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Original article

Improving sperm quality and spermatogenesis through a bioactive marine compound: an experimental study

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Abstract. Dietary lipids may affect sperm membrane structure, fluidity and its susceptibility to oxidative phenomena which may lead to altered sperm viability and proper binding to eggs. Given the recently demonstrated beneficial effects of fish oil diets on turkey fertility and embryo viability, the aim of this study was to test a caviar-derived marine product on spermatogenesis and sperm quality. Sixty mice were divided into four different groups and fed for 3 weeks with normal chow (group A), added with LD-1227 at the dosage of either 5 mg/day (B1) or 10 mg/day (B2) while Group C received standard chow added with 10 mg of a DHA-rich mixture. At sacrifice tests/body weight ration and spermatogenesis was checked. No toxicity, histological sign or body or testes growth abnormality was noted, irrespective of the treatment. As compared to control, all supplements showed to increase sperm counting and motility although the effect of LD-1227 10 mg was significantly higher than DHA alone ($p<0.05$). Viability was improved by DHA ($p<0.05$) but not by low LD-1227 dosage while higher dosage performed better than DHA ($p<0.05$). Morphology was unaffected by any of the employed supplements. Taken altogether, these data suggest that LD-1227 has a remarkable effect on quali-quantitative parameters of spermiogenesis, some of them being more effective than high dosage DHA. These findings may prove to be of interest in clinical practice. (www.actabiomedica.it)

Key words: LD-1227, sturgeon estract, spermatogenesis, sperm count, sperm morphology

Introduction

Approximately 15 percent of couples are infertile and more than 90% of male infertility cases are known to be due to low sperm quality, as a most recent large survey reports (1). Besides overt cases of health problems such as chronic disease, lack of nutrition or genetic abnormality, in 30-40% cases of sperm abnormality and consequent fertility failure, the main cause remains unexplained as yet. Stress and its related biochemical counterpart, subtle micronutrients deficiency and environmental xenobiotic interference are further areas still partly investigated. Moreover, it has already been suggested that in countries with low and falling fertility rates among young women should consider the possibility that semen quality of their younger male cohorts may also have deteriorated (2). Various modalities have been used to protect spermatozoa against the free radical-induced injury through dietary provision of essential antioxidant components such as vitamin C, vitamin E and carotenoids but also through nutraceuticals. However, such intervention seems to affect the overall systemic redox system while, more specifically, Surai et al. (3) has hypothesized that dietary lipids abnormalities may affect sperm membrane structure, fluidity, or sperm suscep-

tibility to peroxidation by changing specific phospholipids, fatty acids and/or n-6:n-3 ratios. One or more of these modifications may effectively modify fertility rates via affecting sperm viability or binding to the egg. For example, dietary n-3 polyunsaturated fatty acids (PUFA) are successfully transferred to spermatozoa of chicken fed fish oil (4). In addition, a significant increase of n-3 fatty acids and lower n-6:n-3 ratio in sperm with resulting improved viability has been described in turkeys when fed a fish diet (5) while, as compared with corn oil, dietary salmon oil similarly lowers n-6:n-3 ratio and improves fertility in roosters (6). The beneficial effects of fish oil diets on turkey fertility and embryo viability has also been shown to increase with age (7). More recent studies in bigger mammals have confirmed that long-term dietary supplementation of fish oil, regardless of the EPA/DHA ratio is able to beneficially modify the fatty acid compositions in testis (8). Given the uniqueness of the composition of sturgeon eggs as compared to other marine species as for PUFA and phospholipid moieties and in consideration of our recent work proving the efficacy of a controlled caviar-derived homogenate (LD-1227, Caviarlieri, LabDom, Switzerland) as an antioxidant/anti-inflammatory modulator in the brain, a typical lipidic mielieu, we tested this compound on spermatogenesis and sperm quality

