TITLE

Effect of dietary supplementation with a highly pure and concentrated docosahexaenoic acid (DHA) supplement on human sperm function.

SHORT TITLE

Effect of DHA in human sperm.

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ABSTRACT

The aim of this study was to evaluate the possible beneficial effects of diet supplementation with a highly concentrated and purified docosahexaenoic acid (DHA) formula on human sperm function. We performed a prospective, randomized, double blind, placebo-controlled intervention study. One-hundred eighty human semen samples from sixty infertile patients of IVI Sevilla clinic (an assisted reproduction center) were included. All samples were examined according to World Health Organization guidelines. We analyzed macroscopic and microscopic sperm parameters, oxidative stress, apoptosis, lipid peroxidation, mitochondrial membrane potential and DNA fragmentation before and after supplementation with different DHA daily doses (0.5, 1 and 2 g) or placebo for 1 and 3 months. No differences were found in traditional sperm parameters except for progressive sperm motility, with a significant increase after DHA ingestion after the first month with 1 or 2 g doses and after 3 months with 0.5 g of DHA. This effect was more evident in asthenozoospermic patients. No differences were found in any molecular semen parameter except oxidative stress, in which a slightly benefit is observed after DHA treatment. In conclusion, this study support previous indications that highlight the importance of DHA supplementation as a means of improving sperm quality in asthenozoospermic men.

Keywords: sperm; DNA; oxidative stress; spermatozoa; DHA

1. INTRODUCTION

Infertility has become a major medical and social problem worldwide, affecting around 15% of couples of reproductive age, and approximately 40-50 % of the cases are due to male factors [1-3]. It has been observed a significant decline in sperm counts, motility and morphology during the last 50-70 years [4]. In many cases, a multifactorial origin is suspected, involving genetic disorders, food habits, lifestyle changes and environmental contamination among others [5, 6].

A key player in the production of deficient spermatozoa is the presence of free radicals such as reactive oxygen species (ROS). In sperm, moderate levels of ROS are required in primary functions such as defence against infections, capacitation, hyperactivation, acrosome reaction or sperm-oocyte interaction [2, 3, 7-11]. However, additional free radicals may be formed under certain physiological conditions such as obesity, extreme exercise, diseases or high blood sugar levels as well as in response to external factors such as pollution, smoking, radiation, heat, alcohol abuse or medications [12]. An overload of free radicals beyond physiological levels can be deleterious and damage essential biomolecules (ie. DNA, lipids and proteins) in a process known as oxidative stress (OS). OS plays a major role in the development of well-known chronic and degenerative diseases as well as in sperm dysfunction [2, 13, 14]. Almost 50% of infertile men have increased levels of ROS in their seminal plasma [2, 15] and a clear association has been established between supraphysiological ROS levels and male infertility [16].

Sperm lipid membrane contain a particularly high percentage of polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) [17], which give the plasma membrane the necessary fluidity to facilitate the membrane fusion events associated with fertilization. Eslamian et al. [18] have found a positive correlation between the composition and fatty acid content and the odds of having asthenozoospermia. DHA benefits go beyond sperm quality and integrity, as it also increases the production of endogenous antioxidants (i.e. glutathione or catalase), preserves DNA integrity, restores synaptic plasticity, improves cognitive and visual functions, produces anti-inflammatory mediators and improves insulin sensitivity [19-22]. Its six double bonds contribute to a peculiar folding of its molecule that gives a very characteristic fluidity and flexibility to sperm cell membranes

[23, 24]. Many observational studies have related seminal antioxidants levels with infertile men. Seminal plasma is rich in antioxidants (ie. vitamin C, Vitamin E, glutathione, albumin, carotenoids or uric acid) which prevent sperm damage by ROS following ejaculation [11, 25], although under high OS conditions, these might be insufficient. To counteract the deleterious effects that an excessive free radical load can have in biological systems, adequate levels of antioxidants are required, as they constitute the main defence mechanism against ROS [14, 25].

Because of this, dietary supplementation with antioxidants has gained much attention in recent years [26] and numerous antioxidant supplements are available to address this need. Many of them contain DHA but in different concentrations and/or in combination with other molecules, which makes it difficult to assess the individual effect of DHA on seminal quality and the functional status of spermatozoa. The present study was intended to elucidate the effect of a highly concentrated and purified DHA supplement in sperm quality.

2. MATERIAL AND METHODS

2.1. Study population

This study was approved by our institutional review board (protocol no. 1303-SEV-00-CG, NCT02889341), and all patients signed a written informed consent. Sixty subjects were recruited for this prospective, randomized, double blind and placebo-controlled intervention study. The inclusion criteria for this study were men aged between 18 and 50 years with a previous history of infertility of at least one year and whose semen analysis met the following criteria: (a) Sperm count greater than 10 million per mL, (b) Sperm progressive motility of less than 60%, (c) Normal sperm morphology of less than 2% [16]. Subjects were randomly assigned to either the placebo group (n=15) or to one of the following DHA groups: the 0.5 g DHA group (n=15), the 1 g DHA group (n=15), or the 2 g DHA group (n=15) using NuaDHA[®], a highly concentrated DHA-TG formula (that is, as triglyceride; with a minimal 90% of omega-3). These quantities (0.5 g, 1 g and 2 g) represent daily doses. DHA was provided by Nua Biological Innovations SL (Vizcaya, Spain). This supplement complies with a very rigorous quality standard (5 star IFOS Program), which guarantees

that the oil is practically free from heavy metals (mercury, cadmium, lead and arsenic), it is almost free from polychlorinated biphenyls, furans or dioxins, it is not oxidised and has the actual amount of active substance indicated on the label. Treatment duration was 3 months, and seminal and functional tests were carried out in semen samples collected prior to the treatment, 1 month after the beginning of diet supplementation and 3 months after the beginning of diet supplementation. The placebo group received 0.5 g of primrose oil daily. The number of patients assessed for eligibility, study enrollment and dose allocation is shown in Fig. 1.

