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Maternal and postnatal high-fat diets with high ω6 : ω3 ratios affect the reproductive performance of male offspring in the mouse

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Abstract. High-fat diets (HFDs) are an acknowledged risk factor for male subfertility, but the underlying mechanisms remain unclear. In the present study we compared the effects of two HFDs with different ω6 : ω3 ratios, one enriched with soy oil (SOD; ω6 : ω3 = 9.62) and another enriched with sunflower oil (SFOD; ω6 : ω3 = 51.55), with those of a commercial diet (CD; ω6 : ω3 = 19.87), supplied from pregnancy to adulthood, on morphometric parameters and reproductive performance in adult male mice (recommended ω6 : ω3 for rodents = 1–6). Bodyweight was significantly higher in the SFOD than CD group, and relative testicular weight was significantly lower in the SFOD than the other two groups. SFOD altered sperm performance: it reduced sperm viability (mean ± s.e.m.; 76.00 ± 1.35% vs 82.50 ± 1.45% and 80.63 ± 1.00% in the SFOD vs CD and SOD groups respectively; $P < 0.05$) and increased the percentage of immature spermatozoa (71.88 ± 7.17% vs 51.38 ± 5.87% and 48.00 ± 5.72% in the SFOD vs CD and SOD groups respectively; $P < 0.05$). The epididymal ω6 : ω3 ratio was higher in the SFOD versus CD and SOD groups, whereas the unsaturation index was higher in the SOD and SFOD groups than in CD group. Sperm membrane integrity was diminished in both the SOD and SFOD groups, but there was no difference in sperm reactive oxygen species production in these two groups compared with the CD group. The fertilisation rate was lower in the SFOD compared with the CD and SOD groups. In conclusion, although both HFDs affected sperm quality, the fertilising ability was more altered by the excessive dietary ω6 : ω3 ratio than by the net ω6 content.

Additional keywords: epididymal sperm quality, male fertility, polyunsaturated fatty acids, sperm maturity.

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Introduction

Fetal programming explains the influence of multiple environmental and nutritional factors that determine new morphological and functional characteristics in critical moments of fetal development (Barker 2004). There are many parental factors that interfere with fetal programming, such as exposure to toxins, undernutrition, overnutrition, obesity and high-fat (HFD) or low-protein diets (Vehaskari *et al.* 2001; Gluckman *et al.* 2008). It has been shown that exposure to an HFD adversely affects insulin production, the mass of pancreatic β-cells, blood glucose, hepatic accumulation of fat and reproductive health in offspring (Fullston *et al.* 2015; Ornellas *et al.* 2015).

In previous studies, we demonstrated that the growth of male mice is slower when they are exposed to an HFD with ω6 excess from gestation until adulthood. In addition, there were delays in some physical and neurobiological characteristics, including puberty onset. These results show that maternal nutrition during pregnancy and lactation has significant effects on the development of their offspring (Santillán *et al.* 2010). In this paper we focus on the reproductive capacity of males exposed to HFDs from gestation until adulthood.

The importance of lipid composition, in particular phospholipids in the sperm membrane, for sperm function has long been recognised (Lenzi *et al.* 2000). In mammalian spermatozoa,

long-chain polyunsaturated fatty acids (PUFAs) are important for the integrity of the sperm membrane, motility and viability (Robinson *et al.* 2006; Esmaeili *et al.* 2015). It has been suggested that the proportion and degree of unsaturation of PUFAs may modify the physical properties of the sperm membrane, including fluidity and flexibility (Miller *et al.* 2004; Gholami *et al.* 2010). Recently, it was proposed that lipid concentrations may affect semen parameters, with this effect being more pronounced on the morphology of the sperm head (Schisterman *et al.* 2014).

The structural integrity of the sperm cell membrane plays a pivotal role in successful fertilisation. This is because both the acrosome reaction and sperm–oocyte fusion are associated with the sperm membrane fatty acid (FA) profile (Gulaya 2003). Thus, PUFAs are studied as markers of sperm pathophysiology.