Materials and Methods

Study Design

Animals were acclimatized for 14 days under normal conditions with 12 h daylight and 12 h darkness, with free access to a balanced diet food pellets (28% crude protein, 14% crude fat) and water. A total of 60 mice were selected and randomly divided into three different groups with two subgroups, of fifteen in each set fed for 21 days as follows. Animals of Group A (controls) were force fed with distilled water. Animals of Group B received LD-1227 at the dosage of either 5 mg/day (B1) or 10 mg/day (B2) while Group C received standard chow added with 10 mg of a DHA-rich mixture. The ingested food was regularly checked on a daily basis and mice failing to ingest the required food allowance within 2 days were taken out of the study if not totally consumed within the next day. Moreover, mice were weighed individually everyday and before the test animals to be sacrificed at the end of the study. All procedures regarding handling of the test animals were in accordance with the existing guidelines of the for good care and use of laboratory animals (Guide for the care and use of laboratory animals, NIH, The National Academies Press, 2011).

Testes to body weight ratio, kidney and liver examination

Under light ether anesthesia testes were removed from the animals. Testes were immediately cleaned of superficial fatty layer, weighed and then transferred into 10% formalin solution. The testes-body weight ratio was determined according to the formula described by Yakubu et al. i.e. Organ:BW ratio = weight of testes/weight of the animal (9). Liver and kidney were also removed and weighed. All these tissues were fixed in a Bouin's solution for 24 hours, washed in running tap water, dehydrated through graded concentration of ethanol, embedded in paraffin wax, sectioned at 5 m thicknesses and stained with Hematoxylin and Eosin (H&E).

Sperm quali-quantitative assessment: counts, morphology, viability, motility and membrane integrity

The cauda epididymis was finely minced by anatomical scissors in 5 ml of Bigger-Whitten - Whittingham medium in a petri dish prior to incubation with 5% CO2 for 30 min at 37°C to allow the migration of spermatozoa to the medium. Afterwards, samples were homogenated in 1 mL cold Phosphate Buffer Glucose Saline (PBGS: NaCl, 50 mM/L; Na_2HPO_4, 200 mM/L; glucose, 20 mM/L; KH_2PO_4, 26 mM/L) and stored at 4°C for 24 h to allow sperm to be cleared from the walls. Sperm count was done strictly according to the recommended protocol of the 1999 WHO manual. Sperm number was estimated using Neubauer counting chamber by counting the number of cells in the five fields of 1 mm squares in 10^1 dilution (10 μL sperm suspension in 1 mL PBGS) under the light microscope and computed as concentration/mL = dilution factor × count in 5 squares × 0.05 × 10^6. Sperm

viability and morphology was evaluated according to the procedure of Bjorndahl et al. by staining 10 μL of epididymal sperm suspension with eosin counter stained with nigrosin smeared on a glass slide and dried overnight at room temperature (11). They were then examined under the micro-scope at 400 X magnification. Heads of the spermatozoa which showed red coloration indicated non-viable cells. To test sperm motility, the sperm cell suspensions were then observed using an inverted microscope to record sperm motility. Sperm motility was assessed using the WHO (1999) classification system, with only the three grades (a, b and c) reported; rapid forward progression, medium forward progression and slow forward progression. Each sample was assessed twice. It was then expressed as percentage of motile cells moving in any direction and at any speed. The experimental error was ascertained to be within 5%. For consistency, all readings were carried out at 37°C (WHO, 1999). Sperm with intact membrane do not undergo staining, which shows the integrity of cell membrane (10). Thus, it was calculated the percentage of alive (unstained) and dead (stained) cells over at least counting 200 cells.

Statistical analysis

All data were subjected to a one-way analysis of variance (ANOVA) to determine the level of significance between control and the treated groups. A difference was considered significant at $p<0.05$. Data are reported here as mean ± standard deviation (±SD).

Results

None of the animals died from the time of acclimatization until the end of the experiment, nor showed any overt signs of stress behaviour. Furthermore, animals did not exhibit any sign of toxicity, such as weight loss, poor grooming, irrespective of the supplementation given. As compared to control, no histological changes appeared in supplemented rats, whatever the scheduled dosage employed either in kidney and liver (data not shown) and in testes (Fig. 1). There was also a significant increase in the average daily body weight till the end of the study in all groups at a comparable extent and irrespective of the supplementation employed (data not shown).

