Docosahexaenoic acid supplementation fully restores fertility and spermatogenesis in male delta-6 desaturase-null mice

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Abstract Delta-6 desaturase-null mice ($^{-/-}$) are unable to synthesize highly unsaturated fatty acids (HUFAs): arachidonic acid (AA), docosahexaenoic acid (DHA), and n6-docosapentaenoic acid (DPAn6). The $^{-/-}$ males exhibit infertility and arrest of spermatogenesis at late spermiogenesis. To determine which HUFA is essential for spermiogenesis, a diet supplemented with either 0.2% (w/w) AA or DHA was fed to wild-type ($^{+/+}$) and $^{-/-}$ males at weaning until 16 weeks of age (n = 3–5). A breeding success rate of DHA-supplemented $^{-/-}$ was comparable to $^{+/+}$. DHA-fed $^{-/-}$ showed normal sperm counts and spermiogenesis. Dietary AA was less effective in restoring fertility, sperm count, and spermiogenesis than DHA. Testis fatty acid analysis showed restored DHA in DHA-fed $^{-/-}$, but DPAn6 remained depleted. In AA-fed $^{-/-}$, AA was restored at the $^{+/+}$ level, and 22:4n6, an AA elongated product, accumulated in testis. Cholesta-3,5-diene was present in testis of $^{+/+}$ and DHA-fed $^{-/-}$, whereas it diminished in $^{-/-}$ and AA-fed $^{-/-}$, suggesting impaired sterol metabolism in these groups. Expression of spermiogenesis marker genes was largely normal in all groups. In conclusion, DHA was capable of restoring all observed impairment in male reproduction, whereas 22:4n6 formed from dietary AA may act as an inferior substitute for DHA.—Roqueta-Rivera, M., C. K. Stroud, W. M. Haschek, S. J. Akare, M. Segre, R. S. Brush, M-P. Agbaga, R. E. Anderson, R. A. Hess, and M. T. Nakamura. **Docosahexaenoic acid supplementation fully restores fertility and spermatogenesis in male delta-6 desaturase-null mice. *J. Lipid Res.* 2010.** 51: **360–367.**

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Delta-6 desaturase (D6D) is the first and rate-limiting enzyme for highly unsaturated fatty acid (HUFA) synthesis that consists of a series of elongation and desaturation reactions (1). The dietary essential fatty acids 18:2n-6 (linoleic acid) and 18:3n-3 (α-linolenic acid) are substrates for D6D and precursors of physiologically important HUFAs, such as 20:4n-6 [arachidonic acid (AA)], 22:5n6 [docosapentaenoic acid (DPAn6)], and 22:6n3 [docosahexaenoic acid (DHA)]. D6D is also required for the final desaturation step for the synthesis of DPAn6 and DHA.

These HUFAs are present in high concentration in testes and sperm of mammals. DPAn6, a HUFA derived from AA, dramatically increases in rat testes during the sexual maturation stage (2). In mice, AA, DPAn6, and DHA are abundant in membrane phospholipids of round spermatids (3) and mature mouse spermatozoa (4), suggesting an important role for these fatty acids for proper spermatogenesis. In humans, DHA is the main HUFA in sperm (5). DHA is specifically high in the sperm tail when compared with the sperm head in monkeys (6), implying a role of DHA in sperm tail function. AA may also have a role in male fertility as a precursor to eicosanoids. Prostaglandin E2, for example, has been shown to increase sperm motility (7), while inhibition of cycloxygenase-2 in mouse vas deferens results in a decrease of sperm motility and fertil-

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Abbreviations: AA, arachidonic acid; CD, cholesta-3,5-diene; D6D, Δ6 desaturase; DHA, docosahexaenoic acid; DPAn6, docosapentaenoic acid; FAME, fatty acid methyl esters; HUFA, highly unsaturated fatty acid; VLPUFA, very-long-chain polyunsaturated fatty acid.

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ity (8). In addition to the presence of HUFAs in mammalian testis and spermatozoa, there are also very-long-chain polyunsaturated fatty acids (VLPUFAs) that contain C26-C38 hydrocarbon chains (9–11). These VLPUFAs are elongation products of the C20 and C22 chain HUFAs (12). These VLPUFAs are incorporated mainly into sphingomyelin and ceramides in the sperm head (9, 10). These sphingolipids are suggested to be involved with capacitation of sperm (13, 14).

In the study reporting discovery of essential fatty acids, testicular degeneration and a low breeding success rate were among the deficiency symptoms in rats fed a fat-free diet (15). A later study reported that rats receiving a diet deficient in all essential fatty acids had a lower epididymal sperm concentration (16). However, these previous studies were unable to demonstrate the essentiality of HUFAs for male fertility because these animals also had severe growth retardation and dermatitis. In order to deplete tissue HUFAs in these studies, the D6D enzyme substrates, linoleic acid and α-linolenic acid, as well as all products were eliminated from the diet. However, linoleic acid is required for skin water barrier function (17). Thus, deficiency of linoleic acid resulted in severe growth retardation and dermatitis, confounding the study into the physiological roles of HUFAs, including male fertility (15, 18).