The essential FAs linoleic acid (LA; 18:2 ω6) and α-linolenic acid (ALA; 18:3 ω3) obtained from the diet are converted into their long-chain derivatives (LC-PUFAs) by the liver and then incorporated into tissues (Cook 1996). Testicular cells are also able to convert essential FAs to LC-PUFAs (Retterstøl *et al.* 2000; Tran *et al.* 2003). In recent years, several efforts have been made to increase the proportion of sperm LC-PUFAs ω3 series by supplementing animal diets with ALA (Mourvaki *et al.* 2010; Gürler *et al.* 2015) or LC-PUFAs ω3 (Cerolini *et al.* 2006; Zaniboni *et al.* 2006; Gliozzi *et al.* 2009). Yan *et al.* (2013) demonstrated that the ω3 : ω6 ratio in the diet has a considerable effect on sperm quality traits and reproductive performance.

By increasing ω3 and decreasing ω6 in the diet to yield a lower ω6 : ω3 ratio, health benefits can be achieved (Simopoulos 2016). In humans, because many of the prevalent chronic diseases in Western countries are multigenic and multifactorial, recommendations for the ω6 : ω3 ratio vary from 1 : 1 to 4 : 1 for suppressive effects on diseases such as cardiovascular disease, rheumatoid arthritis or colorectal cancer (Simopoulos 2004). Despite that, Simopoulos (2011) considered that an optimal ω6 : ω3 ratio between 2 : 1 and 1 : 1 should be the goal for human nutrition. Conversely, the World Health Organization (WHO)/Food and Agriculture Organization of the United Nations (FAO) dietary recommendations considered that the overall intake of each ω6 and ω3 FA is more important than the ratio, as long as basic dietary requirements are covered (WHO/FAO 2010). Currently, the ω6 : ω3 ratio in Western human populations is in the range 10–20 : 1, primarily due to decreased fish consumption and the presence of food derived from animals fed diets rich in ω6 and poor in ω3 (Simopoulos 2011). This imbalance, in addition to the excess fat, may be one of the many factors affecting male fertility in Western countries. Efforts to achieve the proper ω6 : ω3 ratio in the diet are increasing in order to improve reproductive success, both in humans and animals. Nevertheless, there is still a lack of research data about the effects of different ω6 : ω3 ratios on sperm quality and fertility, although both positive and negative actions are theoretically possible. Thus, the aim of the present study was to determine the effects of two HFDs supplemented with two of the most widely used vegetable oils in the Western human diet, namely soy oil (ω6 : ω3 balanced) and sunflower oil (ω6 : ω3 exceeded), on semen quality, testosterone plasma concentrations and *in vivo* fertilising ability of spermatozoa from mice exposed to those diets from gestation until adulthood.

Materials and methods

Animals

All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals from the Medicine School of the Universidad Nacional de Córdoba (UNC-RHCS 674/09). The Animal Ethics Committee of the Universidad Nacional de Córdoba approved the study protocols (Protocol number: 055/15).

Albino Swiss mice were maintained under a standard 14-h light–10-h dark photoperiod and controlled temperature (24 ± 2°C), with food and water provided *ad libitum*. Female mice (10 weeks old, 24–26 g) were mated individually with a male in suspended cages and monitored daily to detect a vaginal sperm plug. Once the plug was detected, females (F_0 generation) were placed in separate cages and randomly assigned to one of the three dietary conditions throughout gestation and lactation. The offspring (F_1 generation) continued receiving the same diet after weaning (Postnatal Day (PND) 21) until adulthood. The parameters evaluated in the dams and their offspring until the onset of puberty have been published previously (Santillán *et al.* 2010).

Bodyweight was measured daily using an electronic balance to an accuracy of ±0.01 g (Mettler Electronics). Body length and abdominal circumference were measured just before mice were euthanised.

At adulthood (60 days of age), males were euthanised by decapitation to collect blood for hormone analyses. Samples were immediately centrifuged at 420*g* for 10 min at room temperature; the plasma was removed and stored at −20°C until analysis.

Epididymides, testes, seminal vesicles and the liver were excised and allocated in Petri dishes with Tyrode's solution. After surrounding adipose tissue had been removed, organs were weighed. Organ weight relative to bodyweight was calculated by dividing organ weight by bodyweight and multiplying by 100.

