipid peroxidation of the spermatozoa. The semen was diluted in Beltsville Thawing Solution (BTS) extender and stored at 17°C, and the sperm motility was assessed at 12, 36, 72, and 120 h of storage. The 6.6 n-6:n-3 dietary ratio increased the contents of n-3 polyunsaturated fatty acids (PUFAs) and docosahexaenoic acid (DHA) and improved the membrane integrity and membrane fluidity of the spermatozoa, resulting in notably increased total motility, sperm progressive motility, and velocity parameters of fresh semen. Feeding diet with Vitamin E (400 mg kg^{-1}) prevented sperm lipid peroxidation, and resulted in higher total motility and sperm progressive motility in fresh and liquid stored semen. In conclusion, the adjustment of n-6:n-3 ratio (6.6) and supply of Vitamin E (400 mg kg^{-1}) successfully improved sperm motility characteristics and thus may be beneficial to the fertility of boars, which might be due to the modification of the physical and functional properties of spermatozoa membrane in response to dietary supplementation.

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INTRODUCTION

Sperm production is the number of good-quality sperm cells ejaculated within a certain time, which is subsequently determined by the percentage of living and progressively motile sperm cells.[1] Sperm production is a crucial determinant of the number of mating via artificial insemination.[2] A major constraining factor of sperm production is the poor sperm motility[3] resulted from a multistep process.[4] Sperm motility is a central index of fertility strongly influenced by environmental factors, with diet being one of the most important modifying agents.[5] Among dietary factors, numerous experimental data from human and animal studies indicate a positive effect of fish-derived n-3 polyunsaturated fatty acids (PUFAs) on sperm motility and fertility.[6–9] For example, although the n-6:n-3 ratio in the fatty acid of porcine commercial diets is often >10:1 with no n-3 PUFA content, Mitre *et al.*[7] found that n-3 PUFA-enriched diets that contained docosahexaenoic acid (DHA; 22:6n-3) reversed the n-6:n-3 ratio in spermatozoa and improved sperm motility. There was one research suggesting that dietary n-6:n-3 ratio in cockerels[10] improved the fertilizing ability of spermatozoa. However, the underlying mechanism through which dietary n-6:n-3 ratio influences sperm functions remains unknown.

PUFAs of the n-6 and n-3 series are classified according to the distance of the first double bond to the methyl terminal. PUFAs cannot be synthesized *de novo* by animals and thus need to be provided in diets as they have been suggested to be important for the motility and functions of spermatozoa.[11] As for n-6 fatty acids (FA), to the best of our knowledge, only three studies in chicken,[8] bulls,[12] and rams[13] have reported a greater effect of dietary n-6 PUFA on the quality of spermatozoa. The porcine commercial diets provide adequate n-6 FA,[6,14] which probably can satisfy the boar's requirement. Whereas dietary n-3 PUFA modifies spermatozoa FA profile[7,15] and promotes the susceptibilities of membrane PUFAs to lipid peroxidation,[16,17] resulting in changes of membrane integrity and function, and subsequently the changes of sperm motility and fertility.[17,18] These results imply that dietary n-3 may help to improve sperm functions, especially motility, by modifying membrane properties as well as decreasing spermatozoa lipid peroxidation. As a fat-soluble antioxidant, Vitamin E can exert antioxidative effects and affect boar semen quality *in vivo*[16] as well as *in vitro*.[17] Therefore, Vitamin E was included in premix to reverse the negative impact of spermatozoa lipid peroxidation of PUFA addition.[6,18]

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However, it is still unclear which component improves sperm quality due to the simultaneous supplementation of n-3 PUFA and Vitamin E, and the physiological effects of the altered membrane properties have not been investigated in boar by adjustment of dietary n-6:n-3 ratio and inclusion of Vitamin E. Thus, the experiments in this work were designed to find out the impacts of reducing the n-6:n-3 ratio to below 10:1 and the inclusion of Vitamin E in boar diets on the sperm motility characteristics, and the physical and functional properties of spermatozoa membrane.