2.2. Semen samples and sperm preparation

Semen samples were obtained by masturbation after 3–4 days of sexual abstinence and processed immediately after liquefaction as described previously [27]. Quantitative, manual semen analysis was performed on undiluted semen according to 2010 World Health Organization [16] guidelines using light microscopy. After liquefaction, 5 μ L of semen were loaded in a Makler Counting Chamber (Sefi Medical Instruments, Haifa, Israel) and total sperm count ($\times 10^6$ /mL) and percentage motility were measured. Samples were also examined for volume and morphology. A minimum of 200 cells were counted per 5 μ L droplet, and at least three droplets were studied per sample and by two different people. The semen samples were analyzed by a team of experienced technicians who are tested periodically to ensure homogeneity in their criteria. The motility pattern of sperm cells was defined as progressive motility (PR), non-progressive motility (NP) and immobility (IM) and measured as a percentage of the total (PR+NP+IM), which was considered as 100%. Liquefied semen samples were washed with modified human tubal fluid medium (mHTF, Irvine Scientific, Santa Ana, CA, USA) supplemented with 5% human serum albumin at 37°C. The diluted sperm was concentrated by centrifugation (400 g for 10 min) and the pellets were gently reconstituted in 3.5 mL of fresh mHTF supplemented with HSA. 0.5 mL of the sample were fixed for sperm DNA fragmentation study.

2.3. Flow Cytometry

Evaluation of oxidative stress, apoptosis, mitochondrial membrane potential (MMP), lipid peroxidation and DNA fragmentation was performed by flow cytometry as previously reported [28]. Detection of oxidative stress ROS levels was done using the CellROX Green and CellROX Orange Kit (Molecular Probes, Eugene, OR). MMP and apoptosis were analyzed with Mitotracker Red and Alexa Fluor 488 annexin V by using the Mitochondrial Membrane Potential/Annexin V Apoptosis Kit (Molecular Probes). The assessment of lipid peroxidation was made with Image iT Lipid Peroxidation Kit (Molecular Probes). Immediately after semen preparation, sperm samples were adjusted to a concentration of 2×10^6 cells/mL and divided into different aliquots. A minimum of 500,000 cells were incubated with the corresponding dye for 30 min at 37° in 5% CO₂, according to the manufacturer instructions. Cumene hydroperoxide was used as a positive control of lipid peroxidation, H₂O₂ was used as a positive control of ROS, apoptosis and MMP; and TO-PRO3 iodide was used in all experiments as supravital fluorescent stain. Fluorescence data from at least 20,000 events per sample were captured on a BD Accuri C6 flow cytometer (BD Biosciences, San José, CA) and analyzed using CFlow Plus software. Fluorescence from live spermatozoa was determined by subtraction of dead sperm cells background fluorescence in each histogram.

Evaluation of DNA fragmentation was performed by using a TUNEL assay (In situ Cell Death Detection Kit, Fluorescein, Roche). Immediately after semen preparation, sperm samples were adjusted to a concentration of 2×10^6 cells/ml and fixed in 2% paraformaldehyde in phosphate buffered solution. Sperm cells were then permeabilized in 0.1% sodium citrate plus 0.1% Triton X-100 and negative and positive aliquots prepared for flow cytometry following manufacturer instructions. Fluorescence data from at least 20,000 events per sample were captured on a BD Accuri C6 flow cytometer and analyzed using CFlow Plus software.

2.4. Statistical analysis

The variables are summarized as mean and SD at the start (0 months), after 1 month and after 3 months of treatment. Changes in the different variables after 1 or 3 months were examined using a paired *t* test showing the difference and its 95% confidence interval. Statistical significance was established as $p < 0.05$.

To avoid random associations, we estimated that there was a possible effect only when a consistent significant difference in more than one dose was found. The variables with a possible effect (progressive motility and ROS) were stratified by sperm quality (normal or asthenozoospermic) to explore possible differences in the effect of the treatment in different patients.

3. RESULTS

3.1. Macroscopic and microscopic semen parameters in placebo and DHA-treated groups

The distribution of different semen parameters among the four groups is shown in Table 1. The volume, concentration and morphology of the samples were not affected by DHA treatment in comparison to the placebo group. In contrast, sperm motility was significantly affected by 0.5 g DHA after 3 months treatment, and by 1 or 2 g DHA after the first month and maintained during the 3 months of the study without significant variations. DHA increased the proportion of progressive motile sperm together with a parallel decrease in the percentage of non-progressive motile sperm, while the proportion of immotile sperm was unaffected (Table 1).

On this basis, the effect of DHA treatment was further analysed by separating patients into two groups, differentiating asthenozoospermic patients from those who were not. As shown in Table 2, the effect of DHA was particularly evident in patients with reduced progressive motility, lower than 32%.