Epididymal, retroperitoneal and subcutaneous adipose depots were dissected and weighed as described previously (Johnson and Hirsch 1972). The cauda epididymides were cut into small pieces and incubated in Tyrode's solution for 10 min at 37°C under a 5% CO_2 atmosphere, as described previously (Puechagut *et al.* 2012).

Diets

The diets used in the present study were a commercial diet (CD), a soy oil-enriched diet (SOD) and a sunflower oil-enriched diet (SFOD). The CD was a pelleted chow (Grupo Pilar-Gepsa), without any oil enrichment, that was fed to the control group. Table 1 lists the FA composition, energy content and the kilocalories from fat as a percentage of total energy for each diet. The diets used in the present study were the same as those used by Santillán *et al.* (2010). Briefly, the SOD and SFOD were prepared by adding 5 g commercial soy oil (Sojola; 100% pure soy oil; Aceitera General Deheza) or sunflower oil

Table 1. Diet composition (g per 100 g food), calories (Kcal/g) and percentage of Kcal as fat of total dietary energy of the diets
CD, control diet; SOD, soy oil-enriched diet; SFOD, sunflower oil-enriched diet

Nutrients	CD	SOD	SFOD
Carbohydrates (g per 100 g food)	42.00[A]	39.90	39.90
Proteins (g per 100 g food)	18.00[A]	17.10	17.10
Fats (g per 100 g food)	3.90[A]	8.70	8.70
Calories (kcal g^{-1})	2.75	3.06	3.06
% Calories (kcal) as fat	12.76	25.56	25.56
Saturated fatty acids[B] (g per 100 g food)			
Palmitic 16:0	0.65	1.17	0.92
Stearic 18:0	0.25	0.46	0.43
Arachidonic 20:0	0.01	0.04	0.03
Behenic 22:0	0.00	0.03	0.04
Lignoceric 24:0	0.00	0.01	0.02
Total	0.91	1.71	1.44
Monounsaturated fatty acids[B] (g per 100 g food)			
Palmitoleic 16:1	0.10	0.10	0.10
Oleic 18:1	1.17	2.17	2.38
Eicosenoic 20:1	0.02	0.04	0.04
Erucic 22:1	0.00	0.00	0.00
Total	1.29	2.31	2.52
Polyunsaturated fatty acids[B] (g per 100 g food)			
Linoleic 18:2 ω6	1.59	4.14	4.64
α-Linolenic 18:3 ω3	0.08	0.43	0.09
Total	1.67	4.57	4.73
ω6 : ω3 ratio	19.87	9.62	51.55

[A]Data from Gepsa Feeds. Grupo Pilar SA Ratón-rata autoclavable.
[B]Quantified by gas liquid chromatography in the Departamento de Química Biológica, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba.

(Natura; 100% pure sunflower oil; Aceitera General Deheza) respectively to 95 g of the CD. The CD had an ω6 : ω3 ratio of 19.87; the ω6 : ω3 ratio in the SOD was nearly adequately balanced at 9.62, whereas at 51.55, the ω6 : ω3 ratio in the SFOD exceeded nutritional requirements for laboratory rodents (between 1 and 6; Bourre *et al.* 1989; Reeves *et al.* 1993). Both enriched diets are high in fat because their lipid content is double that of the control diet. Diets were prepared weekly and stored in a refrigerator (4°C). Fresh diet was provided daily to further protect against oxidation. Food consumed by the mice in each cage was weighed daily (g) throughout the experimental period and cumulative food intake was calculated for each animal.

The FA composition of the diets (oils and CD) was evaluated using gas chromatography at the Department of Química Biológica, Facultad de Ciencias Agropecuarias, Instituto Multidisciplinario de Biología Vegetal (IMBIV)-Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

The isoflavones in the CD were daidzein (D; mean ± standard error of the mean (s.e.m.) content 6.5 ± 1.3 mg kg^{-1}) and genistein (G; 20.3 ± 1 mg kg^{-1}). The isoflavones were identified and quantified using high-performance liquid chromatography (HPLC)– in triplicate (Barnes *et al.* 1994; Fabani *et al.* 2013). The levels of these compounds in the diets used in the present study were considerably lower than those in another study (Thigpen *et al.* 1999), in which the lowest proportion of G + D in a 5-g sample of diet per day per animal was 0.4 mg compared with 0.134 mg in the CD and less in the oil-enriched diets.

