

ORIGINAL ARTICLE

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Dietary fatty acids influence sperm quality and function

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SUMMARY

Recently, obesity has been linked to male infertility. In animal models the administration of a high-fat diet caused a reduction in sperm quality, by impairing gamete energy metabolism. The aim of this study was to investigate a possible effect of dietary fatty acids supplementation in the modulation of sperm energy metabolism and, in turn, in the improvement of sperm quality in rats fed a high-fat diet. Sexually mature male Sprague–Dawley rats were divided into four groups and fed for 4 weeks a standard diet (control group), a high-fat diet (enriched in 35% of fat and 15% sucrose), a high-fat diet supplemented with 2.5% olive oil (a source of monounsaturated fatty acids) or a high-fat diet supplemented with 2.5% krill oil (a source of n-3 polyunsaturated fatty acids). Liver and adipose tissue weight, plasma glucose, insulin and lipid concentrations were determined. Activities of enzymes involved in sperm energetic metabolism were evaluated by spectrophotometric assays. Sperm mitochondrial respiratory efficiency was also assayed. The obtained results suggest that olive oil partially counteracts the negative effects of a high-fat diet on sperm quality, by increasing gamete motility, by reducing oxidative stress and slightly improving mitochondrial respiration efficiency. On the other hand, krill oil determines an increase in sperm concentration and motility, an increase in the activities of lactate dehydrogenase, Krebs cycle enzymes and respiratory chain complexes; a parallel increase in the cellular levels of ATP and a reduction in oxidative damage were also observed. These results suggest that dietary fatty acids are able to positively influence sperm quality and function.

INTRODUCTION

In the Western World, obesity is often associated to other diseases, such as insulin resistance, abnormal blood lipid levels, hypertension and liver alterations (Ferramosca *et al.*, 2014; Jahangir *et al.*, 2014; Asrih & Jornayvaz, 2015; Yoo & Choi, 2015). In the last years, growing evidence has linked abdominal obesity, insulin resistance and dyslipidemia to male infertility (Kasturi *et al.*, 2008; Morrison & Brannigan, 2015). Several studies suggest that alterations in lipid metabolism may affect semen quality and fertility (Shalaby *et al.*, 2004; Saez Lancellotti *et al.*, 2010; Bobjer *et al.*, 2012; Hagiuda *et al.*, 2014). In this context, the model of diet-induced obesity in rats has been recently used to investigate sperm dysfunction (Ferramosca *et al.*, 2016). In particular, rats fed a diet with a high amount (35%) of fat, containing also 15% sucrose, showed defects in sperm energy metabolism, regarding both glycolytic and mitochondrial reactions capable of generating ATP.

It is known from the literature that fatty acids are able to modulate lipid metabolism by complex mechanisms involving a sequence of interdependent and cross-regulated molecular events (Ferramosca & Zara, 2014a,b). With regards to this aspect, several authors demonstrated that monounsaturated fatty acids (MUFA) increase lipid oxidation and decrease insulin resistance (Soriguer *et al.*, 2006; Ferramosca *et al.*, 2008a; Assy *et al.*, 2009). In these studies MUFA are essentially supplied in the form of olive oil (OO), a basic component of the Mediterranean diet, which mainly contains oleic acid, a MUFA of the n-9 series.

On the other hand, dietary polyunsaturated fatty acids (PUFA) of the n-3 and n-6 series are potent inhibitors of hepatic fatty acid synthesis (Ferramosca & Zara, 2014a,b). However, some studies performed in rodent models suggested that not all PUFA exert the same effect. In particular n-3 PUFA, such as eicosapentaenoic acid (EPA, 20 : 5) and docosahexanoic acid (DHA, 22 : 6), are known to have a variety of health benefits against

cardiovascular diseases, insulin resistance and fatty liver (Siriwardhana *et al.*, 2012). Among the source of these n-3 PUFA, krill oil (KO) is a novel dietary supplement, which has become increasingly popular as a food supplement during the last decade. In fact, the ratio of EPA to DHA present in KO is higher than that present in fish oil, the most common source of n-3 PUFA (Burri *et al.*, 2012). It has been proposed that this peculiar characteristic of KO composition in fatty acids, along with a high amount of phospholipids, renders this oil more efficient in the modulation of activity and expression of many enzymes involved in lipid metabolism (Ferramosca & Zara, 2014b).

Although a great deal of data is currently available on the dietary modulation of lipid metabolism, little is known about the nutritional regulation of sperm energetic metabolism. The aim of this study was therefore to investigate if and how fatty acids are able to modulate sperm energetic metabolism in rats fed a high-fat (HF) diet, a commonly used experimental model to investigate obesity. The HF diet was supplemented with OO, a source of MUFA, or with KO, a source of n-3 PUFA.