To overcome this confounding problem, we and others created mice with the D6D gene disabled (19, 20). The D6D knockout ($^{-/-}$) mouse is unable to synthesize HUFAs, thus allowing us to specifically create AA deficiency without depleting tissue linoleic acid or to create DPAn6 and DHA deficiency without depleting tissue AA. The D6D-null mouse developed intestinal ulcers and severe dermatitis by 5 months of age despite an adequate supply of linoleic acid and α-linolenic acid from diet (19). Moreover, the male $^{-/-}$ mouse became infertile before manifestation of dermatitis. Histology of the D6D $^{-/-}$ mouse revealed disrupted spermiogenesis, the last stage of spermatogenesis in which spermatids develop to spermatozoa (19, 20). Although the essentiality of HUFAs in spermiogenesis and male fertility has been demonstrated by these studies, the specific role of each HUFA for spermatogenesis has not been elucidated. Thus, the objective of this study was to determine if dietary AA and DHA can restore spermatogenesis in the D6D-null mouse and to elucidate the role of these HUFAs in spermiogenesis.

EXPERIMENTAL PROCEDURES

Animal study

All animal work was approved by the University of Illinois Institutional Animal Care and Use Committee. Mice used in the experiment were produced by breeding heterozygous males and females of mixed strain (129S6/SvEvTac/C57BL/6J) fed a standard rodent chow. At weaning, a total of 30 male mice, nine wild-type ($^{+/+}$), nine heterozygote ($^{+/-}$), and 12 D6D null ($^{-/-}$) were distributed to receive one of the following diets: control diet (AIN93G), AIN93G supplemented with 0.2% (w/w) AA, or AIN93G with 0.2% (w/w) DHA. The AIN93G diet is a purified, nutritionally adequate diet that contains sufficient linoleic acid and α-linolenic acid but no D6D products (21). DHASCO (45% DHA, <0.1% AA, and <0.1% 20:5 n-3) and ARASCO (44% AA and 0.11% 20:5 n-3) oils (Martek Biosciences, Columbia, MD) were used for supplementation of DHA and AA, respectively. All dietary groups consisted of three mice with exception of the AA supplemented $^{-/-}$ (n = 4) and DHA supplemented $^{-/-}$ (n = 5). Mice were single housed at weaning and received the diet until 4 months of age.

Male fertility

Fertility was evaluated by breeding single housed males with either a $^{+/+}$ or $^{+/-}$ female for 4 days at four different time points: 6, 9, 12, and 15 weeks of age; different females were used at each time point; at least 12 mating attempts were done per dietary group for each genotype. Copulatory behavior was confirmed in all mice. The percentage of successful matings as indicated by pregnant females and viable litters was noted.

Tissue collection and histology

Animals were euthanized by carbon dioxide inhalation at 4 months of age. Left testis and left epididymis were removed and weighed; left testis was then frozen for HUFA and RNA analysis, while left epididymis was used for sperm collection from cauda. Right testis and epididymis were fixed in Davidson's fixative and transferred to 10% neutral buffered formalin after 24 h. Tissues were trimmed for paraffin embedding. Sections were cut at 3 μm and stained with hematoxylin and eosin for histological evaluation.

Sperm count and motility

The cauda epididymis was cut with a surgical blade, minced with small scissors, and placed in 2 ml of dmKBRT buffer at 37°C for 15 min. The dmKBRT buffer contained 120 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 10 mM $NaHCO_3$, 0.36 mM NaH_2PO_4 H_2O, 1.2 mM $MgSO_4$, 5.6 mM glucose, 1.1 mM Na pyruvate, 25 mM TAPSO, 18.5 mM sucrose, and 6 mg/ml BSA. The sperm cell suspensions were then observed using an inverted microscope to record sperm motility. Epididymal sperm counts were done by hemocytometer from epididymal sperm in 2 ml of dmKBRT buffer.

Gene expression

RNA was analyzed with a slight modification of a method previously described (22). Testis was homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) and total RNA extracted. MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA), along with random hexamers, were used to synthesize cDNA. Real-time quantitative PCR, using SYBR Green fluorescent dye (Applied Biosystems), was used to analyze RNA relative to a rRNA L7a. Oligonucleotides used for real-time quantitative PCR were mTISP69-F, 5′-CGGACGCTCAGGTTAACTTGA -3′, mTISP69-R, 5′-CCACAGGAACCCCAAGCA-3′, mTISP50-F, 5′-ACCTTTGGCATCAAGCATTC -3′, mTISP50-R, 5′-GCACATTTCTGTGGGAGGAT- 3′, mAkap3-F, 5′-CGCAAAGACCTGGAGAAAAG-3′, mAkap3-R, 5′-ACCTTTTCGTTGGTCCACTG-3′, mCttn-F, 5′-AGGTGCCATCTGCCTATCA-3′, mCttn-R, 5′-TCTCGGCTTCTGCCTTCC-3′, mTnp1-F, 5′-ATGTCGACCAGCCGCAAGC-3′, mTnp-R, 5′-CCACTCTGATAGGATCTTTGG-3′, mSfap-F, 5′-AGAAGGGAAGCTCAGATCCA-3′, and mSfap-R, 5′-AGAAGGGAAGCTCAGATCCA-3′.