3.2. Molecular semen parameters in placebo and DHA-treated groups

The distribution of different molecular and functional semen parameters (oxidative stress, apoptosis, mitochondrial membrane potential (MMP), lipid peroxidation and DNA fragmentation) among the four groups is shown in Table 1. No significant differences were found in these parameters, except for oxidative stress (OS). Regarding OS, those men who took DHA showed a significant benefit since the first month of treatment and it was maintained during the 3 months intake. The results obtained showed that DHA does not increase apoptosis or lipid peroxidation, which agrees perfectly with the fact that the mitochondrial membrane potential does not vary either, demonstrating that the product is non-toxic and does not harm spermatozoa, but it can benefit patients with low percentages of motility. When analysing the effect on ROS, after differentiating

asthenozoospermic patients from men with motility within the normal range, it was found a beneficial effect of DHA in both groups, appearing sooner in patients with normal progressive motility, and requiring a 3 months treatment period in asthenozoospermic patients. Unfortunately, it was not possible to define at which dose the best effect was obtained (Table 3).

4. DISCUSSION

Studies in mice have shown that DHA is essential for acrosome reaction and a DHA deficiency results in abnormal sperm morphology, loss of motility and infertility; which can be restored by dietary DHA supplementation [29, 30]. Most scientists agree that the proportion of PUFAs in human sperm membranes –especially DHA– is positively correlated with semen quality. However, some authors consider that these results are controversial [5, 24, 31-33]. This may be due to the wide variety of antioxidant preparations that exist in the market, which usually contain omega-3 fatty acids together with other antioxidant molecules, either in different concentrations or with different oil purities (i.e. 65% vs 85% omega-3). Highly pure and concentrated omega-3 preparations such as the one used in the present study have not been available in the market until recent years. It is important to note that working with higher omega-3 concentrations, results in a higher uptake and a more potent effect than formulations with lower omega-3 concentrations [34].

Conventional sperm analysis provides important information on male fertility and, despite its limitations; it is currently the most useful technique to determine the fertility status of men. According to this, the effect of DHA supplementation was first analyzed on standard sperm parameters determined according to WHO guidelines [16]. DHA did not affect macroscopic semen parameters (volume, viscosity and pH) and had no effect on sperm concentration or morphology. However, progressive sperm motility was significantly increased after DHA supplementation, in accordance with previously published studies [33, 35, 36]. This increase was observed in all 3 doses assayed (0.5 g, 1 g and 2 g), although the lower dose (0.5 g) needed 3 months to show a significant effect, whilst both the middle and high doses (1 g and 2 g) showed a significant improvement in motility after just one month of treatment. When comparing normal versus asthenozoospermic

patients, in this last population higher DHA doses were required to obtain an increase in the percentage of progressive motile spermatozoa, in agreement with Martinez-Soto et al, 2016. A recent case-report study [18] also showed an inverse and dose-dependent association between asthenozoospermia and the intake of ω -3 PUFAs. However, Conquer and colleagues [31], despite publishing that spermatozoa from asthenozoospermic males showed lower levels of DHA when compared to normozoospermic population, did not found a direct effect on motility after supplementation with 400 mg and 800 mg of DHA per day during 3 months. A possible explanation could be that the dose used was insufficient since other authors have reported that a minimal daily dose of 1 g is needed in order to obtain beneficial effects on sperm quality. In any case, our results are in line with previous cross-sectional analyses of dietary fatty acids intakes and semen quality in men.

Since vigorous sperm motility includes the transition from progressive to hyperactivated motility (necessary to reach the oocyte and penetrate the zona pellucida), infertile men with asthenozoospermia and low omega-3 FAs concentrations could be the optimal candidates who would benefit from DHA supplementation in a therapeutical approach [5, 18]. In addition, it is of special interest the fact that DHA supplement could protect spermatozoa against the damage caused by the cryopreservation process. As pointed out by Martínez-Soto and colleagues [37], DHA supplementation in humans is positively correlated with sperm viability and motility before and after freezing.

Several sperm molecular markers, including MMP (mitochondrial membrane potential), ROS and DNA fragmentation have recently been described as accurate predictors of sperm function that can be used in combination with the semen analysis to evaluate sperm quality [38-40]. Increased OS due to a ROS overload can often result in decreased MMP and increased DNA fragmentation, thus hindering sperm viability [41]. Consequently, these parameters were also considered in the present study.

Mitochondrial membrane potential is a major parameter that reflects mitochondrial functionality and act as an indicator of mitochondrial energy status. Previous studies have shown that

spermatozoa with high motility and viability show the highest values of MMP whilst infertile patients have lower MMP values than healthy individuals. It is widely accepted that sperm motility depends on the ATP energy produced by mitochondria located in the spermatozoa mid-piece, which can explain the positive correlation that exists between increased motility and MMP [38, 39, 42-44]. Hence, MMP could be considered a potential regulator and indicator of sperm motility and could be directly related to male fertility. In the present study, no significant changes were observed in MMP values following DHA supplementation. This was expected, since MMP values were already normal at the beginning of the study.

