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Original article

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

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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 recruited in a private 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 slight benefit was 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.

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 defense 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 (i.e., 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

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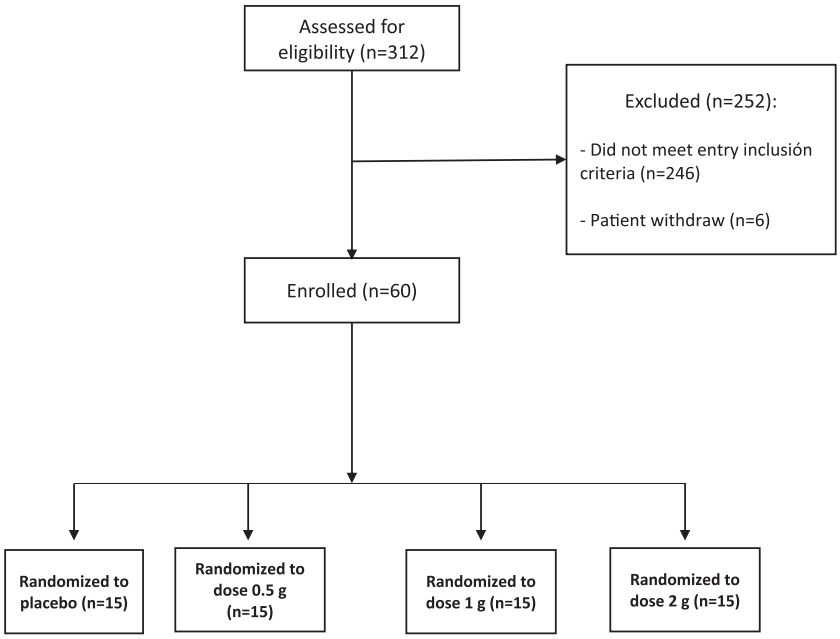

Fig. 1. Enrollment of patients administered placebo or DHA to improve sperm quality and functionality. Most of the initial patients were excluded because they did not meet entry criteria. No patients were lost to follow-up, and no patients discontinued the intervention. The outcome data for all patients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

asthenozoospermia. DHA benefits go beyond sperm quality and integrity, as it also increases the production of endogenous antioxidants (like gluthatione 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 (e.g., vitamin C, vitamin E, gluthatione, 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 defense 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 on sperm quality. To this end, we have analyzed the effect of different doses of DHA in comparison to placebo on a population of infertile men, regarding macroscopic, microscopic and molecular characteristics of the seminal sample.

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%; and (c) normal sperm morphology of less than 2% [16]. The sperm count needed to be at least 10 million per mL in order to apply the molecular and functional tests of this study. Motility limit was set at 60% to select a patient population that could particularly benefit from antioxidant treatment and to assess to what extent a patient with a fair sample –with indication for IVF– could be a candidate for IUI after 3 months of DHA treatment. Morphology limit was set at 2% in order to assess if any improvement could be obtained in patients with moderate to severe teratozoospermia after DHA treatment.

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). These quantities (0.5 g, 1 g and 2 g) represent daily doses. The randomization list was generated using Randomization.com [http://www.randomization.com] with randomly permuted blocks of 60 subjects randomized into four blocks. The list was kept in a locked drawer in the administration office, to which the clinical staff who enrolled the participants in the study had no access; group allocation was requested by telephone. Physicians and patients were blinded to the assigned study intervention.

In this study, we have used NuaDHA®, a highly concentrated DHA-TG formula (that is, a triglyceride; with a minimal 90% of omega-3) 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 of heavy metals (mercury, cadmium, lead and arsenic). It is almost free of polychlorinated biphenyls, furans or dioxins. Furthermore, it is not oxidized 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.

Regarding placebo group, primrose oil was considered an appropriate candidate to be administered to this group. Primrose oil has a fatty acid profile similar to sunflower oil, which has been traditionally used as a placebo in many studies. Both primrose oil and sunflower oil are vegetable oils, have a low saturated fat content and have a high polyunsaturated fat content with linoleic acid as its main component. The amount of primrose oil taken by the participants was of 0.5 g a day. This dose is very small and can only be considered as a nutritional dose of a vegetable oil.

The number of patients assessed for eligibility, study enrollment and dose allocation is shown in Fig. 1.

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.

	Parameter (unit)	Basal	After 1 month	After 3 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
	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
MMP (%)	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 (−29.3 to 7.2)	0.21
Apoptosis (%)	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
	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)	0.2
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
	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
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

MMP: Mitochondrial membrane Potential.
ROS: Reactive Oxygen Species.
* Significant difference.