Fatty acid extraction and GC-MS analysis

Total lipids were extracted from frozen testis according to the method of Folch, Lees, and Sloane Stanley (23). Very-long-chain highly unsaturated fatty acid methyl esters were prepared with a slight modification of a method previously described (12). A mixture of pentadecanoic acid (15:0), heptadecanoic acid (17:0), heneicosanoic acid (21:0), pentacosanoic acid (25:0), and heptacosanoic acid (27:0) was added as an internal standard to the lipid extracts from the testis. The extracts were derivatized to fatty acid methyl esters (FAMEs) with HCl in methanol at 85°C overnight. After extracting with hexane, FAMEs were separated on TLC plates with hexane:ether (80:20) to remove cholesterol. Absolute ethanol was added to the scraped bands, which was then sonicated for 10 min. FAMEs were extracted with hexane after adding water. GC-MS analysis was performed as previously described (12).

Statistical analysis

Statistical analysis with Statview version 5.01 for Windows was conducted using one-way ANOVA with Fisher's PLSD post-test (Table 1; Fig. 1B), the Wilcoxon sum rank test (Fig. 1A), and the Student's *t*-test (Table 2). Data are presented as mean ± SD; $P < 0.05$ was considered as statistically significant.

RESULTS

Fertility, sperm counts, and motility were restored by dietary DHA

One of three nonsupplemented $^{-/-}$ males was able to impregnate a female at the first time point of 6 weeks of age. Beyond 6 weeks of age, all nonsupplemented $^{-/-}$ males (AIN) failed to impregnate females of $^{+/+}$ or $^{+/-}$ genotype. The rate of successful matings (**Fig. 1A**) for nonsupplemented $^{-/-}$ (8%) was significantly lower ($P < 0.05$) when compared with nonsupplemented $^{+/+}$ (67%) and $^{+/-}$ (50%). Both dietary AA and DHA supplemented $^{-/-}$ had significantly higher success rates, 38% and 61%, respectively ($P < 0.05$), than the nonsupplemented $^{-/-}$ (Fig. 1A).

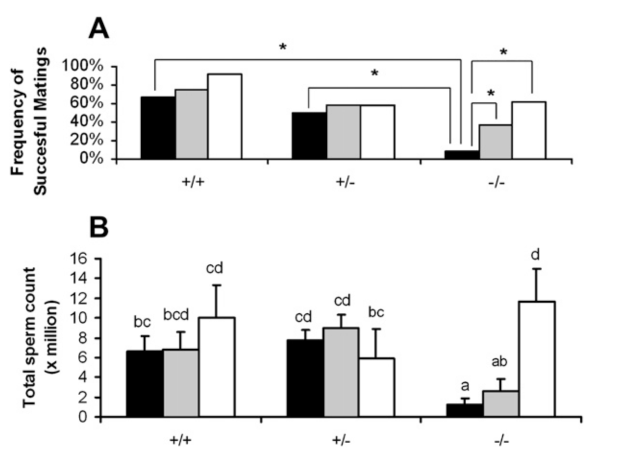

Fig. 1. A: Fertility of $^{+/+}$, $^{+/-}$, and $^{-/-}$ male mice fed AIN93G diet (black), 0.2% AA-supplemented (gray), or 0.2% DHA-supplemented (white) to the AIN93G diet (n = 3–5) . Fertility is expressed as frequency of matings resulting in impregnated females and a viable litter; 12 to 18 attempts per group. * $P < 0.05$ by Wilcoxon rank sum test. B: Total mature sperm count of epididymis from 16-week-old male mice. Mean ± SD. Groups without a common letter are statistically different by Fisher's PLSD after one-way ANOVA ($P < 0.05$).

Wild-type ($^{+/+}$) and heterozygote ($^{+/-}$) mice from all dietary groups presented normal total sperm numbers stored in epididymis (Fig. 1B). On the other hand, nonsupplemented $^{-/-}$ animals showed a drastic decrease in sperm count (18% of $^{+/+}$). Spermatozoa in nonsupplemented $^{-/-}$ had abnormal morphology with a condensed rounded head (globozoospermia; Fig. 3B, inset). DHA supplementation to $^{-/-}$ males fully restored sperm count ($11.62 \pm 3.0 \times 10^6$) to wild-type levels, while AA supplementation only partially restored sperm counts ($2.62 \pm 1.2 \times 10^6$) (Fig. 1B). Epididymal sperm was motile in all AA- and DHA-supplemented $^{-/-}$ animals, while sperm in $^{-/-}$ animals fed the control diet completely lacked motility.