The obtained results led us to depict a possible framework for the molecular action of fatty acids on sperm quality during dys-metabolic conditions.

MATERIAL AND METHODS

This study was carried out in strict accordance with the European Committee Council 106 Directive (86/609/EEC) and with the Italian animal welfare legislation (art 4 and 5 of D.L. 116/92). The Italian Ministry of Health specifically approved this study.

Materials

Bio-Rad protein assay kit was purchased from Bio-Rad; sodium pyruvate, oxaloacetate, citrate, phosphoenolpyruvate, malic acid, succinic acid, cytochrome *c*, ascorbic acid, N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD), rotenone, antimycin A, ADP, NADH, NAD⁺, NADP⁺, 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), acetyl-CoA, isocitrate dehydrogenase, lactate dehydrogenase and pyruvate kinase were from Sigma (St. Louis, MO, USA); Virgin OO was from a local supplier, whereas KO was a generous gift of Aker BioMarine ASA (Oslo, Norway). Kits for serum biochemical analysis, Luciferase ATP assay kit and Lipid Hydroperoxide (LPO) assay kit were from Sigma. All other reagents were of analytical grade.

Animals and diets

Sexually mature male Sprague–Dawley rats were obtained from Harlan and housed individually in animal cages at a temperature of 22 ± 1 °C with a 12 : 12 h light–dark cycle and 30–40% humidity. After acclimatization, 24 rats were divided into four groups of six animals each and fed with four different diets for 4 weeks. The first group (control group) received a standard natural-ingredient diet (Global Diet 2018S from Harlan Teklad), containing 6.2% fat, 44.2% carbohydrate (5% sucrose) and 18.6% protein. The second group (HF group) received a diet (Diet TD.03584 from Harlan Teklad) with 35.2% fat, 36.1% carbohydrate (15% sucrose) and 20.4% protein. Approximate fatty acid profile of this diet was: 40% saturated (SFA), 50% MUFA, 10% PUFA. The third and the fourth groups of animals were fed with the above reported HF diet supplemented with 2.5% (w/w) olive

oil (HF+OO group) or krill oil (HF+KO group), respectively. Diet composition is shown in Table 1.

Animals were allowed *ad libitum* access to diet and water. The calories intake did not differ significantly between the four treatment groups during the study (Control: 65.0 ± 10.7 kcal/day; HF: 75.5 ± 11.2 kcal/day; HF + OO: 68.5 + 8.2 kcal/day; HF ± KO: 78.0 + 9.6 kcal/day).

For the determination of plasma lipid, glucose and insulin concentrations, control and treated rats were starved overnight before sacrifice. Tryglicerides, cholesterol and glucose concentrations were determined using commercial kits. Plasma insulin concentration was analyzed with an enzyme-linked immunosorbent assay kit. Each experiment was repeated three times with each animal. After the rats were sacrificed, liver and adipose tissue were excised and weighed.

Sperm cells analysis

The epididymides cauda were quickly placed in a Petri dish with 2 mL of saline solution and cut into pieces. Sperm number was immediately evaluated using a microscope with a Makler counting chamber. Non-motile sperm numbers were first determined, followed by counting of total spermatozoa. Sperm motility was expressed as a percentage of motile spermatozoa of the total spermatozoa counted under a microscopic field (×1000) for 200 cells in the Makler chambers.

Sperm preparation procedures

Caudal epididymal spermatozoa were flushed from the excised epididymis with isotonic salt medium (2 g/L BSA, 113 mM KCl, 12.5 mM KH₂PO₄, 2.5 mM K₂HPO₄, 3 mM MgCl₂, 0.4 mM EDTA and 20 mM Tris adjusted to pH 7.4 with HCl). Rat spermatozoa were collected by centrifugation at 800 g for 10 min at room temperature and washed three times by resuspension in isotonic salt medium. Sperm samples were then processed as described below for enzymatic spectrophotometric assays or kept in ice-chilled hypotonic medium (potassium

Table 1 Composition of diets (%)

	Control	HF	HF+OO	HF+KO
Proteins	18.6	20.4	19.9	19.9
Lipids	6.2	35.2	36.8	36.8
Fatty acids				
14 : 0	–	0.5	0.5	0.6
16 : 0	0.7	8.7	8.7	9.0
18 : 0	0.2	4.3	4.3	4.3
16 : 1	–	–	–	0.1
18 : 1 (n9-7-5)	1.2	15.8	17.2	16.0
18 : 2 (n6)	3.1	3.5	3.6	3.5
18 : 3 (n3)	0.3	–	–	–
20 : 5 (n3) - EPA	–	–	–	0.3
22 : 6 (n3) - DHA	–	–	–	0.2
∑ SFA	0.9	13.5	13.5	13.9
∑ MUFA	1.3	15.8	17.3	16.1
∑ PUFA	3.4	3.5	3.6	4.1
∑ PUFA n-3	0.3	–	–	0.6
Carbohydrates	44.2	36.1	35.2	35.2
kcal/100 g	310	540	549	549