MATERIALS AND METHODS

Animals and dietary treatments

Forty Duroc boars were allocated by age (591.5 ± 9.2 d) and live weight (272.9 ± 47.6 kg) to four treatment groups, with 10 animals for each treatment. The basal diets were formulated to be isoenergetic and isonitrogenous and to meet the nutritional requirements[19] (**Table 1**). The boars were provided *ad libitum* access to water. The experiment followed a 2 × 2 factorial (n-6:n-3 ratio and Vitamin E level) design. The experimental rations contained either soybean oil or soybean oil together with fish oil. The soybean oil (Shandong Bohi Industry Co., Ltd, Shandong, China) was the main source of n-6 FA leinoleic acid (LA, C18:2 n-6). The fish oil (Rongcheng Ayers Ocean Bio-Technology Co., Ltd., Rongcheng, Shandong, China) included 10% eicosapentaenoil acid (EPA, C20:5 n-3), 25% DHA and 35% total n-3 FA. The fish oil was stabilized with an antioxidant (500 ppm of Butylated Hydroxytoluene [BHT], Bayer, China). As the dietary n-6:n-3 ratio in commercial production of boars is usually higher than 10:1, the 14.4 n-6:n-3 ratio diet, which was composed of 60 g d^{-1} soybean oil and 2.5 kg d^{-1} basal diet, was used as the control diet; whereas 6.6 n-6:n-3 ratio diet consisted of 45 g d^{-1} soybean oil, 15 g d^{-1} fish oil and 2.5 kg d^{-1} basal diet. The FA profiles of 14.4 and 6.6 n-6:n-3 ratio diets are reported in **Table 2**. The second treatment was a dietary inclusion of Vitamin E (vE, α-tocopherol acetate, Zhejiang NHU Co., Ltd., Zhejiang, China) at two levels: 200 and 400 mg kg^{-1} of feed. The following experimental diets were formulated: 14.4 n-6:n-3 ratio diet with 200 mg vE kg^{-1}; 14.4 n-6:n-3 ratio diet with 400 mg vE kg^{-1}; 6.6 n-6:n-3 ratio diet with 200 mg vE kg^{-1}; 6.6 n-6:n-3 ratio diet with 400 mg vE kg^{-1}. The experiment lasted for 16 weeks.

Semen evaluation

Semen was collected in thermo container (37°C) from all the boars (on 1 day in each week) at weeks 0, 8, 12 and 16 of the experiment by the gloved-hand technique.[20] After collection, the semen was diluted (1:9, v/v) in a Beltsville Thawing Solution (BTS, Wuhan Jinli, Wuhan, China) to a final concentration of 2.5×10^9 spermatozoa $dose^{-1}$. The diluted sperm-rich fraction was split into 80 ml semen doses. The first dose was stored at 17°C for 120 h, and aliquots were removed at hours 12, 36, 72, and 120 for motion analysis. The second dose was cooled and immediately transported at 17°C in an insulated container from the farm to the university laboratory within 24 h after extraction according to Yeste *et al.*[18] to assess the sperm membrane properties (structural membrane integrity, function membrane integrity, and membrane fluidity). Aliquots of semen containing 3×10^9 and 3×10^8 sperm were centrifuged at 850 ×*g* for 20 min at 20°C. The obtained sperm pellets were re-suspended in 0.85% NaCl, washed twice by centrifugation and frozen at −80°C for fatty acid analysis and lipid peroxidation (malondialdehyde, MDA) assay.

Sperm motility

Before analyzing the sperm motility, semen samples were incubated at 37°C for 15 min. Then, a computer-assisted semen analyzer (CASA sperm analyzer; Songjiang, China) was used. The analysis was based on the examination of 30 consecutive digitalized images per second using a 10 × negative phase-contrast objective. Several fields of view were captured, and at least 1000 spermatozoa were counted in each analysis. The system provided data of the following 6 motility parameters: total motility (MOT, %); progressive motility (PMOT, %); curvilinear velocity (VCL, μm s^{-1}), which is the average velocity measured over the actual point-to-point track followed by the cell; average path velocity (VAP, μm s^{-1}), which corresponds to the average velocity of the smoothed cell path; straight line velocity (VSL, μm s^{-1}), which represents the average velocity measured in a straight line from the beginning to the end of a track; and amplitude of lateral head displacement (ALH, μm).

Table 1: Composition of basal diet

Ingredient	*Content (%)*
Corn	66.9
Soybean meal	21.9
Wheat bran	7.5
Limestone	1.1
$CaHPO_3$	1.3
Salt	0.3
Premix*	1
Calculated values	
Digestable energy (Mcal kg^{-1})	3.17
Crude protein (%)	16.47
Ether extract (%)	3.10
Lysine (%)	0.78
Methionine (%)	0.24
Methionine + cystine (%)	0.55
Threonine (%)	0.60
Tryptophan (%)	0.18
Ca (%)	0.80
TP (%)	0.60
AP (%)	0.35

*Supplied per kilogram of diet: 5 mg Cu, 80 mg Fe, 50 mg Zn, 20 mg Mn, 0.14 mg I, 0.15 mg Se, 15 000 IU Vitamin A, 2400 IU Vitamin D_3, 50 μg 25(OH)D, 0.48 mg menadione, 2 mg thiamin, 7.2 mg riboflavin, 3.6 mg pyridoxine, 25 μg Vitamin B_{12}, 0.48 mg biotin, 25 mg pantothenic acid, 4 mg folic acid, 400 mg niacin, Vitamin E was supplied with two levels of 200, 400 mg. TP: total phosphorus; AP: available phosphorus