Regarding oxidative stress, all semen samples showed a rising tendency in ROS levels although it was not possible to define at which dose the best effect is obtained. This beneficial effect appeared sooner in patients with normal progressive motility, while in the asthenozoospermic group it required the 3 months treatment period in order to be detected. The increase in ROS production has been associated with an increase in ATP production by mitochondria located in the sperm headpiece, which results in an increased motility, as observed in this study. Free radicals are known to be key signalling molecules in sperm physiology and a moderate amount of ROS is required for sperm primary functions [2, 3, 7-11, 45]. However, an excessive increase in sperm ROS levels can be deleterious and cause subfertility [1, 2, 46]. One of the first negative effects of an excessive ROS load is lipid peroxidation (LPO) of the unsaturated fatty acids of the sperm membrane, which directly affects its fluidity and integrity and can contribute to fertilisation failure, since most sperm functions are dependent on membrane functionality [2, 47, 48]. Considering that DHA treatment was accompanied by an increase in ROS levels, we examined the potential effects of this by analysing lipid peroxidation before and after DHA supplementation. We found that DHA did not induce LPO, in fact, it was slightly lower after DHA treatment. This is consistent with the fact that DHA can also improves the endogenous antioxidant defences, by means of increasing the catalase, glutathione peroxidase and glutathione reductase levels [19]. Therefore, we can conclude that the DHA formula used in this study caused a 'positive' oxidative stress. The observed moderate increase in ROS levels

272 did not have a detrimental effect on the sperm, as there was no increase in peroxidation products
273 upon treatment.

274 Apart from lipid peroxidation, an excessive free radical load can compromise sperm integrity
275 in other ways including DNA fragmentation and activation of programmed cell death or apoptosis
276 [2, 11, 40, 45, 49, 50]. These processes can impair sperm function and hinder sperm-oocyte fusion
277 and embryo development, causing infertility. Therefore, apoptosis and DNA fragmentation tests
278 were also performed to assess changes occurred upon DHA supplementation. The studies performed
279 by Martínez-Soto [51] and Alvarez [24] are of the few ones to test DNA fragmentation upon DHA
280 supplementation in human semen and contrary to our results, they observed a reduction in DNA
281 fragmentation. This can be explained by the fact that DNA fragmentation rates of patients at the
282 beginning of the aforementioned studies were already high ($> 18\%$), whilst in the present study they
283 were within normal values right from the beginning ($< 18\%$, average 8.2%). It should be reminded
284 that not all low motilities are necessarily associated to high DNA fragmentation rates, but rather,
285 high DNA fragmentation rates do result in low motilities [52].

286 Regarding apoptosis, cells initiating this process suffer translocation of phosphatidylserine
287 molecules to the outer surface of the plasma membrane phospholipids. One characteristic of these
288 phospholipids is that they bind with high affinity to annexin V, which is a marker of early apoptosis
289 [2]. When we studied the effect of excessive production of ROS and DHA supplementation, no
290 significant changes in apoptosis were observed by the end of the treatment, which further supported
291 that the OS levels obtained with the treatment was positive for sperm function.

292 In conclusion, the present data show that dietary supplementation with a highly pure and
293 concentrated DHA composition at doses of 0.5 g, 1 g and 2 g per day had beneficial effects on sperm
294 function without producing any adverse effects, obtaining more immediate results with higher doses.
295 DHA was perfectly tolerated by patients and no adverse effects were reported.

296 A novel outcome of the present study was a clear indication of DHA supplementation for
297 patients with asthenozoospermia, suggesting that dietary DHA supplementation at 1 g/day would be
298 particularly beneficial for this infertile population. In addition, the increase in motility –although

associated with an increased ROS production– did not cause an increase in lipid peroxidation, DHA fragmentation or apoptosis, therefore reinforcing the positive nature of the OS generated. Overall, our data support previous reports that highlight the importance of DHA supplementation as a means of improving men sperm quality.

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AUTHOR CONTRIBUTIONS

CG and LC designed and coordinated the study. CG and MF were responsible of the patient’s recruitment. FP and DM were in charge of analyzing the sperm samples. All authors were responsible for the data collection, analysis, and interpretation presented in the manuscript. CG, MA, VB, ES and LC wrote, edited and reviewed the manuscript. All authors read and approved the final manuscript.