The control group of animals received a standard diet (Global Diet 2018S from Harlan Teklad). The HF group received a diet with 35% fat (Diet TD.03584 from Harlan Teklad); the HF+OO and the HF+KO groups were fed with the above reported HF diet supplemented with 2.5% of OO or KO, respectively. Fatty acids were extracted from the three diets and analyzed by gas-liquid chromatography.

phosphate 10 mM, pH 7.4, with 2 g/L BSA) for 1.5 h, washed three times using isotonic salt medium, pH 7.4, and used in oxygenographic studies.

Enzymatic assays

Lactate dehydrogenase C4 isoenzyme (LDH-C4) activity was measured spectrophotometrically. 6×10^6 /mL of sperm cells were sonicated three times in 500 μ L Tris-HCl (0.1 M, pH 7.0) for 10 sec, and centrifuged at 10,000 g at 4 °C for 10 min. 10 μ L of the extract were added to 1 mL of reaction buffer (0.05 M Na_2HPO_4 , pH 7.0, 0.1 mg/mL NADH and 27.5 μ g/mL pyruvate). LDH-C4 activity was calculated as the change in absorbance at 340 nm over a period of 1 min.

To evaluate the activity of mitochondrial enzymes, sperm cells were resuspended in the required volume of 20 mM potassium phosphate buffer (pH 7.0) to give a final concentration of 2×10^8 spermatozoa/mL and then disrupted by freeze-thawing before analysis.

Citrate synthase (CS) activity was determined in a medium containing 100 mM Tris-HCl (pH 8.1), 0.1 mM DTNB and 0.25 mM acetyl-CoA. The reaction was started by adding 0.25 mM oxaloacetate and followed by monitoring changes at 412 nm for 3 min.

Aconitase and fumarase activities were determined as described in (Razmara *et al.*, 2008) and activity ratio of aconitase to fumarase was calculated as an indicator of mitochondrial ROS production.

Mitochondrial respiration measurements

Oxygen uptake by rat spermatozoa (2×10^7 sperm cells) was measured using a Clark-type oxygen probe (Hansatech oxygen-graph; Hansatech Pentney, King's Lynn, UK) immersed in a magnetically stirred, 1 mL sample chamber in a water bath as described in (Ferramosca *et al.*, 2008b). Sperm cells were temperature-equilibrated at 36 °C for 15 min prior to respiratory substrates or inhibitor addition. The addition of different substrates, inhibitors and artificial acceptors of electrons, permitted to determine the specificity of particular enzymatic reactions and the entry of substrates in the respiratory chain. In particular, mitochondrial respiration was evaluated when respiratory complexes I (10 mM pyruvate and 10 mM malate which has a stimulatory role on pyruvate oxidation), II (10 mM K-succinate, 15 μ M rotenone) or IV (10 mM ascorbate, 1 mM TMPD, 15 μ M rotenone,

2 μ M antimycin A and 13 mM cytochrome c) were stimulated. After 2 min, 0.76 μ M ADP was added. The oxidative activity of respiratory chain complexes was measured using the respiratory control ratio (RCR), which was calculated by dividing rate of oxygen uptake measured in the presence of substrates + ADP (V_3) by rate of oxygen uptake measured with substrates alone (V_4).

Mitochondrial respiratory complexes activities were also evaluated spectrophotometrically, essentially as described in (Ferramosca *et al.*, 2015a).

ATP levels in sperm cells were determined using the luciferase ATP assay kit. The concentration of ATP in the samples was calculated from interpolation of a standard curve prepared with known amounts of ATP. Results were expressed as nmoles of ATP/ 10^8 spermatozoa.

Statistical analysis

Experimental data represent the means \pm SD of data obtained from six animals. The data were analyzed by one-way ANOVA and a Tukey–Kramer *post hoc* analysis was used to detect significant differences between the means at a level of $p < 0.05$.