Table 2: Fatty acids composition (g per 100 g of fatty acids) of the diets*

Fatty acids	*n-6*	*n-6 and n-3*
14:0	0.13	0.27
16:0	16.20	13.59
18:0	2.05	5.10
SFA**	18.38	18.97
16:1n-7	0.12	3.09
18:1n-9	18.74	20.84
MUFA***	18.86	23.93
18:2n-6	56.96	45.88
18:3n-3	4.02	2.51
20:5n-3	0.00	1.57
22:6n-3	0.00	3.29
PUFA****	60.98	53.25
Total n-6	56.96	45.88
Total n-3	4.02	7.37
Ratio n-6:n-3	14.16	6.24

*Fatty acid percentage was analyzed using gas chromatography; **SFA: saturated fatty acids percentage is the sum of 14:0, 16:0 and 18:0; ***MUFA: monounsaturated fatty acids percentage is the sum of 16:1n-7 and 18:1n-9; ****PUFA: polyunsaturated fatty acids percentage is the sum of 18:2n-6, 18:3n-3, 20:5n-3 and 22:6n-3.

Sperm membrane properties

Structural membrane integrity
The proportion of spermatozoa with intact plasma membrane was estimated using eosin Y (Solarbio, Beijing, China) staining with the observation of at least 200 spermatozoa at ×400 magnification. The unstained spermatozoa were considered to have an intact plasma membrane, whereas partly or completely purple spermatozoa were considered to have lost their plasma membrane.

Functional membrane integrity
Functional membrane was assessed using hypo-osmotic swelling test (HOS),[21] which was performed by incubating an aliquot (50 μl) of semen sample with 1 ml of hypo-osmotic solution prepared by mixing 7.35 g sodium citrate $2H_2O$ (Sinopharm, Beijing, China) and 13.51 g fructose (Sinopharm, Beijing, China) in 1 L of distilled H_2O at 37°C for 30 min. After incubation, a minimum of 200 spermatozoa were evaluated for coiled tails by counting in at least five different fields under a phase-contrast microscope at ×400 magnification.

Membrane fluidity
Membrane fluidity determination followed the basic principle that the alterations in lipid packing change the mobility of a membrane-bound fluorophore. The mobility of the fluorophore can be monitored by exciting the fluorophore with a polarized light and measuring the emitted light in two planes parallel and perpendicular to the polarization plane of the excitation light. Fluorescence polarization (FP) is defined as the following ratio: $r = (I_{vv} - G\ I_{vh})/(I_{vv} + 2G\ I_{vh})$, $G = (I_{hv}/I_{hh})$, where I_{vv} and I_{vh} are the fluorescence intensities measured in the parallel and perpendicular channels, and G is the grating correction factor. Membrane fluidization increases the mobility of the dye and decreases the intensity of the emitted parallel component. Fluorescence measurements (excitation λ = 365 nm; emission λ = 430 nm) were performed in a PerkinElmer LS 55 Luminescence Spectrometer equipped with polarizer. Aliquots of fresh spermatozoa (200–300 μl) were diluted in BTS and centrifuged for 20 min at 800 ×*g*. The washed spermatozoa were suspended at a concentration of 1×10^6 spermatozoa ml^{-1} in 3 ml of PBS (phosphate buffered saline) with the fluorescent lipophylic molecule 1,6-diphenyl-1,3,5-hexatriene (DPH, Sigma, USA, 10^{-6} mol l^{-1}, prepared from a DPH stock solution of 2×10^{-3} mmol l^{-1} in tetrahydrofurane). The suspension was incubated for 30 min at room temperature.[22] The DPH/phospholipid molar ratio was lower than 1/2000 in order to minimize the probe-to-probe interactions and probe-induced disturbance of the lipid bilayer. Sperm suspensions containing no DPH were similarly assessed to check light scattering. An increased value of *r* was interpreted to be a decrease of membrane fluidity, and the converse with decreased *r* value.

Lipid peroxidation assay
Cellular content of MDA (nmol 10^{-8} Sperm cells) was determined with commercial test kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). Briefly, the measurement of MDA was accomplished through binding of thiobarbituric acid (TBA) to the MDA molecule, which produced a colorimetric reaction that can be measured on a spectrophotometer at a wavelength of 532 nm.

Sperm fatty acid analysis
Lipids were extracted from diets and sperm samples after homogenization in a suitable excess of chloroform–methanol (2:1, v/v).[23] Fatty acid methyl esters (FAMEs) were prepared for gas chromatography determination using KOH/methanol.[24] The CP-3800 gas chromatography (Varian Inc., USA) equipped with a 1177 injector, a flame ionization detector and a capillary chromatographic column CPSil88 (Varian Inc., USA) for FAME (50 m × 0.25 mm id 0.2 μm) was used in this experiment. The injector and detector temperatures were kept between 250°C and 270°C. Nitrogen was used as carrier gas. A total of 40 saturated, monounsaturated and polyunsaturated fatty acid standards (NU-CHEK, Prep, UK) were used. Peaks were identified by retention times relative to individual fatty acid standard.