DHA was required for sperm head elongation and flagellum formation

Testis and epididymis weights did not differ among groups in either absolute weight or in percentage weight relative to body weight (data not shown). Testis histology showed all stages of spermatogenesis in $^{+/+}$ and $^{+/-}$ genotypes regardless of the dietary treatments (**Fig. 2A**). Consistent with our previous study (19), all animals of nonsupplemented $^{-/-}$ had disrupted spermatogenesis specifically at Step 9 of spermiogenesis, where round spermatids are elongated. Spermatogonia, spermatocytes, and round spermatids were present, while elongated spermatids and spermatozoa were absent (Fig. 2B). AA supplementation partially restored spermatogenesis (Fig. 2C), while DHA supplemented $^{-/-}$ show all stages of spermatogenesis from spermatogonia to spermatozoa (Fig. 2D).

All $^{+/+}$ mice had spermatozoa in the lumen of the epididymis (**Fig. 3A**). Nonsupplemented $^{-/-}$ epididymis contained mostly sloughed round spermatids and spermatocytes, cells from an earlier stage of spermatogenesis than spermatozoa (Fig. 3B). A closer examination revealed that spermatozoa in the epididymis of $^{-/-}$ exhibited globozoospermia (Fig. 3B, inset). Partial restoration of spermatogenesis by AA supplementation is indicated by a mix of mature spermatozoa, spermatocytes, and round spermatids present in the epididymal lumen (Fig. 3C). The DHA-supplemented $^{-/-}$ group presented only spermatozoa in epididymal lumen (Fig. 3D), the same as in the $^{+/+}$ (Fig. 3A).

Expression of genes analyzed were largely unchanged in $^{-/-}$

Spermatogenesis did not proceed successfully beyond the round spermatid phase (Step 9 of spermiogenesis) in $^{-/-}$ males; therefore, we measured gene expression of late spermiogenesis markers in testis (**Table 1**). There was a 45% decrease in sperizin (Znrf4, TISP69) RNA in $^{-/-}$ males of all dietary groups. Two other genes that encode sperm flagellar proteins, Shippo1 (Odf3, TISP50) and A-kinase anchoring protein (Akap3), had a mild (20%) but statistically significant decrease in RNA expression in $^{-/-}$ fed the nonsupplemented diet. Other spermiogenesis markers analyzed were transition protein 1 (Tnp1), cortactin (Cttn), and sperm flagellum associated protein (Sfap1), all of which were normally expressed in $^{-/-}$ with

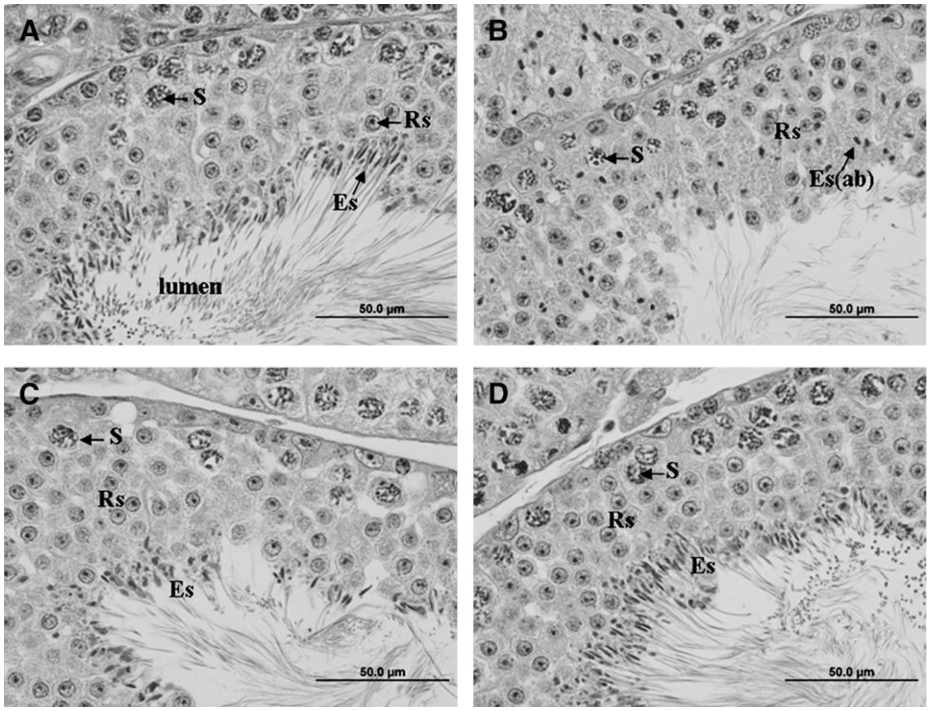

Fig. 2. Testis histology of $^{+/+}$ and $^{-/-}$ male mice fed AIN93G diet with or without 0.2% AA or 0.2% DHA supplementation from weaning until 16 weeks of age. A: Seminiferous tubule in testis of $^{+/+}$ without supplementation, showing stage VII of spermatogenesis with normal elongated spermatids. B: Seminiferous tubule from nonsupplemented $^{-/-}$ shows impairment of spermiogenesis, with abnormal elongation of spermatid head. C: AA supplementation to $^{-/-}$ partially restores spermatogenesis. D: DHA supplementation to $^{-/-}$ completely restores spermatogenesis showing no difference when compared with $^{+/+}$. H and E stain. S, spermatocytes. Rs, round spermatids. Es, elongated spermatids; Es(ab), abnormal elongated spermatids.