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REFERENCES

- [1] Agarwal A, Mulgund A, Sharma R, Sabanegh E. Mechanisms of oligozoospermia: an oxidative stress perspective. *Syst Biol Reprod Med*. 2014;60:206-16.
- [2] Agarwal A, Virk G, Ong C, du Plessis SS. Effect of oxidative stress on male reproduction. *World J Mens Health*. 2014;32:1-17.
- [3] Du Plessis SS, Agarwal A, Halabi J, Tvrdá E. Contemporary evidence on the physiological role of reactive oxygen species in human sperm function. *J Assist Reprod Genet*. 2015;32:509-20.
- [4] Merzenich H, Zeeb H, Blettner M. Decreasing sperm quality: a global problem? *BMC Public Health*. 2010;10:24.
- [5] Giali L, Mohammadmoradi S, Javidan A, Sadeghi MR. Nutritional modifications in male infertility: a systematic review covering 2 decades. *Nutr Rev*. 2016;74:118-30.
- [6] Singh K, Jaiswal D. Human male infertility: a complex multifactorial phenotype. *Reprod Sci*. 2011;18:418-25.
- [7] de Lamirande E, Gagnon C. A positive role for the superoxide anion in triggering hyperactivation and capacitation of human spermatozoa. *Int J Androl*. 1993;16:21-5.
- [8] de Lamirande E, Jiang H, Zini A, Kodama H, Gagnon C. Reactive oxygen species and sperm physiology. *Rev Reprod*. 1997;2:48-54.
- [9] Iommiello VM, Albani E, Di Rosa A, Marras A, Menduni F, Morreale G, et al. Ejaculate oxidative stress is related with sperm DNA fragmentation and round cells. *Int J Endocrinol*. 2015;2015:321901.
- [10] Rivlin J, Mendel J, Rubinstein S, Etkovitz N, Breitbart H. Role of hydrogen peroxide in sperm capacitation and acrosome reaction. *Biol Reprod*. 2004;70:518-22.
- [11] Tremellen K. Oxidative stress and male infertility--a clinical perspective. *Hum Reprod Update*. 2008;14:243-58.
- [12] Rahman K. Studies on free radicals, antioxidants, and co-factors. *Clin Interv Aging*. 2007;2:219-36.
- [13] Saleh RA, Agarwal A. Oxidative stress and male infertility: from research bench to clinical practice. *J Androl*. 2002;23:737-52.
- [14] Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol*. 2007;39:44-84.
- [15] Lanzafame FM, La Vignera S, Vicari E, Calogero AE. Oxidative stress and medical antioxidant treatment in male infertility. *Reprod Biomed Online*. 2009;19:638-59.
- [16] WHO. WHO laboratory manual for the examination and processing of human semen: Geneva: World Health Organisation; 2010.
- [17] Koppers AJ, Garg ML, Aitken RJ. Stimulation of mitochondrial reactive oxygen species production by unesterified, unsaturated fatty acids in defective human spermatozoa. *Free Radic Biol Med*. 2010;48:112-9.
- [18] Eslamian G, Amirjannati N, Rashidkhani B, Sadeghi MR, Baghestani AR, Hekmatdoost A. Dietary fatty acid intakes and asthenozoospermia: a case-control study. *Fertil Steril*. 2015;103:190-8.
- [19] Hashimoto M, Hossain S, Al Mamun A, Matsuzaki K, Arai H. Docosahexaenoic acid: one molecule diverse functions. *Crit Rev Biotechnol*. 2017;37:579-97.
- [20] Molfino A, Gioia G, Rossi Fanelli F, Muscaritoli M. The role for dietary omega-3 fatty acids supplementation in older adults. *Nutrients*. 2014;6:4058-73.
- [21] Tanaka K, Farooqui AA, Siddiqi NJ, Alhomida AS, Ong WY. Effects of docosahexaenoic Acid on neurotransmission. *Biomol Ther (Seoul)*. 2012;20:152-7.
- [22] Wu A, Ying Z, Gomez-Pinilla F. Docosahexaenoic acid dietary supplementation enhances the effects of exercise on synaptic plasticity and cognition. *Neuroscience*. 2008;155:751-9.
- [23] Sanocka D, Kurpisz M. Reactive oxygen species and sperm cells. *Reprod Biol Endocrinol*. 2004;2:12.

- [24] Alvarez J. Utilidad del ácido docosahexaenoico en el tratamiento de la infertilidad masculina. *Rev Int Androl* 2011;9:138-44.
- [25] Kefer JC, Agarwal A, Sabanegh E. Role of antioxidants in the treatment of male infertility. *Int J Urol*. 2009;16:449-57.
- [26] Showell MG, Mackenzie-Proctor R, Brown J, Yazdani A, Stankiewicz MT, Hart RJ. Antioxidants for male subfertility. *Cochrane Database Syst Rev*. 2014:CD007411.
- [27] Ravina CG, Seda M, Pinto FM, Orea A, Fernandez-Sanchez M, Pintado CO, et al. A role for tachykinins in the regulation of human sperm motility. *Hum Reprod*. 2007;22:1617-25.
- [28] Cejudo-Roman A, Pinto FM, Subiran N, Ravina CG, Fernandez-Sanchez M, Perez-Hernandez N, et al. The voltage-gated sodium channel nav1.8 is expressed in human sperm. *PLoS One*. 2013;8:e76084.
- [29] Roqueta-Rivera M, Abbott TL, Sivaguru M, Hess RA, Nakamura MT. Deficiency in the omega-3 fatty acid pathway results in failure of acrosome biogenesis in mice. *Biol Reprod*. 2011;85:721-32.
- [30] Roqueta-Rivera M, Stroud CK, Haschek WM, Akare SJ, Segre M, Brush RS, et al. Docosahexaenoic acid supplementation fully restores fertility and spermatogenesis in male delta-6 desaturase-null mice. *J Lipid Res*. 2010;51:360-7.
- [31] Conquer JA, Martin JB, Tummon I, Watson L, Tekpetey F. Effect of DHA supplementation on DHA status and sperm motility in asthenozoospermic males. *Lipids*. 2000;35:149-54.
- [32] Esmaeili V, Shahverdi AH, Moghadasian MH, Alizadeh AR. Dietary fatty acids affect semen quality: a review. *Andrology*. 2015;3:450-61.
- [33] Safarinejad MR. Effect of omega-3 polyunsaturated fatty acid supplementation on semen profile and enzymatic anti-oxidant capacity of seminal plasma in infertile men with idiopathic oligoasthenoteratospermia: a double-blind, placebo-controlled, randomised study. *Andrologia*. 2011;43:38-47.
- [34] Bryhn M, Hansteen H, Schanche T, Aakre SE. The bioavailability and pharmacodynamics of different concentrations of omega-3 acid ethyl esters. *Prostaglandins Leukot Essent Fatty Acids*. 2006;75:19-24.
- [35] Aksoy Y, Aksoy H, Altinkaynak K, Aydin HR, Ozkan A. Sperm fatty acid composition in subfertile men. *Prostaglandins Leukot Essent Fatty Acids*. 2006;75:75-9.
- [36] Tavailani H, Doosti M, Abdi K, Vaisiraygani A, Joshaghani HR. Decreased polyunsaturated and increased saturated fatty acid concentration in spermatozoa from asthenozoospermic males as compared with normozoospermic males. *Andrologia*. 2006;38:173-8.
- [37] Martinez-Soto JC, Landeras J, Gadea J. Spermatozoa and seminal plasma fatty acids as predictors of cryopreservation success. *Andrology*. 2013;1:365-75.
- [38] Marchetti C, Obert G, Deffosez A, Formstecher P, Marchetti P. Study of mitochondrial membrane potential, reactive oxygen species, DNA fragmentation and cell viability by flow cytometry in human sperm. *Hum Reprod*. 2002;17:1257-65.
- [39] Wang X, Sharma RK, Gupta A, George V, Thomas AJ, Falcone T, et al. Alterations in mitochondria membrane potential and oxidative stress in infertile men: a prospective observational study. *Fertil Steril*. 2003;80 Suppl 2:844-50.
- [40] Wang X, Sharma RK, Sikka SC, Thomas AJ, Jr., Falcone T, Agarwal A. Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. *Fertil Steril*. 2003;80:531-5.
- [41] Ghaleno LR, Valojerdi MR, Hassani F, Chehrazi M, Janzamin E. High level of intracellular sperm oxidative stress negatively influences embryo pronuclear formation after intracytoplasmic sperm injection treatment. *Andrologia*. 2014;46:1118-27.
- [42] Agnihotri SK, Agrawal AK, Hakim BA, Vishwakarma AL, Narender T, Sachan R, et al. Mitochondrial membrane potential (MMP) regulates sperm motility. *In Vitro Cell Dev Biol Anim*. 2016;52:953-60.