Statistical analysis
All data were analyzed using Statistical Analysis Software (SAS, version 9.1, SAS Institute, Inc., Cary, NC, USA). Data were examined for normality and homogeneity of variance using the Kolmogorov–Smirnov normality test and Bartlett tests. Four transformations of data (square root [x], $\log_{10}$[x], arcsine square root[x] and 1/[x]) were effected when the distribution was not normal and/or heteroscedastic (x), and the most suitable transformation was chosen. The transformed data were used to calculate *P* values. The corresponding least squares mean and standard deviation of the nontransformed data were presented in the results for clarity. Data were subjected to analysis of variance (ANOVA) according to the GLM procedure of the Statistical Analysis System (SAS Institute, Inc., Cary, NC, USA). The model included dietary n-6:n-3 ratio, Vitamin E, two-way interaction of dietary n-6:n-3 ratio and Vitamin E as possible sources of variation. Vitamin E effect was not significant ($P > 0.05$) and thus was excluded in the final model for the fatty acid composition analysis of spermatozoa. Hence, the data were analyzed using an ANOVA for repeated measurements, with boar within dietary n-6:n-3 ratio as the subject of the repeated effect of time (i.e., week of experiment), and with dietary n-6:n-3 ratio, time, and two-way interaction of n-6:n-3 ratio and time as fixed effects. When ANOVA revealed a significant treatment effect, means were compared using Duncan's multiple range tests. Pearson correlations were calculated to determine the associations between viability, HOS, lipid peroxidation (MDA), CASA results and fatty acid composition in spermatozoa. In all statistical analyses, the significance level was set at 5%.

RESULTS

Semen motility characteristics
On the ground that the n-6:n-3 fatty acid ratio is generally higher than 10:1 in commercial porcine diets, 14.4 n-6:n-3 ratio diet was used as the control. To ascertain whether there was any effect of dietary n-6:n-3 ratio on sperm motility, CASA was used to evaluate the sperm motility characteristics. The results showed that the 6.6 dietary n-6:n-3 ratio resulted in considerably enhanced total motility, sperm progressive motility and kinematic parameters (VSL, VAP, and VCL) at week 12 and week 16 (**Table 3**). Compared with 200 mg kg^{-1} supplementation of Vitamin E, 400 mg kg^{-1} supplementation of Vitamin E notably increased the progressive sperm motility. In addition, to evaluate the influence of dietary n-6:n-3 ratio and Vitamin E on sperm motility in response to cooling (17°C), the semen was diluted with BTS at hours 12, 36, 72, and 120 at 17°C. The results showed that high Vitamin E diet resulted in higher total motility and sperm progressive motility than low Vitamin E diet (**Figure 1**), and dietary n-6:n-3 ratio had no effect on the motility of stored sperm (data not shown). The interaction between n-6:n-3 ratio and Vitamin E was not significant for all motility characteristics ($P > 0.05$).

Sperm membrane properties
The maintenance of membrane integrity and membrane fluidity of boar spermatozoa is important for achieving high sperm motility, because the motility of the spermatozoon partly depends on the transport of compounds across its membrane. At 0 and 8 week, sperm membrane

Table 3: Effect of dietary n-6:n-3 ratio (n6:n3) and Vitamin E on boar fresh sperm motility characteristics*