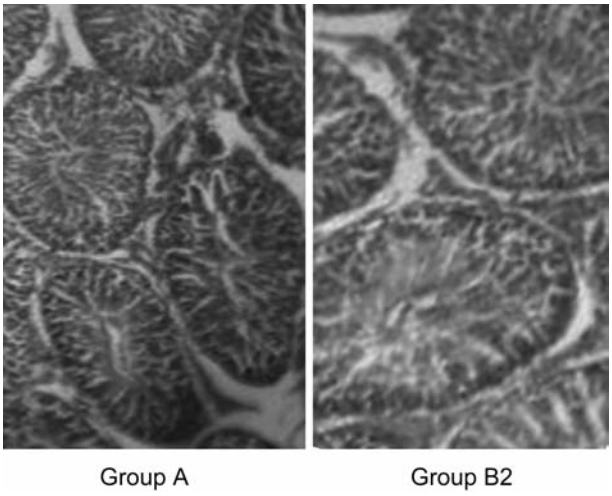

Figure 1. Testes histology in control and in high-dosage LD-1227. H & E staining (400 X magnification). No difference appeared among the groups

Organ (testes and liver) weight

Analysis of the liver to body weight ratio showed that there was no significant difference between the control and the experimental groups. In particular, administration of 5 mg to 10 mg of LD-1227 or DHA did not show any significant change either in the weight of the testes and of the liver (Tab. 1). The same applied when considering both right and left kidney to body weight ratios separately (data not shown). Histological analysis however was not performed. Moreover, whatever supplementation employed, no statistically significant difference was noted as for testes and separate cauda epididymis to body weight ratio (Tab. 2).

Table 1. Kidney and liver weight: effect of supplementation with DHA and LD-1227

Group	Kidney (g)	Liver (g)
Control	0.21±0.04	1.82±0.18
5 mg LD-1227	0.23±0.02	1.86±0.21
10 mg LD-1227	0.22±0.01	1.89±0.10
10 mg DHA	0.22±0.02	1.88±0.13

No statistical difference appeared among the groups

Table 2. Testes to body weight ratio: effect of supplementation with DHA and LD-1227

Group	Cauda epididymis		Testes
	Right	Left	
Control	0.12±0.01	0.12±0.01	0.62±0.08
5 mg LD-1227	0.11±0.01	0.11±0.02	0.64±0.01
10 mg LD-1227	0.12±0.02	0.12±0.01	0.63±0.06
10 mg DHA	0.11±0.02	0.12±0.01	0.62±0.02

No statistical difference appeared among the groups

Sperm morphology, counting and quality assessment

Eosin and nigrosin cell viability staining revealed no significant difference between the number of living and dead cells at the time of eosin infiltration with 100% living cells observed in 200 cells counted. There were no specific aberrations such as curled distal part of the flagella nor observable signs of aberration in both right and left cauda epididymis as compared to control, whatever the supplement and the dosage employed (data not shown).

Table 3 shows the sperm count in the left and right cauda epididymis. Unlike DHA, sperm count in the left and right cauda epididymis was significantly affected by LD-1227. Indeed, rats administered with the lowest dose in both right and left epididymis showed a higher mean as compared to the control ($p<0.05$) and this effect was significantly more pronounced in the group supplemented with highest LD-1227 dose ($p<0.05$ vs all the other groups).

When examining the sperm count/testes weight ratio, it appeared the only the higher dosage of LD-1227 yielded a statistically significant increased value ($p<0.01$ vs control) and this was even higher than what obtained with high dosage DHA ($p<0.05$, table 4). The right and left cauda epididymis of the experimental group revealed comparable ratios.

Table 4. Sperm morphology and quality assessment: effect of supplementation with DHA and LD-1227

Group	Sperm Morphology (%)	Sperm Viability (%)
Control	85±0.1	0.82±0.09
5 mg LD-1227	81±0.08	0.83±0.14
10 mg LD-1227	94±0.08	0.89±0.09**
10 mg DHA	83±0.06	0.85±0.13*

*$p<0.05$ vs control; **$p<0.01$ vs DHA and vs control

The effects of DHA and of different doses of LD-1227 on sperm viability and morphology are presented in table 4. Sperm morphology remained normal in all the groups of animals, no matter the treatment. Differently from low dosage of LD-1227, high dosage DHA significantly improved sperm viability ($p<0.05$ vs control and vs LD-1227 5 mg). However, higher dosage supplementation performed even better ($p<0.05$ vs DHA).