the nonsupplemented diet. These results suggest that a gene expression sequence still proceeds to the spermiogenesis stage in $^{-/-}$.

Testis DHA and cholesta-3,5-diene were restored by dietary DHA

Testis fatty acid analysis (**Table 2**) shows significant changes due to genotype and dietary treatments. Consistent with the previous study (19), nonsupplemented $^{-/-}$ presented low levels of DHA (35% of $^{+/+}$), AA (8% of $^{+/+}$), near depletion of DPAn6, and accumulation of 20:3Δ7,11,14, a product of linoleic acid desaturated by delta-5 desaturase and elongated to 20 carbons (Table 2). As expected, AA supplementation to $^{-/-}$ males restored AA to the level of $^{+/+}$ animals, but not DHA and DPAn6, whereas 22:4n6 and 24:4n6, elongation products of AA, accumulated in the AA supplemented $^{-/-}$ group. Testis DHA was restored in $^{-/-}$ receiving DHA supplementation, while AA in testis was low, similar to nonsupplemented $^{-/-}$. DPAn6 was nearly depleted in $^{-/-}$ (Table 2), indicating that DPAn6 is dispensable for spermatogenesis and male fertility in the presence of sufficient DHA.

The VLPUFAs ($C \geq 26$) in testis of all $^{+/+}$ groups were elongation products of DPAn6 (26:5n6, 28:5n6, 30:5n6) and an elongation product of DHA (30:6n3), all of which were quantitatively minor (**Fig. 4A**). In nonsupplemented $^{-/-}$, these VLPUFAs became undetectable, except for 30:6n3, while an AA-elongated product, 26:4n6, appeared. AA-supplemented $^{-/-}$ had AA elongation products 28:4n6 and 30:4n6 but did not present the DPAn6-elongated VLPUFAs found in $^{+/+}$. The DHA-supplemented $^{-/-}$ lacked n6 VLPUFAs; however, 28:5n3, 28:6n3, and 30:6n3 were present.

VLPUFA analysis revealed presence of a large peak at 49 min in $^{+/+}$ and DHA-supplemented $^{-/-}$ (Fig. 4A). The peak diminished in nonsupplemented and AA-supplemented $^{-/-}$. The major species in the peak was identified as cholesta-3,5-diene (CD; Fig. 4B), a dehydration product of the alcohol group at the 3 position of cholesterol.

DISCUSSION

In this study, we determined the effects of dietary AA and DHA on the fertility and spermatogenesis of the D6D-null males. Supplementing 0.2% DHA alone was able to fully restore male fertility, spermiogenesis, sperm morphology, and sperm count in $^{-/-}$ males. This restoration occurred despite very low AA and near depletion of DPAn6, the major HUFA present in $^{+/+}$ males. Considering the variable ratios of DPAn6 and DHA among species (24), DPAn6 and DHA may be interchangeable for sperm function; therefore, DPAn6 is dispensable for sperm function in mice as long as sufficient DHA is present. It was a little unexpected that DHA supplementation alone can fully