RESULTS

Tissue weight, serum biochemical markers and sperm analysis

Table 2 shows that the administration of a HF diet caused an increase of 27% in body weight and predisposed rats to obesity, as epididymal, inguinal, mesenteric and retroperitoneal white adipose tissue was enlarged (from 25 to 38%) in the HF group compared with the control group. Although the administration of OO did not influence fat accumulation, the supplementation of the HF diet with 2.5% KO significantly prevented this effect and hence fat levels in HF+KO rats were comparable to those observed in control animals. No statistically significant variations were found in the liver weight/body weight ratio of animals fed with the different experimental diets.

A strong increase in the levels of glucose and insulin was found in the plasma of HF and HF+OO animals, suggesting the appearance of typical insulin resistance (Table 2). The blood glucose concentration increased by 54% and 46% in HF and HF+OO animals, respectively. Such an increase was less evident in the case of the HF+KO fed animals (35% in comparison to control group). A massive increase in the levels of insulin was

Table 2 Effect of experimental diets on tissue weight, serum biochemical markers and sperm quality

	Control	HF	HF+OO	HF+KO
Tissue weight				
Body weight (g)	241.5 \pm 12.5	305.8 \pm 11.2*	295.5 \pm 13.4*	249.2 \pm 20.4**
Epididymal adipose tissue (%)	1.9 \pm 0.3	2.4 \pm 0.2*	2.3 \pm 0.3*	2.0 \pm 0.2**
Inguinal adipose tissue (%)	4.4 \pm 0.4	6.0 \pm 0.4*	5.7 \pm 0.2*	4.6 \pm 0.4**
Mesenteric adipose tissue (%)	2.0 \pm 0.3	2.5 \pm 0.2*	2.4 \pm 0.2	2.1 \pm 0.2**
Retroperitoneal adipose tissue (%)	2.9 \pm 0.3	4.0 \pm 0.5*	3.7 \pm 0.3*	3.1 \pm 0.4**
Liver weight /body weight ratio (%)	3.8 \pm 0.4	3.7 \pm 0.3	3.5 \pm 0.3	3.5 \pm 0.4
Serum biochemical analysis				
Glucose (mg/dL)	58.8 \pm 4.5	90.7 \pm 8.5*	85.7 \pm 4.2*	79.5 \pm 6.0***
Insulin (ng/mL)	1.9 \pm 0.3	5.4 \pm 0.2*	5.0 \pm 0.4*	2.8 \pm 0.2***
Serum triglycerides (mg/dL)	145.5 \pm 9.2	167.2 \pm 10.9*	159.8 \pm 8.1*	149.2 \pm 5.1**
Total cholesterol	100.3 \pm 6.2	102.2 \pm 4.7	97.0 \pm 4.8	98.8 \pm 4.5
Sperm analysis				
Concentration ($\times 10^8$ /mL)	1.7 \pm 0.3	0.9 \pm 0.2*	1.1 \pm 0.2*	1.5 \pm 0.3**
Motility (%)	67.2 \pm 8.5	39.3 \pm 11.3*	53.7 \pm 13.3***	62.0 \pm 4.5**

Each value represents the mean \pm SD for 6 animals. * $p < 0.05$ vs. rats fed a control diet. ** $p < 0.05$ vs. rats fed a HF diet.

revealed in the plasma of HF and HF+OO animals. Insulin levels were 2.8-fold in HF rats and 2.6-fold in HF+OO animals, in comparison to control rats. KO supplementation was able to counteract the hyperglycemic effects, preventing the strong increase in the plasma insulin concentration. In fact, in HF+KO animals, insulin concentration was 1.5-fold respect to control animals.

In HF and HF+OO animals plasma triglyceride levels were also significantly higher than those of control rats; however, KO supplementation to the HF diet reversed this effect. The assay of plasma cholesterol did not reveal any significant variation among the different dietary groups.

At the same time, in HF animals we observed a significant reduction in sperm concentration (47%) and motility (42%) in comparison to the control group. Interestingly, OO supplementation produced a significant increase of 37% in sperm motility in comparison to HF animals, without improving sperm concentration. On the other hand, KO administration caused a strong increase in sperm concentration and motility.

LDH-C4, CS, aconitase and fumarase activity

LDH-C4 is the sperm lactate dehydrogenase isoenzymatic form, which catalyzes the reversible conversion of pyruvate to lactate with the concomitant oxidation of NADH to NAD⁺ (Burgos *et al.*, 1995; Ferramosca & Zara, 2014c). We found a significant decrease in the LDH-C4 activity in HF (29%) and in HF+OO (20%) animals, in comparison to the control group (Fig. 1A). The supplementation with KO of the HF diet reversed this effect, thereby keeping LDH-C4 activity similar to that found in control rats.

A decrease of 33% and 23% in CS activity was also observed in the HF and HF+OO rats, respectively, in comparison to the control group (Fig. 1B). This decrease was completely reversed by KO administration (Fig. 1B).