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 μL of semen were loaded in a Makler Counting Chamber (Sefi Medical Instruments, Haifa, Israel) and total sperm count (x10^6 /mL) and motility percentage were measured. Samples were also examined for volume and morphology. A minimum of 200 cells were counted per 5 μL 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% CO_2, according to the manufacturer instructions. Cumene hydroperoxide was used as a positive control of lipid peroxidation, H_2O_2 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 stablished 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 and concentration of the samples were not affected by DHA treatment or placebo administration. In contrast, there was a significant increase in the percentage of normal morphology in the placebo group and the DHA 0.5 g group after 3 months of treatment and in the DHA 1 g and DHA 2 g groups after 1 and 3 months of treatment. Sperm motility was significantly affected in the DHA 0.5 g after 3 months of treatment. It is also significantly affected in the DHA 1 g group after the first month of treatment and maintained during the 3 months of the study without significant variations. In the case of the DHA 2 g group, the progressive motility is significantly increased after the first month of treatment. After three months of treatment, there is an increase in motility which is almost significant ($p = 0.06$). 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 analyzed 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 the percentage of reactive oxygen species (ROS). There was a significant increase in ROS percentage in those men who took 0.5 g DHA and 2 g DHA daily. However, the increase was only significant after one month of treatment, while the increase was not significant after three months of DHA intake. When analyzing the effect on ROS, after differentiating asthenozoospermic patients from men with motility within the normal range, it was found an increase in ROS percentage in both groups, appearing sooner in patients with normal progressive motility (after 1 month of DHA treatment), and requiring a 3 months DHA treatment period in asthenozoospermic patients. Unfortunately, it was not possible to define at which dose the major effect was obtained since all DHA doses were unified in a sole group (0.5 g – 2 g) (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. 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. We have also observed a significant increase in the percentage of normal morphology in all study groups, but we have no explanation for the manifestation of this improvement in morphology which also occured in the placebo group. In any case, all values remain under 4% so no group has normal morphology according to WHO 2010 criteria. 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

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.

			Months			Mild effect		No effect	
	Progressive motility (%)	n	0	1	3	Difference 1-0 (95% CI)	p(1-0)	Difference 3-0 (95% CI)	p(3-0)
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 – 2 g	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 patients	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
	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 to 31.7)	0.02*
	0.5 – 2 g	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.

			months						
	ROS (%)	n	0	1	3	Difference 1-0 (95% CI)	p(1-0)	Difference 3-0 (95% CI)	p
Normal patients	Placebo	8	69	68.1	72.1	−0.8 (−25.9 to 24.2)	0,94	3.2 (−26.6 to 32.9)	0,81
	0.5 g	6	45.3	60.7	45.8	15.3 (−6.6 to 37.3)	0,13	0.4 (−26.7 to 27.6)	0,97
	1 g	8	60	67.3	67.1	7.4 (−3.2 to 18.0)	0,14	7.1 (−12.9 to 27.2)	0,43
	2 g	6	54.8	73.3	54.8	18.6 (−9.9 to 47.1)	0,16	0.6 (−40.7 to 41.9)	0,97
	0.5 – 2 g	20	54	67.1	57.2	13.1 (3.8 to 22.4)	001*	3.2 (−10.1 to 16.5)	0,62
Asthenozoospermic patients	Placebo	6	71.7	68.7	64.2	−3.0 (−19.8 to 13.8)	0,67	−7.5 (−27.8 to 12.8)	0,39
	0.5 g	8	39.4	48.5	50.3	9.2 (−5.6 to 23.9)	0,19	11.0 (−8.9 to 30.8)	0,23
	1 g	6	43	47.5	60.6	4.5 (−28.4 to 37.4)	0,74	17.6 (−13.3 to 48.5)	0,2
	2 g	6	37.6	50.3	63	12.8 (−11.2 to 36.7)	0,23	25.4 (−5.0 to 55.8)	0,08
	0.5 – 2 g	20	39.9	48.8	57.2	8.8 (−1.8 to 19.5)	0,1	17.3 (5.0 to 29.6)	001*

0.5 – 2 g group: this group includes all DHA doses (groups DHA 0.5 g, DHA 1 g and DHA 2 g).
* Significant difference. n: sample size.

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 analysis 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 therapeutic approach [5,18]. In addition, it is of special interest the fact that DHA supplements 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 midpiece, 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 signaling 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 fertilization 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 analyzing 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 defenses, 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 did not have a detrimental effect on the sperm, as there was no increase in peroxidation products upon treatment.

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

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

Limitations are present in this study. We are aware that semen analysis is a test with variable results and some of the changes observed could be due to the physiologic variance in time inherent to sperm samples. Furthermore, the duration of the treatment with DHA is limited to three months. It could be interesting to perform new studies to analyze how sperm characteristics evolve after stopping DHA administration and check whether outcome variables return to their initial values. The sample size is also limited (n = 60) and additional studies might be necessary to confirm this data or to perform a meta-analysis.

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

A novel outcome of the present study was a clear indication of DHA supplementation for patients with asthenozoospermia, suggesting that dietary DHA supplementation at 1 g/day would be 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.

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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Declarations of interest

None.

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