Sperm functional activity

Sperm concentration and motility were quantified in a Makler counting chamber (Makler 1978; Sefi-Medical Instruments) under an inverted microscope (CK2; Olympus) at a magnification of ×200. Motility is expressed as the percentage of motile cells (progressive plus non-progressive gametes). No fewer than 100 gametes were examined.

Viability was evaluated using supravital staining with Hoechst 33258 (H258; Calbiochem) as described previously (Yelian and Dukelow 1992; Martini *et al.* 2009; Luque *et al.* 2010). Under appropriate ultraviolet fluorescence optics (Axiolab; Zeiss), spermatozoa with brightly fluorescent nuclei were considered dead, whereas those without fluorescence were considered viable. No fewer than 100 spermatozoa were evaluated.

The percentage of immature gametes was determined by quantifying the spermatozoa that showed flagellar angularity and those with persistent cytoplasmic drop (c.d.) (Bedford *et al.* 1973; Cooper *et al.* 1988). Results are expressed as the percentage of total gametes. The total percentage of immature gametes was calculated as the percentage of spermatozoa with only one of flagellar angularity and persistent c.d. plus those gametes exhibiting both of features (Martini *et al.* 2007).

Membrane functional integrity was evaluated using the hypo-osmotic swelling test (HOST). Briefly, a sperm suspension (0.1 mL) was mixed with a hypo-osmotic solution and incubated at 37°C for 45 min. The hypo-osmotic solution (100 mOsmol L^{-1}) was prepared with fructose and sodium citrate in distilled water (Jeyendran *et al.*1984; Ruiz *et al.* 1996). Evaluations were made using a phase contrast microscope at a magnification of ×400; at least 100 cells were evaluated and the percentage of spermatozoa with swollen tails was calculated.

Acrosomal status was determined with *Pisum sativum* agglutinin stain labelled with fluorescein isothiocyanate, as described by Cross *et al.* (1986) with minor modifications (Fiol de Cuneo *et al.* 1994). The viability and acrosomal status of at least 100 spermatozoa were assessed. Under blue wavelength fluorescein-appropriate excitation and at a magnification of ×1000, spermatozoa with brightly fluorescent acrosomes were considered acrosome intact, whereas those with no fluorescence over the equatorial region were considered acrosome reacted. The filter was then changed to ultraviolet and each cell was further scored as viable or non-viable. Results are expressed as the percentage of acrosome-reacted spermatozoa of all viable cells.

Reactive oxygen species (ROS) production was evaluated by determining the intracellular oxidation of dihydroethidium (DHE) within spermatozoa, as reported previously (Henkel *et al.* 2003; Smith *et al.* 2013). A 100-μL aliquot of caudal epididymal spermatozoa was diluted 1 : 2 with phosphate-buffered saline (PBS) and centrifuged at 300*g* for 10 min at room temperature. The supernatant was discarded and the remaining pellet was resuspended in PBS to a final concentration of 20×10^6 spermatozoa mL^{-1}. Then, 125 μL of 10 μM

Table 2. Food intake of mice fed a commercial diet (CD), a soy-oil enriched diet (SOD) or a sunflower-oil enriched diet (SFOD)
Results are expressed as mean ± s.e.m. Within rows, different superscript letters indicate significant differences ($P < 0.05$). GD, gestational day; PND, postnatal day

	Cumulative food consumption (g)		
	CD	SOD	SFOD
Maternal ($n = 4$ in each group)			
GD1–GD19	82.57 ± 4.56	87.54 ± 3.84	78.72 ± 4.35
PND1–PND21	298.78 ± 4.86	293.41 ± 7.28	289.79 ± 3.31
Offspring PND22–PND60 ($n = 16$ in each group)	139.85 ± 2.45[a]	122.95 ± 3.40[b]	115.22 ± 2.99[b]

DHE solution (Molecular Probes) was added to 125 µL cell suspension and samples incubated for 20 min at 37°C. After incubation, the sperm suspensions were washed by centrifugation with PBS (300*g* for 5 min at room temperature) to eliminate residual DHE. Washed spermatozoa were fixed with 1% formaldehyde for 20 min at room temperature. A droplet of this suspension was smeared on a slide and dried at room temperature. Slides were mounted with Vectashield (Vector Laboratories). The presence of red–orange fluorescing spermatozoa was evaluated under an epifluorescence microscope (488 nm excitation) at a magnification of ×1000. At least 400 cells were analysed and staining scored as positive or negative.