Mitochondrial aconitase activity is a functional indicator of mitochondrial levels of ROS, because the iron-sulfur core of this enzyme is oxidized by superoxide, reducing its activity. As shown

in Fig. 1C, we found a strong decrease of 44% in aconitase activity in HF animals in comparison to the control ones. OO and KO administration was able to reverse this effect. Differently from aconitase activity, fumarase is not affected by mitochondrial ROS. We found a significant decrease in the fumarase activity in HF animals (26%) in comparison to the control group. OO administration caused a slight, but not significant increase in the fumarase activity with respect to HF animals. Interestingly, this enzymatic activity measured in the HF+KO group was similar to that found in control animals. Finally, we used the ratio of mitochondrial aconitase to fumarase activity as a functional indicator of ROS production. Higher aconitase/fumarase ratios were found in HF+OO and HF+KO animals in comparison with HF rats, suggesting a decrease in ROS production after the addition of OO and KO to a HF diet.

Mitochondrial respiratory function

ROS may lead to lipid peroxidation. LPO levels were measured in sperm cells from treated rats (Fig. 2A). We found a significant increase (about 30%) in lipid peroxidation in HF animals in comparison to the control ones. OO and KO supplementation to the HF diet was able to efficiently abolish this effect.

To understand the biological implications of a possible mitochondrial chain impairment, we measured at first ATP concentration in sperm cells from the four groups of animals. Figure 2B shows that a HF diet produced a 40% decrease in sperm ATP content. Such a decrease was less pronounced (20%) after OO addition to a HF diet. ATP levels found in sperm cells from HF+KO animals were similar to those found in animals fed a control diet.

Mitochondrial function was then tested by polarographic and spectrophotometric methods

When we added respiratory substrates for mitochondrial complexes I, II and IV (Table 3), a significant decrease in RCR values was observed in sperm cells from HF group in comparison to control animals. OO administration was able to increase RCR

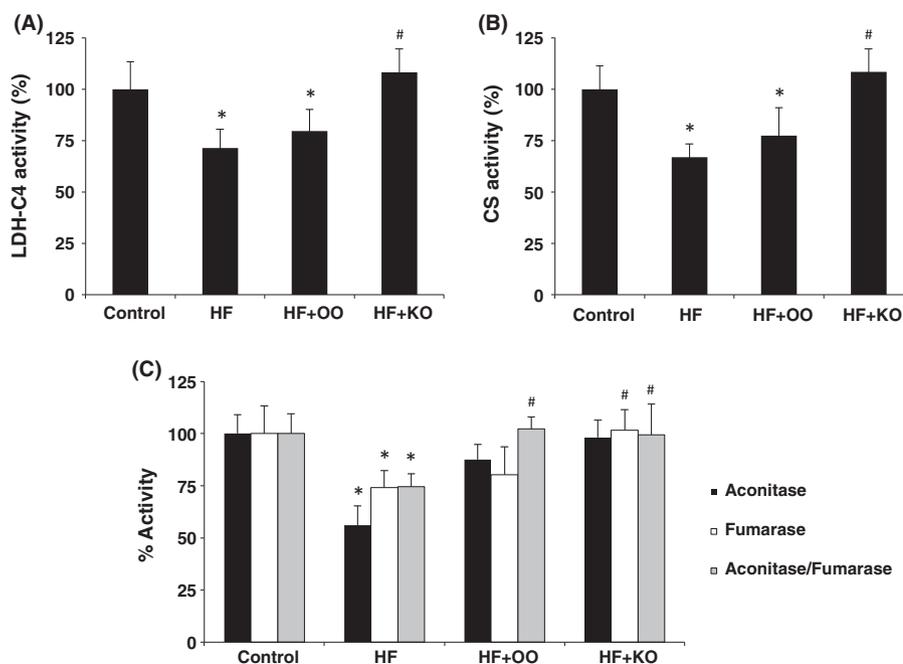
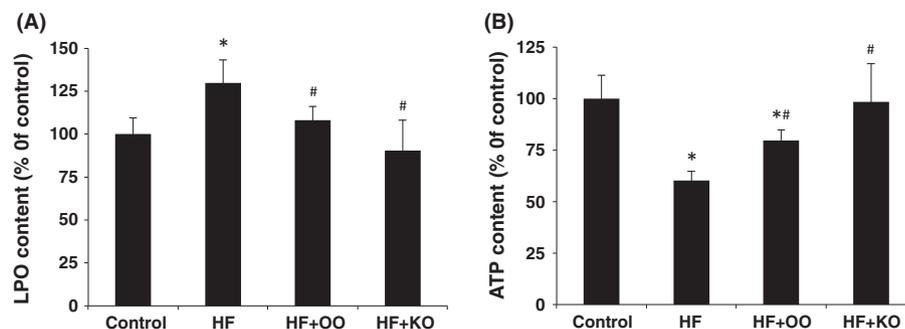


Figure 1 Effect of HF, HF+OO and HF+KO diets on the activity of LDH-C4 (A), CS (B), aconitase and fumarase (C) activities. The values reported in the figure represent the means \pm SD ($n = 6$). * $p < 0.05$ vs. rats fed a control diet; # $p < 0.05$ vs. rats fed a HF diet.