Item	*n6:n3 (14.4)*		*n6:n3 (6.6)*		*P*		
	Vitamin E (200 mg kg⁻¹)	*Vitamin E (400 mg kg⁻¹)*	*Vitamin E (200 mg kg⁻¹)*	*Vitamin E (400 mg kg⁻¹)*	*n6:n3*	*Vitamin E*	*n6:n3 × Vitamin E*
Total motility (%)							
0 week	90.9±8.3	91.9±5.6	91.4±8.6	88.2±7.0	-	-	-
8 weeks	94.4±3.3	93.7±4.2	91.1±5.2	93.5±2.5	0.15	0.55	0.32
12 weeks	87.4±5.3	90.8±6.0	91.8±4.6	95.6±1.6	<0.01	0.01	0.78
16 weeks	95.6±1.5	95.5±3.8	94.6±4.3	97.3±1.4	0.27	0.07	0.71
Progressive motility (%)							
0 week	81.6±14.6	82.5±8.7	81.4±12.6	79.3±9.8	-	-	-
8 weeks	86.8±7.4	85.3±8.4	80.9±9.1	85.1±5.3	0.21	0.63	0.3
12 weeks	77.6±7.6	82.6±7.8	81.8±8.6	87.5±3.5	0.05	0.03	0.92
16 weeks	84.3±8.6	87.0±8.8	89.5±5.7	95.1±1.6	0.03	0.15	0.74
Straight-line velocity (μm s⁻¹)							
0 week	138.5±17.3	140.2±11.0	140.8±13.1	133.7±14.2	-	-	-
8 weeks	142.8±12.1	143.1±9.1	138.9±8.9	144.5±7.0	0.72	0.35	0.39
12 weeks	129.8±8.2	133.1±8.4	134.0±6.8	142.4±6.5	0.01	0.01	0.57
16 weeks	139.4±11.8	142.4±11.0	145.9±9.6	150.4±8.6	0.04	0.28	0.85
Average path velocity (μm s⁻¹)							
0 week	158.1±18.1	162.1±11.2	162.5±12.3	153.9±12.4	-	-	-
8 weeks	166.4±7.5	166.2±7.8	163.8±8.9	166.9±6.3	0.7	0.57	0.49
12 weeks	150.4±9.6	155.0±10.1	159.0±7.1	164.6±5.8	<0.01	0.08	0.89
16 weeks	160.3±11.1	162.3±11.1	163.7±11.3	170.7±3.3	0.05	0.16	0.43
Curvilinear velocity (μm s⁻¹)							
0 week	197.3±24.7	199.7±15.6	200.6±20.2	190.5±18.6	-	-	-
8 weeks	203.4±17.2	203.7±12.9	197.9±12.6	205.7±10.0	0.68	0.35	0.39
12 weeks	176.9±19.5	187.6±13.2	191.3±9.6	203.2±8.8	<0.01	0.02	0.93
16 weeks	198.6±16.8	202.8±15.7	209.1±8.4	211.8±9.5	0.04	0.47	0.84
Amplitude of lateral head displacement (μm)							
0 week	58.5±8.2	59.5±4.6	58.8±5.9	56.1±5.6	-	-	-
8 weeks	60.6±5.1	60.7±3.9	59.0±3.8	61.3±3.0	0.72	0.35	0.39
12 weeks	52.4±5.6	55.8±3.8	57.7±3.5	61.1±2.9	<0.01	0.02	0.89
16 weeks	59.2±5.0	60.4±4.7	60.6±4.1	62.7±3.6	0.58	0.68	0.38

*Data are means±s.d. (*n*=10 replicates/treatment, one ejaculate per replicate). s.d.: standard deviation

integrity parameters and membrane fluidity did not differ among groups (*P* > 0.05, **Figure 2**). When the boars were fed for 12 and 16 weeks, the structural membrane integrity and functional membrane integrity were considerably increased in treatment groups, namely the groups with 6.6 ratio diet and Vitamin E (400 mg kg⁻¹) diet. Increased membrane fluidity was found in the 6.6 n-6:n-3 group at 12 and 16 weeks and in the high Vitamin E group only at week 16. There was no significant effect of the interaction between dietary n-6:n-3 ratio and Vitamin E supplementation on sperm membrane properties (*P* > 0.05).

Lipid peroxidation levels

The lipid peroxidation of sperm can lead to decreased motility and fertilizing ability of spermatozoa. The malondialdehyde (MDA) in the spermatozoa was assessed to validate the effect of dietary n-6:n-3 ratio and Vitamin E on the lipid peroxidation of sperm. Compared with low Vitamin E diet, high Vitamin E diet considerably reduced sperm lipid peroxidation, as measured by MDA levels in sperm. The sperm MDA level was not significantly different between the 6.6 and 14.4 n-6:n-3 groups (*P* > 0.05, **Figure 3**).

Sperm fatty acid composition

Dietary supplementation is known to interfere with the sperm fatty acid, which is a key regulatory factor of sperm membrane properties. To determine whether the sperm fatty acid was affected by the treatments in our experiments, we measured the sperm fatty acid composition. Vitamin E supplementation and the interaction between n-6:n-3 ratio and Vitamin E did not lead to any changes in fatty acid composition, which are thus not presented in **Table 4**. In general, the fatty acid composition of sperm was altered by dietary n-6:n-3 ratio as expected. Notably increased DHA and total n-3 PUFA in boar sperm were founded at the expense of DPA and total n-6 PUFA in 6.6 n-6:n-3 group compared with in 14.4 n-6:n-3 group at weeks 12 and 16 (**Table 4**). Compared with the boars consuming 14.4:1 diets, those consuming 6.6:1 diets had lower ratios of total n-6:n-3 and DPA: DHA in spermatozoa at weeks 12 and 16. The correlation analyses between all the variables demonstrated that the DHA content of spermatozoa was strongly positively correlated with viability, HOS and progressive motility (*r* = 0.38, 0.48, 0.35, *P* < 0.05; *n* = 36).