- [43] Malic Voncina S, Golob B, Ihan A, Kopitar AN, Kolbezen M, Zorn B. Sperm DNA fragmentation and mitochondrial membrane potential combined are better for predicting natural conception than standard sperm parameters. *Fertil Steril*. 2016;105:637-44 e1.
- [44] Marchetti P, Ballot C, Jouy N, Thomas P, Marchetti C. Influence of mitochondrial membrane potential of spermatozoa on in vitro fertilisation outcome. *Andrologia*. 2012;44:136-41.
- [45] Aitken RJ. Molecular mechanisms regulating human sperm function. *Mol Hum Reprod*. 1997;3:169-73.
- [46] Dinesh V, Shamsi M, Dada R. Supraphysiological free radical levels and their pathogenesis in male infertility. *Reprod Sys Sexual Disorders* 2012;1:1-15.
- [47] Okuyama H, Orikasa Y, Nishida T. Significance of antioxidative functions of eicosapentaenoic and docosahexaenoic acids in marine microorganisms. *Appl Environ Microbiol*. 2008;74:570-4.
- [48] Sabeti P, Pourmasumi S, Rahiminia T, Akyash F, Talebi AR. Etiologies of sperm oxidative stress. *Int J Reprod Biomed (Yazd)*. 2016;14:231-40.
- [49] Oborna I, Wojewodka G, De Sanctis JB, Fingerova H, Svobodova M, Brezinova J, et al. Increased lipid peroxidation and abnormal fatty acid profiles in seminal and blood plasma of normozoospermic males from infertile couples. *Hum Reprod*. 2010;25:308-16.
- [50] Wright C, Milne S, Leeson H. Sperm DNA damage caused by oxidative stress: modifiable clinical, lifestyle and nutritional factors in male infertility. *Reprod Biomed Online*. 2014;28:684-703.
- [51] Martinez-Soto JC, Domingo JC, Cordobilla B, Nicolas M, Fernandez L, Albero P, et al. Dietary supplementation with docosahexaenoic acid (DHA) improves seminal antioxidant status and decreases sperm DNA fragmentation. *Syst Biol Reprod Med*. 2016;62:387-95.
- [52] Boushaba S, Belaaloui G. Sperm DNA fragmentation and standard semen parameters in algerian infertile male partners. *World J Mens Health*. 2015;33:1-7.

Figure 1. Enrollment of patients administered placebo or DHA to improve sperm quality and functionality. Most of the initial patients were excluded because they did not meet entry criteria. No patients were lost to follow-up, and no patients discontinued the intervention. The outcome data for all patients who were randomized were included in the final data analysis.

Table 1. Main results of the study. Characteristics of the seminal samples and sperm functions obtained at the beginning, after 1 months and after 3 months of the dietary supplementation with DHA or placebo.