Figure 2 Effect of HF, HF+OO and HF+KO diets on sperm LPO (A) and ATP (B) levels. The amount of ATP or LPO in the control group was set to 100%. The values reported in the figure represent the means \pm SD ($n = 6$). * $p < 0.05$ vs. rats fed control diet; # $p < 0.05$ vs. rats fed a HF diet.



values of about 30%, whereas KO kept these values very similar to those found in the control group.

In the presence of metabolic substrates for complexes I, II and IV, a strong decrease in V_3 values (also known as the rate of oxygen consumption in the active state of respiration) was observed in the HF group. The decrease was 70% when mitochondrial respiration was activated by pyruvate and malate (therefore measuring complex I activity), 51% in the presence of succinate and rotenone (therefore measuring complex II activity) and 46% when the sperm suspension was incubated with rotenone, antimycin A, TMPD and ascorbate (therefore measuring complex IV activity). In these animals the V_4 values (also known as the rate of oxygen consumption in the resting state of respiration) decreased, but significantly less than V_3 : a decrease of 52%, 22% and 13% was found in the presence of metabolic substrates for complexes I, II and IV, respectively.

The addition of OO to the HF diet was able to increase the V_3 values (39%, 37% and 27% in the presence of substrates for complexes I, II and IV, respectively) with respect to those observed in HF rats, therefore improving RCR values. In the sperm cells from HF+KO animals the V_3 and V_4 values returned to values similar to those measured in control rats.

The activity of the single components of the respiratory chain was also assayed spectrophotometrically as reported in Fig. 3. Also in this case, we found that a HF diet caused a significant decrease of about 30% in the activity of respiratory chain complexes II, III and IV and of ATP-synthase; no significant change was observed in the activity of sperm mitochondrial complex I in the HF group in comparison to the control one. On the other hand, OO and KO improved respiratory function. The effect produced by KO, which completely restored the enzymatic

respiratory activities, was more pronounced than that caused by OO (about 20%).

DISCUSSIONS

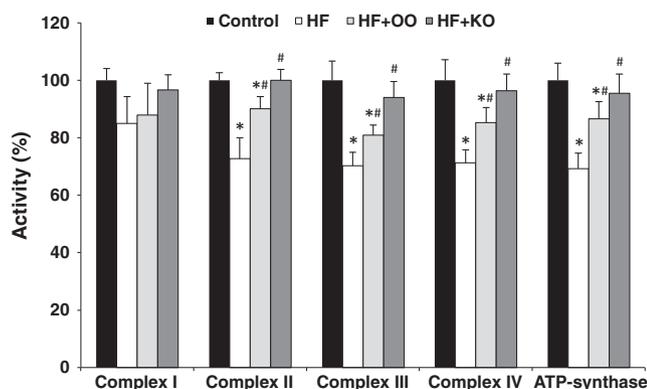
Obesity in men of reproductive age is increasing worldwide, impacting negatively on reproductive potential and assisted reproduction outcomes (Campbell *et al.*, 2015). Adipose tissue depots have an important role in the development of oxidative stress, subsequent to a pro-inflammatory state, which can alter normal reproductive pathways and sperm function (Bachir & Jarvi, 2014; Morielli & O'Flaherty, 2015). Recent studies demonstrated that the increased levels of ROS negatively affect sperm mitochondrial respiration (Ferramosca *et al.*, 2013, 2015b; Rato *et al.*, 2014). In fact, under stress conditions, mitochondria are prone to trigger a cascade of oxidative damages within testicular environment, as these organelles are major producers of ROS. This finding suggests that regulation of mitochondrial function and of molecular mechanisms involved in lipid metabolism could provide useful targets to prevent the development of male infertility related to obesity. Molecules useful to regulate lipid metabolism, along with mitochondrial function, could be dietary fatty acids. Several studies reported that MUFA, especially oleic acid (n-9 MUFA), increase lipid oxidation (Soriguer *et al.*, 2006; Ferramosca *et al.*, 2008a; Assy *et al.*, 2009), while long-chain n-3 PUFA have a strong inhibitory effect on hepatic fatty acid synthesis (Ferramosca & Zara, 2014a,b). In recent years, the use of dietary supplements of MUFA and PUFA has rapidly increased with the aim to prevent human diseases. In this context, OO consumption is encouraged, as it has been associated to a lower prevalence of chronic diseases and hepatic disorders (Sofi & Casini, 2014; Velasco *et al.*, 2014). On the other hand, KO has