DISCUSSION

The results of the present study confirm our hypothesis that 6.6 n-6:n-3 diet contributes to greater sperm motility than 14.4 n-6:n-3 diet, which is in agreement with the observations of boars in previous studies: the sperm motility was enhanced by dietary n-3 PUFA.[6,7,25] However, contradicting results were reported in boars,[15,26,27] which might be caused by variations in the quantities and sources of oil and duration of supplementation. Similar conflicting results about the effect of dietary supplementation with n-3 PUFA on sperm function have been

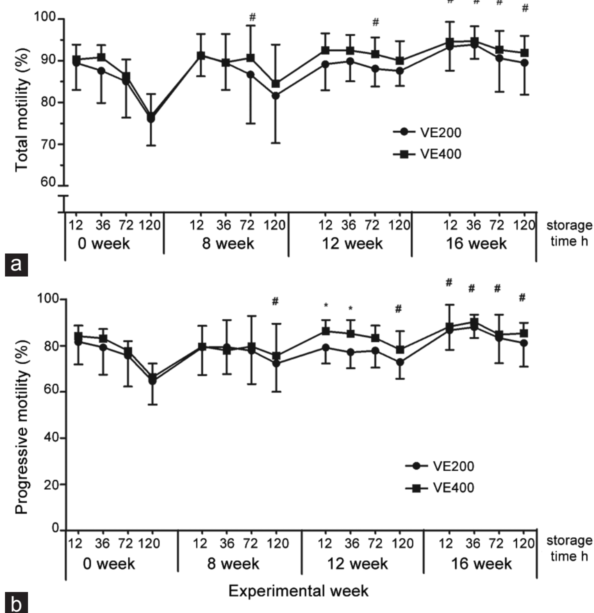

Figure 1: Changes in (**a**) total motility and (**b**) progressive motility of boar lipid stored sperm with dietary Vitamin E during 120-h storage at different experimental week (0, 8, 12 and 16 w). Data are presented as means ± s.d. (n = 10 replicates/vitamin group, one ejaculate per replicate). *P < 0.05, compared with Vitamin E diet (200 mg kg^{-1}). #P < 0.05, compared with week 0 (within the same storage time).

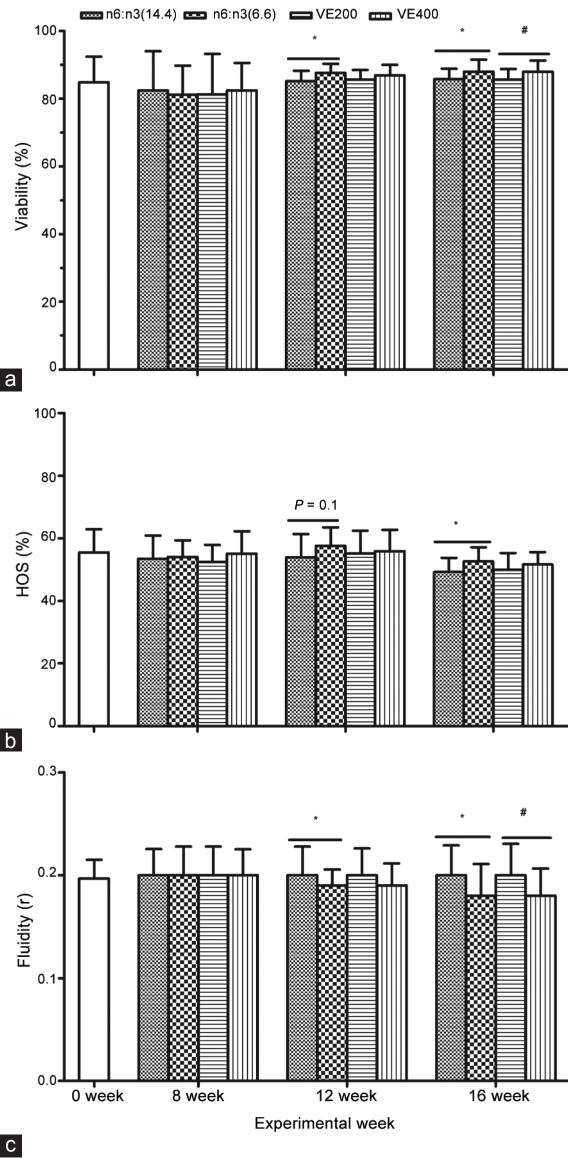

Figure 2: Effect of dietary n-6:n-3 ratio and Vitamin E on boar fresh sperm membrane properties including: (**a**) Viability (%); (**b**) HOS (%) and (**c**) fluidity (r) at different week of experiment (0, 8, 12 and 16 w). Data are main effect means ± s.d. (n = 8–12 replicates/treatment, one ejaculate per replicate). *Difference was from n6:n3 (6.6) versus n6:n3 (14.4) (P < 0.05). #Difference was from Vitamin E (VE) 400 versus Vitamin E (VE) 200 (P < 0.05).