		After 1		After 3				
	Parameter (unit)	Basal	month	months	Difference 1-0 (95% CI)	p	Difference 3-0 (95% CI)	p
Volume (ml)	Placebo	2,9	2,7	2,6	-0,2 (-0,7 to 0,3)	0,44	-0,3 (-0,9 to 0,2)	0,22
	0,5 g	2,9	2,7	3,3	-0,2 (-0,6 to 0,2)	0,23	0,4 (-0,3 to 1,1)	0,22
	1 g	2,7	2,8	2,8	0,1 (-0,5 to 0,6)	0,77	0,2 (-0,5 to 0,8)	0,64
	2 g	3,2	3,3	3,2	0,1 (-0,4 to 0,7)	0,6	0,0 (-0,4 to 0,5)	0,86
Concentration (10 ⁶ cells/ml)	Placebo	34,0	31,2	33,5	-2,8 (-12,3 to 6,6)	0,52	-0,5 (-9,5 to 8,5)	0,9
	0,5 g	24,6	26,5	27,5	1,9 (-7,3 to 11,1)	0,67	2,9 (-1,6 to 7,4)	0,19
	1 g	23,5	25,1	27,1	1,6 (-8,1 to 11,2)	0,73	3,5 (-2,1 to 9,2)	0,2
	2 g	29	28,6	29,1	-0,4 (-7,9 to 7,2)	0,92	0,1 (-8,4 to 8,7)	0,98
Total Sperm count (10 ⁶ cells)	Placebo	100,5	80,8	74,3	19,7 (-58,6 to 19,2)	0,3	-26,2 (-65,3 to 12,8)	0,17
	0,5 g	74,9	69,9	93	-8,0 (-40 to 24)	0,6	18,1 (-3,1 to 39,4)	0,09
	1 g	62,1	66,7	71,3	4,6 (-20 to 29,2)	0,69	9,3 (-8,3 to 26,9)	0,28
	2 g	85,2	89,2	96,6	4,0 (-12,8 to 20,8)	0,62	11,4 (-14,5 to 37,3)	0,36

Progressive motility (%)	Placebo	31,7	33,9	31,7	2,2 (-4,7 to 9,1)	0,51	4,8 (-0,4 to 10,0)	0,07
	0,5 g	31,7	36,7	39,2	5 (-0,3 to 10,2)	0,06	7,5 (0,8 to 14,2)	0,03*
	1 g	28	36,7	36	8,7 (4,0 to 13,5)	0,002*	8,0 (0,6 to 15,4)	0,04*
	2 g	31,9	40,7	41,6	8,7 (1,7 to 15,7)	0,02*	9,7 (-0,5 to 19,8)	0,06*
Non progressive motility (%)	Placebo	13,5	8,5	8,1	-4,9 (16,4 to 6,6)	0,37	-5,3(-18,0 to 7,4)	0,38
	0,5 g	8,5	7,7	7,3	0,9 (-4,4 to 2,7)	0,61	-1,2 (-4,8 to 2,4)	0,48
	1 g	16,2	9,2	9	-7,0 (-14,1 to 0,1)	0,05*	-7,2 (-13,4 to -1,0)	0,03*
	2 g	9,4	7,1	8,1	-2,3 (-5,4 to 0,7)	0,12	-1,3 (-5,6 to 2,9)	0,05*
Immotility (%)	Placebo	60,1	57,5	55,3	-2,6 (-9,4 to 4,2)	0,43	-4,8 (-11,3 to 1,7)	0,14
	0,5 g	59,8	51,3	53,5	-8,5 (-20,5 to 3,6)	0,15	-6,3 (-15,4 to 2,7)	0,16
	1 g	55,8	54,1	55	-1,7 (-10,4 to 6,9)	0,67	-0,8 (-8,0 to 6,4)	0,82
	2 g	58,7	52,3	50,3	-6,4 (-14,4 to 1,6)	0,11	-8,3 (-18,3 to 1,6)	0,09
Morphology (%)	Placebo	1,0	3,5	3,6	2,5 (0,5 to 4,4)	0,02	2,6 (1,4 to 3,8)	<0,001*
	0,5 g	1,4	2,3	3,5	0,9 (-0,1 to 1,8)	0,07	2,1 (0,8 to 3,4)	0,003
	1 g	1,3	3	3,6	1,7 (0,9 to 2,4)	<0,001	2,3 (1,5 to 3,0)	<0,001*
	2 g	1,1	3,2	3,4	2,1 (1,0 to 3,2)	0,001	2,3 (1,1 to 3,5)	0,001

Fragmentation (%)		Placebo	4,8	5,2	9,5	0,7 (-1,2 to 2,6)	0,45	4,7 (-1,0 to 10,4)	0,1
MMP (%)	0,5 g		12,7	11,8	8,6	-0,2(-7,0 to 6,7)	0,96	-4,1 (-10,9 to 2,6)	0,21
	1 g		8,6	10,4	7,8	1,8 (-4,4 to 8,0)	0,54	-0,8 (-5,0 to 3,4)	0,7
	2 g		6,7	5,8	6,2	-0,9 (-4,5 to 2,8)	0,62	-0,5 (-5,7 to 4,6)	0,83
	Placebo		26,3	27,9	34,8	1,6 (-11,5 to 14,7)	0,8	8,5 (-4,6 to 21,6)	0,18
	0,5 g		20,1	27,5	16,9	7,5 (-3,6 to 18,7)	0,17	-3,1 (-13,1 to 6,8)	0,51
	1 g		31,5	28,5	29	-3,0 (-10,7 to 4,8)	0,43	-2,5 (-11,1 to 6,2)	0,55
	2 g		35,1	33,6	24	-1,5 (-18,8 to 15,9)	0,86	-11,0 (-29,2,3 to 7,2)	0,21
	Placebo		29,5	33,7	32,8	4,2 (-5,3 to 13,6)	0,36	3,3 (-2,7 to 9,2)	0,26
	0,5 g		34,3	35,5	31,8	1,2 (-5,8 to 8,1)	0,72	-2,5 (-9,7 to 4,8)	0,48
Apoptosis (%)	1 g		34,8	34,7	38,1	-0,1 (-8,6 to 8,5)	0,99	3,3 (-5,1 to 11,7)	0,41
	2 g		25,8	31	34,5	5,2 (-0,9 to 11,3)	0,09	8,8 (1,7 to 15,8)	0,02
	Placebo		70,1	68,4	68,7	-1,8 (-15,7 to 12,2)	0,79	-1,4 (-18,3 to 15,5)	0,86
	0,5 g		41,9	53,7	48,4	11,8 (1,0 to 22,6)	0,03*	6,4 (-7,6 to 20,5)	0,34
	1 g		52,7	58,8	64,3	6,1(-6,3 to 18,6)	0,31	11,6 (-3,3 to 26,6)	0,12
	2 g		46,2	61,8	59,2	15,7 (0,3 to 31,0)	0,05*	13,0 (-9,5 to 35,5)	0,23
	Placebo								
	0,5 g								
	1 g								
	2 g								
ROS (%)		Placebo	70,1	68,4	68,7	-1,8 (-15,7 to 12,2)	0,79	-1,4 (-18,3 to 15,5)	0,86
ROS (%)	0,5 g		41,9	53,7	48,4	11,8 (1,0 to 22,6)	0,03*	6,4 (-7,6 to 20,5)	0,34
	1 g		52,7	58,8	64,3	6,1(-6,3 to 18,6)	0,31	11,6 (-3,3 to 26,6)	0,12
	2 g		46,2	61,8	59,2	15,7 (0,3 to 31,0)	0,05*	13,0 (-9,5 to 35,5)	0,23
	Placebo								
	0,5 g								
	1 g								
	2 g								
	Placebo								
	0,5 g								