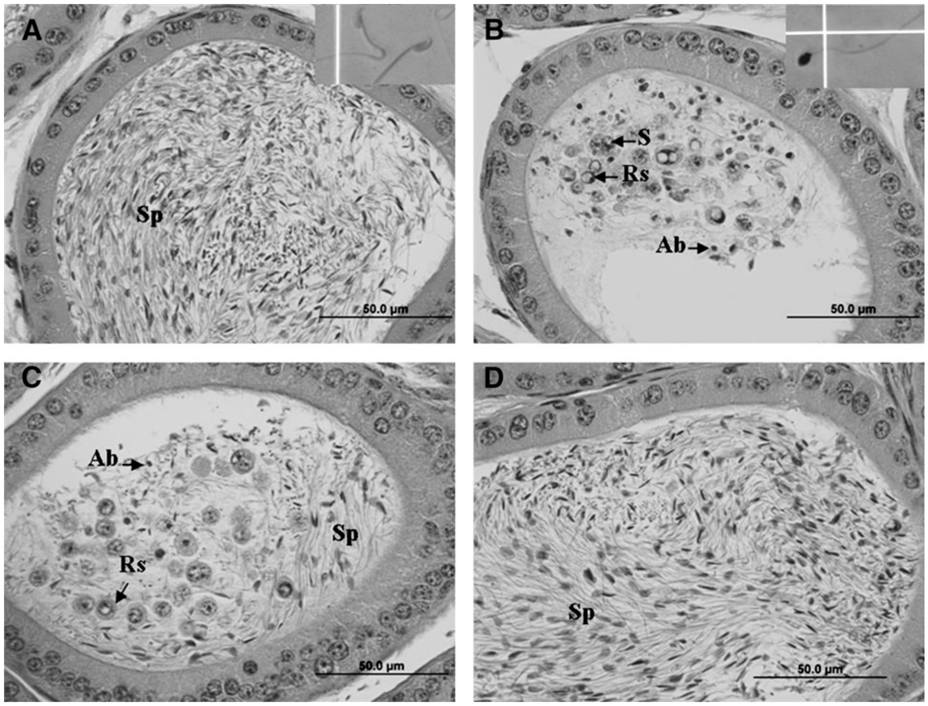

Fig. 3. Epididymis histology of $^{+/+}$ and $^{-/-}$ male mice fed AIN93G diet with or without 0.2% AA or 0.2% DHA supplementation from weaning until 16 weeks of age. A: Epididymis of $^{+/+}$ mouse without supplementation contains viable, mature spermatozoa (Sp), with normal heads and tails. Inset: magnification of sperm showing normal morphology. B: Epididymis from nonsupplemented $^{-/-}$ mouse contains sloughed round spermatids (Rs) and spermatocytes (S) and abnormal rounded head sperm (Ab). Inset: magnification of sperm showing globozoospermia (rounded head sperm). C: AA-supplemented $^{-/-}$ epididymis shows a combination of sloughed round spermatids (Rs), some abnormal rounded head sperm (Ab), as well as spermatozoa (Sp), with normal morphology. D: DHA-supplemented $^{-/-}$ epididymis has only spermatozoa (Sp) and shows no difference compared with $^{+/+}$. H and E stain.

restore male reproduction and spermiogenesis because of proposed roles of AA as eicosanoid precursor in male reproductive function (7, 8). Although the underlying mechanism is yet to be elucidated, there could be a functional redundancy that might compensate for the lack of eicosanoids. Alternatively, the residual AA present in the DHA-supplemented group might be sufficient as a precursor of eicosanoids for male reproduction.

In AA-supplemented $^{-/-}$ mice, sperm counts and a breeding success rate were partially restored even though the testis DHA and DPAn6 were as low as the nonsupplemented $^{-/-}$. This partial rescue is unlikely to be due to the restoration of testis AA in the AA-supplemented $^{-/-}$, considering DHA-supplemented $^{-/-}$ had full recovery with AA levels similar to nonsupplemented $^{-/-}$. On the other hand, there was an accumulation of the AA elongated products 22:4n6 and 24:4n6 in testis of AA supplemented $^{-/-}$. Thus, 22:4n6 or 24:4n6 may have acted as a substitute for DHA and DPAn6, although these AA-elongated fatty acids do not seem as effective as DPAn6 or DHA in restoring fertility.

Yet to be elucidated is the mechanism underlying the loss of spermatogenesis in $^{-/-}$ and the remarkable restoration by dietary DHA. In monkey sperm, 99% of DHA is present in flagella (6). Thus, the failure of spermatogenesis at a late stage of spermiogenesis may be at least in

TABLE 1. Spermiogenesis-specific gene expression markers in testis at 16 weeks of age

		Relative mRNA Expression			
Name	Description/Function	$^{+/-}$ AIN	$^{-/-}$ AIN	$^{-/-}$ AA	$^{-/-}$ DHA
Shippo1 (Odf3, TISP50)	Flagellum structural component	1 ± 0.06[a]	0.81 ± 0.06[b]	0.96 ± 0.14[ab]	1.01 ± 0.1[a]
Sperizin (Znrf4, TISP69)	Proteasome-mediated degradation of spermatid proteins?	1 ± 0.03[a]	0.55 ± 0.11[b]	0.63 ± 0.19[b]	0.63 ± 0.02[b]
A-kinase anchoring protein (Akap3)	Flagellum component	1 ± 0.11[a]	0.80 ± 0.12[b]	0.84 ± 0.07[b]	0.88 ± 0.03[ab]
Cortactin (Cttn)	Spermatid-Sertoli cell interaction	1 ± 0.06	1.25 ± 0.20	1.08 ± 0.16	1.06 ± 0.09
Transition protein-1 (Tnp1)	Compaction of sperm head	1 ± 0.16	0.93 ± 0.28	0.93 ± 0.17	1.10 ± 0.15
Sperm flagellum-associated protein (Sfap1)	Flagellum structural component	1 ± 0.11	1.03 ± 0.04	nd	nd

Mean ± SD. Groups without a common letter differ by Fisher's PLSD after one-way ANOVA ($P < 0.05$). nd, not determined; $^{+/+}$ AIN, wild-type fed AIN93G diet; $^{-/-}$ AIN, knockout fed AIN93G; $^{-/-}$ AA, knockout with 0.2% AA supplementation; $^{-/-}$ DHA, knockout with 0.2% DHA supplementation.