reported in rabbit,[28,29] human,[9,26] and ram.[27,30] Regardless of experimental factors, the control diets for boars contained high n-6 FA as they were corn-based (with a n-6:n-3 ratio higher than 10:1), except for the study by Rooke *et al.*[6] which used barley- and wheat-based diet containing rapeseed components (with a n-6:n-3 ratio lower than 10:1). Refined fish oil (25% DHA and 10% EPA) was used for the dietary 6.6 n-6:n-3 ratio in the present study, and the inclusion content of n-3 FA (15 g d^{-1}) was not within the range (40–300 g d^{-1}) previously reported.[7,15,18,31] CASA system assessment demonstrated that dietary 6.6 n-6:n-3 also yielded higher velocity (VSL, VAP, VCL), which has been proven to be useful for estimating the potential fertility of human[32] and boars.[33] Different velocity values of these studies may be attributed to the variations of software algorithms (to obtain VAP etc.) and acquisition settings.[34]

Changes in plasma membrane structure and functional activity appear to be an essential prerequisite for successful motility acquisition, fertilization and embryo development.[35–37] The results of the present study are in agreement with those of the previous studies: the supplementation of n-3 PUFA could improve the functional membrane integrity (HOS values) of fresh semen in rabbit[29] and ram,[38] the structural membrane integrity of fresh semen in boar[39] and the synaptic plasma membrane fluidity in rat.[40] The reason for the improvement of the structural and functional membrane integrity of fresh boar semen found in the present study might be that dietary 6.6 n-6:n-3 ratio (n-3 enriched diet) increased the incorporation of DHA into the sperm head and the principle piece of sperm tail, and the DHA played a structural role and improved sperm membrane flexibility and stability against hypo-osmotic solution.[41] More studies are needed to address the issues of how FAs are distributed in the heads and tails of the ejaculated boar spermatozoa and what are their corresponding fertilizing functions in each section. In addition, it is probable that DHA acyl-chain in sperm membrane phospholipid bilayers was increased by dietary 6.6 n-6:n-3 ratio, resulting in higher sperm flexibility, compressibility, deformability and elasticity and thus enhanced membrane fluidity of the fresh boar semen as measured by DPH polarization.[42,43]

The modification of the sperm membrane FA profile and lipid peroxidation appears to be closely associated with the changes of the physical and functional properties of spermatozoa membrane.[44–46] In the present study, dietary 6.6 n-6:n-3 ratio successfully increased the n-3 PUFA and DHA contents of the sperm, though the total PUFA was not influenced, showing a noticeable responsiveness of boar sperm to dietary manipulation. Our findings are in agreement with those previously reported in boar,[6,7] rabbit,[29] ram[38] and ovine.[47] The boars consuming 14.4:1 n-6:n-3 diet displayed a sperm DPA: DHA ratio more than triple that of the boars consuming 6.6:1 n-6:n-3 diet at weeks 12 and 16. It

is worthy to note that the optimization of DPA: DHA ratio in sperm may be expected to promote successful spermatozoa development and to facilitate the flagellar movement of the tail.[43] Therefore, the above results suggest that the increased n-3 PUFA and DHA and optimized DPA: DHA ratio in sperm may partly explain the modification and stabilization of structural and functional properties of spermatozoa membrane as previously suggested.[28,29,48] On the other hand, previous studies found that the spermatozoa of several species supplied with dietary n-3 PUFA are more easily subjected to lipid peroxidation,[39,49] which negatively affects the membrane structural and functional properties and sperm motility.[50,51] Our results show that the sperm lipid peroxidation (spontaneous MDA levels) had no statistical difference between 6.6 and 14.4 n-6:n-3 groups. In contrast, Strzezek *et al.*[39] stated that dietary supplementation with n-3 PUFA to boar increased the sperm lipid peroxidation (induced MDA levels), which might mask the dietary effect on semen quality. Such differences possibly result from the different analyzed methods (spontaneous *vs* induced lipid peroxidation assay).[52] The present study indicates that dietary n-6:n-3 ratio modifies membrane properties, which is independent of membrane lipid peroxidation.

The results of the present study also confirm the positive effect of high Vitamin E levels on the motility of the fresh and liquid-stored sperm. These findings are in agreement with previous observations in boars.[17,53] The present study confirms the effect of high level Vitamin E on the prevention of lipid peroxidation in spermatozoa, which is in conformity with the result of Marin-Guzman *et al.*[16] As for the changes of membrane properties, only the membrane structural integrity (head membrane) appears to be affected by Vitamin E supplementation, which is probably due to the different distributions of Vitamin E in the head, tail and plasma membrane of spermatozoa as shown in rat spermatozoa.[36]

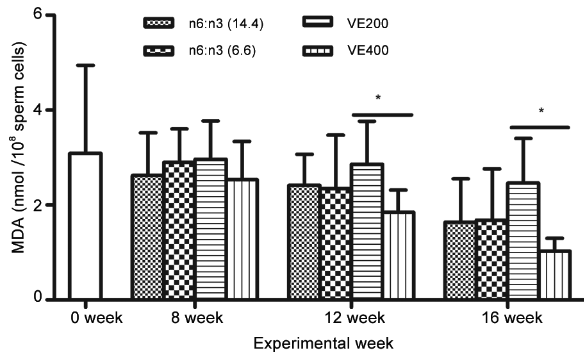

Figure 3: Effect of dietary n-6:n-3 ratio and Vitamin E on boar fresh sperm lipid peroxidation (MDA concentration, nmol 10^{-8} Sperm cells) at different week of experiment (0, 8, 12 and 16 w). Data are main effect means ± s.d. ($n = 10$ replicates/treatment, one ejaculate per replicate). *Difference was from Vitamin E (VE) 400 versus Vitamin E (VE) 200 ($P < 0.05$).