Table 3 Mitochondrial respiratory efficiency

	Control	HF	HF+OO	HF+KO
Pyruvate + malate (Complex I)				
V_3 (nmol O ₂ per mL per min)	6.0 \pm 0.3	1.8 \pm 0.3*	2.5 \pm 0.3***	5.8 \pm 0.3**
V_4 (nmol O ₂ per mL per min)	2.3 \pm 0.2	1.1 \pm 0.2*	1.4 \pm 0.2*	2.0 \pm 0.2**
RCR	3.2 \pm 0.3	1.7 \pm 0.2*	2.2 \pm 0.5***	3.0 \pm 0.3**
Succinate + rotenone (Complex II)				
V_3 (nmol O ₂ per mL per min)	7.2 \pm 0.6	3.5 \pm 0.4*	4.8 \pm 0.3***	7.1 \pm 0.6**
V_4 (nmol O ₂ per mL per min)	2.7 \pm 0.2	2.1 \pm 0.4*	2.2 \pm 0.2*	2.5 \pm 0.3**
RCR	2.7 \pm 0.1	1.7 \pm 0.2*	2.2 \pm 0.1***	2.9 \pm 0.2**
Ascorbate + cytochrome c + TMPD + rotenone + antimycin A (Complex IV)				
V_3 (nmol O ₂ per mL per min)	17.9 \pm 0.7	9.6 \pm 1.1*	12.2 \pm 1.3***	18.4 \pm 1.1**
V_4 (nmol O ₂ per mL per min)	6.7 \pm 0.5	5.8 \pm 0.4*	5.4 \pm 0.5*	7.1 \pm 0.6**
RCR	2.7 \pm 0.2	1.7 \pm 0.3*	2.3 \pm 0.4**	2.6 \pm 0.4**

Respiratory control ratio (RCR) was calculated as the ratio of the rate of oxygen uptake in the presence of added ADP (V_3) to the rate measured with substrates alone (V_4). Each value represents the mean \pm SD for 6 animals. * $p < 0.05$ vs. rats fed control diet. ** $p < 0.05$ vs. rats fed a HF diet.

Figure 3 Effect of HF, HF+OO and HF+KO diets on sperm respiratory complexes and ATP-synthase activity. Enzymatic activities measured in the control group were set to 100%. The values reported in the figure represent the means \pm SD ($n = 6$). * $p < 0.05$ vs. rats fed control diet; # $p < 0.05$ vs. rats fed a HF diet.



become increasingly popular because of its capability of reducing hepatic lipogenesis, stimulating catabolism of fat excess introduced by a HF diet and ameliorating mitochondrial function (Ferramosca *et al.*, 2015a).

Starting from these evidences, we decided to investigate the effects of OO and KO supplementation sperm quality of rats fed with a HF diet, an animal model associated with obesity development. The amount of both dietary fat and carbohydrate, especially sucrose, are important features of this diet, which is essentially used to evaluate the effect of the current western dietary habits. We found that HF animals showed altered sperm parameters, according to other studies (Mendiola *et al.*, 2009; Fernandez *et al.*, 2011; Attaman *et al.*, 2012; Gaskins *et al.*, 2012; Jensen *et al.*, 2013; Ferramosca *et al.*, 2016), along with a significant increase in body weight, hyperglycemia and hyperinsulinemia. Interestingly, OO supplementation significantly increased sperm motility, without affecting serum glycemic profile and fat distribution observed in HF animals. KO administration caused a strong increase in sperm concentration and motility in comparison to HF rats, reversing, at the same time, the adverse effects on body fat and on serum biochemical markers caused by a HF diet.

Although n-3 PUFA supplementation studies on spermatozoa/ semen responses in men are very limited, some investigations suggested that these fatty acids improve antioxidant activity of human seminal fluid and sperm quality (sperm count, motility and morphology) (Safarinejad & Safarinejad, 2012). It is worth noting that PUFA can influence reproductive processes through a variety of mechanisms. On the one hand, these fatty acids may promote loss of body fat and weight reduction, hence preventing the development of infertility related to obesity; on the other, they control specific aspects linked to male fertility (Lenzi *et al.*, 2000; Wathes *et al.*, 2007; Esmaeili *et al.*, 2015; Mahdavi *et al.*, 2015). Moreover, PUFA provide the precursors for eicosanoids synthesis that can modulate the expression patterns of many key enzymes (such as cyclooxygenase and lipoxygenase) involved in both prostaglandin and steroid metabolism; these fatty acids are also structural components of cell membranes, whose fluidity is necessary to promote membrane fusion events associated with fertilization.