TABLE 2. Total fatty acid analysis of testis from 16-week-old D6D knockout mice with and without HUFA supplementation

HUFA	Relative Molar Percentage			
	$^{+/+}$ AIN	$^{-/-}$ AIN	$^{-/-}$ AA	$^{-/-}$ DHA
20:3 (Δ7,11,14)	nd	6.59	0.83	0.35
20:4n6	8.55 ± 3.65	0.68	10.45	0.79 ± 0.53*
20:5n3	0.12 ± 0.02	0.04	0.03	0.26 ± 0.13
22:4n6	1.07 ± 0.43	0.31	6.33	0.1 ± 0.07*
22:5n6	8.49 ± 3.88	0.06	0.05	0.01 ± 0.01*
22:6n3	5.49 ± 2.52	1.91	1.43	7.33 ± 4.58
24:4n6	0.35 ± 0.16	1.306	13.61	0.29 ± 0.19

Mean ± SD. Groups without SD were pooled. $^{+/+}$ AIN, wild-type fed AIN93G diet; $^{-/-}$ AIN, knockout fed AIN93G; $^{-/-}$ AA, knockout with 0.2% AA supplementation; $^{-/-}$ DHA, knockout with 0.2% DHA supplementation; nd, not detected. * $P < 0.05$, significantly different versus $^{+/+}$ AIN by Student's *t*-test.

part due to lack of DHA and DPAn6 for structural components of the flagellar membrane phospholipids. However, the impairment of spermatogenesis is not limited to tail formation. It extends to globozoospermia and possible impairment of sterol metabolism. Globozoospermia is a rare form of infertility in humans, characterized by a rounded sperm head (25). The Jackson Laboratory lists 39 mutant mouse strains under globozoospermia (http://www.jax.org), suggesting multiple causes of this abnormality. Mammalian sperm heads contain ceramides and sphingomyelins with high percentages of VLPUFAs (9, 10). Thus, the loss of VLPUFA in nonsupplemented $^{-/-}$ may play a role in the impaired sperm head function and structure.

Another potentially important finding of this study is decreased CD in the lipid extract of nonsupplemented and AA-supplemented $^{-/-}$ and restoration by dietary DHA. Because of the paucity of literature on CD, it is unclear if CD is present in testis or if it is derived from sterols during sample processing, although presence of CD in cornea has been reported (26). Whichever the case, our data suggest an impairment of sterol metabolism in $^{-/-}$ that was restored by DHA supplementation. Several studies indi-