CONCLUSIONS

Our results demonstrate that dietary 6.6 n-6:n-3 ratio influences the boar sperm fatty acid composition, enhances the sperm membrane properties, and increases sperm motility. Vitamin E (400 mg kg^{-1}) diet prevents sperm lipid peroxidation, directly leading to the improvement of motility parameters. It is suggested that the adjustment of dietary n-6:n-3 ratio and supplementation of Vitamin E can improve sperm motility through modifying the physical and functional properties of spermatozoa membrane.

AUTHOR CONTRIBUTIONS

QL participated in the design of the study, performed the experiment and statistical analysis, and drafted the manuscript. YFZ participated in the statistical analysis and helped to draft the manuscript. RJD participated in the animal experimental studies. HKW participated in the design of the study. JP conceived of the study, and participated in its design and coordination and helped to draft the manuscript. SWJ participated in the design of the study and coordination. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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Table 4: Fatty acid compositions (g per 100 g of fatty acids) of spermatozoa of boars fed diets differing in the ratio of n-6 to n-3 fatty acids from preexperiment*

Items	*n-6:n-3 (14.4) diet*			*n-6:n-3 (6.6) diet*			*P*		
Fatty acids	*0 week*	*12 weeks*	*16 weeks*	*0 week*	*12 weeks*	*16 weeks*	*Diet*	*Time*	*Diet × time*
16:0	24.70±2.12	21.12±5.55	27.12±2.92	24.89±4.39	21.28±2.98	25.06±3.60	0.7	0.01	0.7
16:1	5.59±2.89	6.52±1.78	1.76±1.87	3.95±1.88	6.64±1.93	1.65±1.35	0.56	<0.0001	0.51
18:0	7.64±1.21	7.60±1.71	8.48±1.58	9.58±0.73	8.42±1.32	10.01±1.57	<0.01	0.11	0.64
18:1 (n-9)	2.56±0.31	2.71±0.47	3.18±0.41	2.54±0.48	2.63±0.78	3.41±0.49	0.93	<0.01	0.73
18:2 (n-6)	3.84±0.28	3.06±0.64	3.37±0.68	3.90±0.43	3.82±0.91	4.09±0.71	0.03	0.22	0.35
20:4 (n-6)	3.02±0.32^{a,b}	2.34±1.06^{b}	3.78±0.48^{a}	3.00±0.19^{a,b}	4.15±2.39^{a}	3.04±0.55^{a}	0.31	0.58	0.01
22:4 (n-6)	1.20±0.76	2.77±2.33	1.28±0.17	1.04±0.18	1.18±0.28	1.29±0.47	0.12	0.13	0.18
22:5 (n-6) DPA	19.69±4.25^{c,d}	32.17±5.85^{a}	27.53±3.87^{a,b}	21.70±2.95^{b,c}	16.57±3.62^{b}	15.42±5.41^{d}	<0.0001	0.19	0.0001
22:6 (n-3) DHA	28.05±3.78^{b}	23.23±5.96^{b,c}	21.34±4.94^{c}	28.84±3.07^{b}	35.52±7.33^{a}	34.85±3.79^{a}	<0.0001	0.83	0.01
Total (n-6)	27.77±3.84^{c}	40.35±7.09^{a}	35.98±4.01^{a,b}	29.65±3.21^{a,b,c}	25.72±2.77^{c}	23.85±5.34^{c}	<0.0001	0.1	<0.01
Total (n-3)	28.05±3.78^{b}	23.23±5.96^{b,c}	21.34±4.94^{c}	28.84±3.07^{b}	35.52±7.33^{a}	34.85±3.79^{a}	<0.0001	0.83	0.01
Total (n-6):(n-3)	1.00±0.20^{c}	1.85±0.57^{a}	1.78±0.49^{a}	1.04±0.20^{c}	0.76±0.22^{b,c}	0.70±0.22^{b,c}	<0.0002	0.3	<0.01
DPA:DHA	0.71±0.17^{b}	1.49±0.51^{a}	1.36±0.40^{a}	0.76±0.17^{b}	0.49±0.17^{b}	0.45±0.20^{b}	<0.0001	0.33	0.0001
PUFA	55.83±5.15	63.59±8.20	57.33±4.26	58.50±2.52	61.25±4.76	58.70±3.73	0.78	0.04	0.45

*Values are means±s.d.; n=6. ^{a,b,c,d}Mean values with unlike superscript letters were significantly different (P<0.05). s.d.: standard deviation

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