Lipid peroxidation (%)	Placebo	3,2	3,6	3,3	0,4 (-0,3 to 1,1)	0,23	0,0 (-0,6 to 0,7)	0,98
	0,5 g	3,3	3,1	2,9	-0,2 (-0,6 to 0,2)	0,25	-0,4 (-1,0 to 0,3)	0,27
	1 g	3,7	3,4	3,1	-0,3 (-0,7 to 0,1)	0,1	-0,6 (-1,4 to 0,2)	0,13
	2 g	3,5	3,2	3,1	-0,3 (-0,9 to 0,3)	0,34	-0,4 (-1,5 to 0,7)	0,44

* Significant difference.

MMP: Mitochondrial membrane Potential

ROS: Reactive Oxygen Species

Table 2. Effect on motility. Effect of dietary supplementation with different doses of DHA on sperm progressive motility comparing asthenozoospermic patients from those who are not.

	Progressive motility (%)	n	0	1	3	Difference 1-0 (95% CI)	p(1-0)	Difference 3-0 (95% CI)	p
Normal patients	Placebo	9	42,3	40,7	48,4	-1,7 (-11,7 to 8,3)	0,72	6,1 (-2,1 to 14,3)	0,13
	0,5 g	6	47,7	50,3	52,3	2,7 (-1,9 to 7,2)	0,19	4,7 (-7,0 to 16,4)	0,35
	1 g	8	39,3	49,6	42,3	10,4 (4,1 to 16,7)	0,006*	3,0 (-7,6 to 13,6)	0,52
	2 g	7	46	49,7	46,1	3,7 (-7,6 to 15)	0,45	0,1 (-15,2 to 15,4)	0,98
	0,5 - 2g	21	43,9	49,9	46,4	6,0 (1,8 to 10,1)	0,007*	2,5 (-3,5 to 8,5)	0,39
Asthenozoospermic	Placebo	6	14,3	19,7	14,5	5,3 (-1,9 to 12,6)	0,12	0,2 (-4,4 to 4,7)	0,93
patients	0,5 g	9	21	27,6	30,4	6,6 (-2,5 to 15,7)	0,14	9,4 (-0,6 to 19,5)	0,06
	1 g	7	15,1	22	28,9	6,9 (-2,4 to 16,1)	0,12	13,7 (2,1 to 25,4)	0,03*
	2 g	8	19,6	32,8	37,6	13,1(3,1 to 23,1)	0,02*	18,0(4,3 to31,7)	0,02*
	0,5 - 2g	24	18,8	27,2	32,4	8,3 (3,4 to 13,3)	0,002*	13,5 (7,6 to 19,5)	<0,001*

* Significant difference. n: sample size.

Table 3. Effect on ROS. Effect of dietary supplementation with different doses of DHA on ROS comparing asthenozoospermic patients from those who are not.

	ROS (%)	n	months			p(1-0)	Difference 3 -0 (95% CI)	p
			0	1	3			
Normal patients	Placebo	8	69,0	68,1	72,1	0,94	3,2 (-26,6 to 32,9)	0,81
	0,5 g	6	45,3	60,7	45,8	0,13	0,4 (-26,7 to 27,6)	0,97
	1 g	8	60	67,3	67,1	0,14	7,1 (-12,9 to 27,2)	0,43
	2 g	6	54,8	73,3	54,8	0,16	0,6 (-40,7 to 41,9)	0,97
	0,5 - 2g	20	54	67,1	57,2	0,01*	3,2 (-10,1 to 16,5)	0,62
Asthenozoospermic patients	Placebo	6	71,7	68,7	64,2	0,67	-7,5 (-27,8 to 12,8)	0,39
	0,5 g	8	39,4	48,5	50,3	0,19	11,0 (-8,9 to 30,8)	0,23
	1 g	6	43	47,5	60,6	0,74	17,6 (-13,3 to 48,5)	0,2
	2 g	6	37,6	50,3	63	0,23	25,4 (-5,0 to 55,8)	0,08
	0,5 - 2g	20	39,9	48,8	57,2	0,1	17,3 (5,0 to 29,6)	0,01*

* Significant difference. n: sample size.