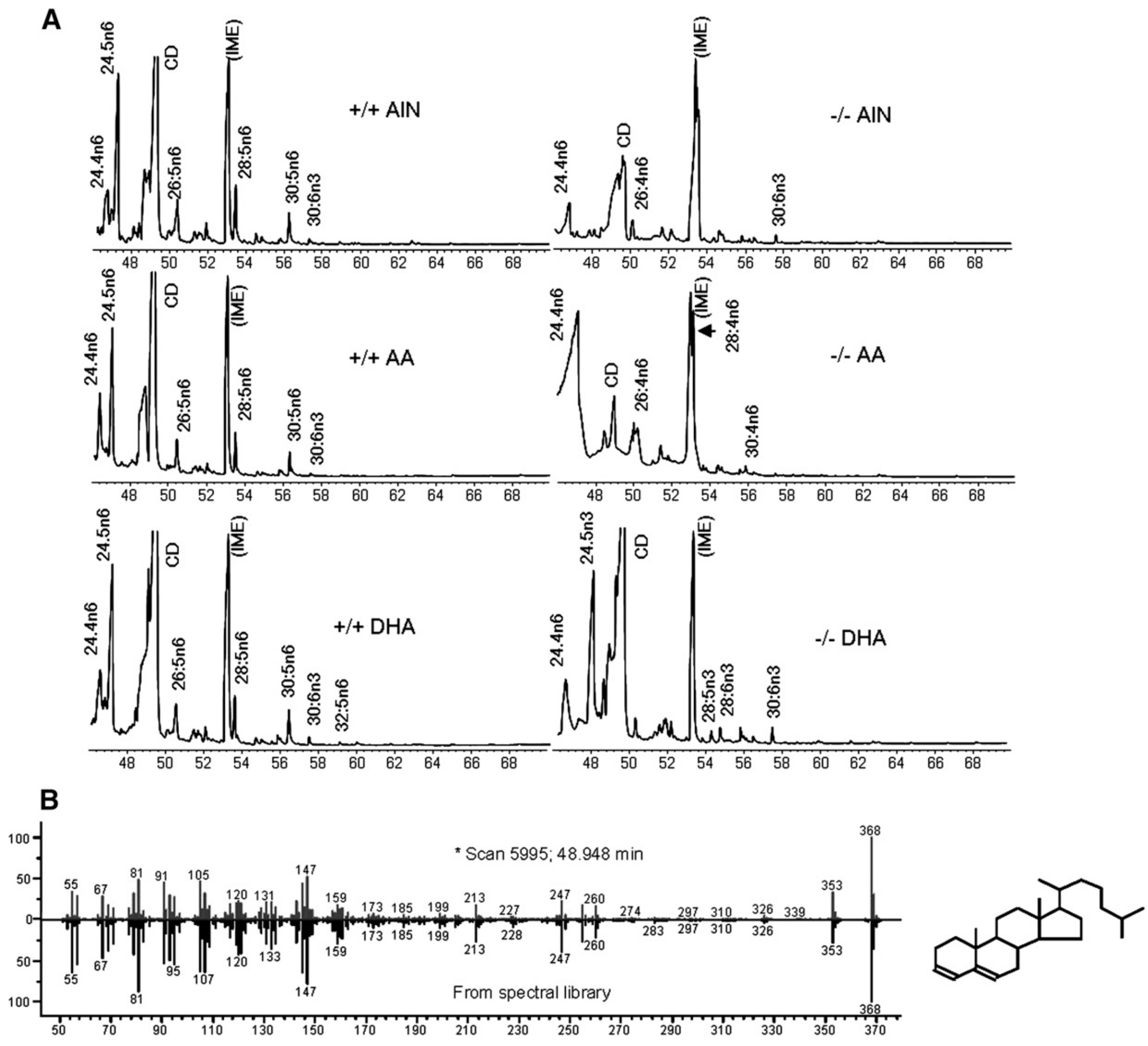

Fig. 4. A: VLPUFA analysis of testis at 16 weeks of age by GC-MS. $^{+/+}$ AIN, wild type fed AIN93G diet; $^{+/+}$ AA, wild type with 0.2% AA supplementation; $^{+/+}$ DHA, wild type with 0.2% DHA supplementation; $^{-/-}$ AIN, knockout fed AIN93G; $^{-/-}$ AA, knockout with 0.2% AA supplementation; $^{-/-}$ DHA, knockout with 0.2% DHA supplementation. IME, isocholesteryl methyl ether (normalizer). B: Mass spectra of the peak at 48.9 min of $^{+/+}$ testis sample matches reference spectra (NIST Mass Spectral Library, Agilent Technologies) of CD (chemical structure shown at the right).

cate importance of sterols in spermatogenesis. Desmosterol (24-dehydrocholesterol), an intermediate metabolite of the last step of cholesterol synthesis, is not present in large quantity in most tissues. However, free desmosterol is the major sterol present in flagella of monkey sperm followed by free cholesterol and cholesteryl esters (6, 27). Furthermore, the major impairment in the hormone-sensitive lipase-null mouse is a complete loss of spermatogenesis (28), a similar phenotype to our D6D-null mice. Because hormone-sensitive lipase is the only esterase that can hydrolyze cholesteryl ester in testis, a loss of the enzyme resulted in accumulation of cholesteryl ester in Sertoli cells and loss of spermatogenesis (28), indicating essentiality of cholesterol in spermatogenesis. Further studies will be warranted to elucidate the role of DHA in sterol metabolism in testis.

Sperizin is a protein highly expressed in spermatids and may act as ubiquitin ligase (29, 30). A study reported complete abolition of sperizin RNA in the testis of D6D-null mice and suggested an arrest of the gene expression sequence at the spermiogenesis stage (20). However, in our study, sperizin showed only a mild decrease in the $^{-/-}$ animals of all three dietary groups, which displayed a drastic difference in spermiogenesis, excluding sperizin as the cause of impaired spermatogenesis. Moreover, several other markers specific to spermiogenesis showed largely normal expression in $^{-/-}$ animals fed different diets, including genes that encode proteins in sperm flagella such as Shippo1 (31), Akap3 (32), and Sfap1 (33). Thus, our RNA analysis suggests that there is no general arrest of gene expression sequence at the spermiogenesis stage, although it is possible that expression of specific genes may be affected by DHA deficiency.

In conclusion, this study demonstrated that DHA supplementation to D6D-null male mice restored spermatogenesis and fertility in the absence of DPAn6 and low AA in testis, while dietary AA was much less effective. The accumulation of 22:4n6 in the AA-supplemented $^{-/-}$ testis suggests that 22:4n6 may act as a lesser substitute for DPAn6 or DHA in spermiogenesis. CD was detected in testis lipid extracts from $^{+/+}$ and DHA-supplemented $^{-/-}$, whereas it greatly decreased in nonsupplemented and AA-supplemented $^{-/-}$, suggesting impairment of sterol metabolism in the latter groups. The expression of spermiogenesis marker genes in $^{-/-}$ animals was largely normal. The mechanism underlying the loss of spermatogenesis in $^{-/-}$ and the rescue by dietary DHA is yet to be elucidated.

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