Discussion

A considerable decline in organ and body weights correlates to the impairment of reproductive functions, irrespective of aging factor. Given that some natural compounds may have antispermatogenic effects with functional and morphological defects in human sperm (11, 12), in the present study it was noteworthy to observe that the ratio of reproductive organ weight to body weight remained similar with the control and the oral treatment of LD-1227 showed a remarkably safe profile. As a comparative interventional compound, we used high dosage DHA. This is because it has been shown that failure of spermatogenesis may be at least in part due to lack of DHA and n6-docosapentaenoic acid (DPAn6) for structural com-

Table 3. Sperm counting and sperm count/testes weight ratio: effect of supplementation with DHA and LD-1227

Group	Sperm count (x106)		Sperm count/testes wt ratio	
	Right	Left	Right	Left
Control	16.6±2.8	19.6±2.8	19.2±5.1	20.1±2.1
5 mg LD-1227	21.9±2.3*	22.9±2.3*	21.1±3.5	22.6±4.2
10 mg LD-1227	36.2±3.5**	38.2±3.5**	24.2±3.0**,§	25.4±6.3**,§
10 mg DHA	17.2±2.6	18.2±2.6	22.4±4.6**	23.4±3.0

*$p<0.05$ vs control; **$p<0.01$ vs control; §$p<0.05$ vs DHA

ponents of the flagellar membrane phospholipids and most of the DHA is indeed present in that part (13). As a matter of fact, delta-6 desaturase-null male mice which are unable to synthesize highly unsaturated fatty acids, arachidonic acid, DHA and DPAn6 exhibit infertility and arrest of spermatogenesis at late spermiogenesis. In this setting, 0.2% DHA was capable of restoring all observed impairment in male reproduction (14). On the other hand, 22:4n6 fatty acids formed from dietary source was shown by these authors to be acting as an inferior alternative for DHA being less effective in restoring fertility, sperm count, and spermiogenesis. On the other hand, a recent study showed that although it modified the fatty acid composition of sperm, supplementation of boars with dietary fish oils, rich in long chain n-3 fatty acids, did not substantially influence semen production or quality (15). In our present study we showed that high dosage LD-1227 performed significantly better than DHA not only as for sperm count but, most important, as for sperm vitality. This is of particular relevance, since semen quality has been regarded to as a fundamental biomarker of overall male health, according to what reported by Jensen et al. (16) who conducted the very large Copenhagen Sperm Analysis Laboratory study. In a different setting, we have proved that this compound exerted a more significant biological activity than DHA (17). Indeed, although different dietary regimens with either corn oil, fish oil or flax seed did not affect membrane phospholipid ratios in sperm membrane, they did modified major fatty acids within certain phospholipids (18). These authors have thus shown that diets containing distinct lipid sources differently change the lipid contents of sperm head and sperm body membranes. In the present study we did not address the issue of testing the percentage of individual fatty acids (C14:0 to C24:1) in total fatty acids as well as the percentage of the whole fatty acid categories subsets. Nonetheless, the present employed sturgeon-derived bioactive compound is expected to contain, besides DHA also a rich array of lipoproteic moieties. and these further components and/or their inner synergy might account for the observed difference. Further tests, however, should be conducted to confirm the specific mechanisms of action of LD-1227 on spermatogenesis such as histological analysis to determine the effects of this marine compound on epithelialization of spermatogonial cells and lumen diameter. The absence of substantial sperm morphology changes under supplementation although yielding a significant viability improvement are somewhat complex to explain. Most of studies in this setting have been carried out in abnormal situations rather than in healthy samples. A most recent study performed in healthy students and comparing a number of sperm parameters with antioxidant-rich diet, found an association with motility changes rather than with morphology (19). Nonetheless, the issue is still prone to some debate. Finally, infertile mice with idiopathic oligoasthenoteratozoospermia could represent a further worthwhile application trial.

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