This study revealed that fatty acid composition of the diet influenced sperm metabolism. In particular, in animals fed the HF diet, which is low in PUFA, LDH-C4 activity was significantly reduced, whereas dietary supplementation of n-3 PUFA in the form of KO reversed this effect. This is the first evidence of a nutritional modulation of LDH-C4, an isoenzymatic form specifically involved in the energy metabolism of spermatozoa. We also found that fatty acid composition of the diet was able to influence the activity of some enzymes of the Krebs cycle, such as CS, aconitase and fumarase. A strong and significant negative correlation of CS with obesity was reported in liver (Ferramosca *et al.*, 2015a) and in human omental adipose tissue (Christe *et al.*, 2013). At the same time, an increase in CS activity was recently observed after KO diet supplementation in rat liver (Ferramosca *et al.*, 2015a), suggesting that a modulation of the activity of this protein, which is the rate-limiting enzyme of the Krebs cycle, could regulate the flux of reducing equivalents to mitochondria.

NADH and FADH₂ generated by dehydrogenase reactions in the Krebs cycle are used in the final common oxydative phosphorylation to generate ATP and this process is coupled with the transfer of electrons along the respiratory chain (Piomboni *et al.*, 2012). Our data indicate that, during HF feeding, a lower coupling between sperm mitochondrial respiration and ATP synthesis occurs. Such an uncoupling between the two processes, suggested by the recorded V₃ and V₄ values, was reversed after OO and KO supplementation to the diet. Interestingly, the effect of KO was more pronounced than that of OO. The presence of a fatty acid modulation of respiratory complexes activity, which was previously observed in liver mitochondria (Ferramosca *et al.*, 2015a), was confirmed by measuring the activity of single components of the sperm respiratory chain spectrophotometrically.

The biological implications of a mitochondrial chain impairment (after a HF diet administration) or improvement (after a HF diet supplementation with OO or KO) result in different ATP concentrations in sperm cells from animals fed the different experimental diets. The observed differences in sperm mitochondrial respiration efficiency in treated animals could reflect a direct effect of the diets on mitochondrial respiration efficiency and ATP production, or alternatively an effect of the experimental treatments on sperm viability. According to this last hypothesis, a recent study reported that a HF diet reduced sperm viability, by inducing apoptosis (Yan *et al.*, 2015). Indeed, a limitation of our study is the lack of evaluation of sperm viability in treated animals. Therefore, the HF diets used in this study could reduce or improve sperm viability, while the mitochondrial function of the individual viable spermatozoa may not be modified.

The observed changes in ATP content in sperm cells from animals fed the HF diets were accompanied by different levels of LPO, which are an oxidative stress marker. However, it is important to underline that the oxidative stress may not be intrinsic to the spermatozoa, but may reflect the antioxidant capacity (or the degree of oxidative stress) of the fluid surrounding the spermatozoa. Moreover, it is important to underline that mechanisms other than ROS may be involved in the observed changes in sperm quality and function.

In conclusion, this study indicates for the first time that a specific fatty acid composition of the diet can counteract the negative effects of a HF diet on sperm cells. In particular, we

found that dietary administration of long-chain n-3 PUFA, besides reducing fat accumulation caused by a HF diet, reduced oxidative damages on sperm cells (as suggested also by the increase in the activity ratio aconitase/fumarase). On the other hand, OO administration partially counteracted the negative effects of a HF diet, without influencing fat deposition. The antioxidant activity of OO could also positively influence sperm function (Mansour *et al.*, 2013).

Therefore, our results contribute to elucidate the molecular mechanism by which MUFA and long chain n-3 PUFA influences many metabolic steps and ameliorates mitochondrial dysfunctions caused by the administration of a HF diet (rich in lipids and sucrose), which often characterizes the nutritional habits of Western populations.

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CONFLICT OF INTEREST

The authors do not have any conflicts of interest to declare.

AUTHORS' CONTRIBUTIONS

AF designed the research study, performed the research, analyzed the data and wrote the manuscript; NM and MDG designed the experiments, performed the research and analyzed the data; VZ designed the research study, analyzed the data and wrote the manuscript. All authors participated in experimental design and read and approved the final manuscript.

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