FA profile in the epididymis and spermatozoa

Total FA profile of the cauda epididymidis and spermatozoa was determined by gas chromatography (GC). Spermatozoa were obtained by making incisions in the isolated caudal portion of the epididymides, allowing the spermatozoa to extrude into 2 mL Tyrode's medium for 10 min ($n = 4$ for each group). Then, the remains of the epididymal tissue were processed to extract the lipids. In contrast, the spermatozoa suspended in Tyrode's solution were pooled so that the amount of lipids extracted from their membranes was sufficient for GC determination. Each pool corresponded to a mixture of two supernatants of the caudal epididymides from two mice. These spermatozoa in 2 mL Tyrode's solution were centrifuged at 420*g* for 10 min at room temperature and the pellets were treated to obtain sperm lipids, as described in the following paragraph.

Lipids were extracted according to the method of Folch *et al.* (1957) and methylated with sodium methoxide. The separation, quantification and identification of FA methyl esters was performed using a polyethylene glycol (SUPELCO) capillary column (20 m × 250 µm id × 0.25 µm) in a Clarus 500 (Perkin-Elmer) gas chromatograph. The FA methyl esters were identified using a commercial standard (Nu-check). All values were expressed as the percentage area of total (Dain *et al.* 2016). The degree of FA unsaturation in the tissues is expressed as the double bound index (DBI), calculated as follows:

$$\mathrm{DBI} = \sum(\mathrm{UF} \times \mathrm{DB})/\mathrm{SF}$$

where UF is the percentage composition of each unsaturated FA, DB is the number of double bonds in that FA and SF is the percentage composition of saturated FAs (Calderón and Eynard 2000).

Testosterone assay

To determine testosterone concentrations, immunoreactivity in mouse plasma samples was measured using a ^{125}I-testosterone radioimmunoassay kit (Total Testosterone, Coat-A-Count; Siemens). The antiserum has less than 5% cross-reactivity with other steroids, except 19-nortestosterone (20%; data provided by Siemens). The interassay CV was <10% and assay sensitivity was 20 ng mL^{-1}.

Fertilisation rate

Fertilisation rate is expressed as the percentage of females that produced a viable litter after confirmation of a vaginal plug. Fertilisation rate was used to evaluate the *in vivo* fertilising ability of the male mice. Mating was conducted 3–5 days before males were euthanised by placing two untreated female albino Swiss mice into a cage with a male mouse. Females were monitored for vaginal plugs on a daily basis and, once the plug was detected, the female was removed from the cage and housed individually. All female mice were fed with the same commercial standard chow during pregnancy. Dams were allowed to deliver spontaneously and both fertilisation rate and litter size were recorded.

Statistical analysis

Data are expressed as the mean ± s.e.m. Results were analysed by two-way repeated-measures analysis of variance (ANOVA) or one-way ANOVA, as appropriate. *Post hoc* testing was performed with Tukey's test. When values did not show Gaussian distribution and/or variance homogeneity, a non-parametric (Kruskal–Wallis) test was used (i.e. when evaluating the percentage of bending spermatozoa). Chi-square test was used to analyse the fertilisation rate. Statistical significance was set at $P < 0.05$. Statistical procedures were performed using Infostat 1.1 (Grupo Infostat, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba).

Results

Food consumption, bodyweight and the weight of adipose depots and organs

There were no significant differences in cumulative maternal food intake during gestation and lactation. Cumulative offspring food intake from PND22 until PND60 was lower in the SOD and SFOD than CD groups (Table 2).

With regard to the bodyweight of male mice in adulthood, those in the SFOD group were significantly heavier than those

Table 3. Bodyweight and the weight of adipose depots and organs in male mice fed a commercial diet (CD), a soy-oil enriched diet (SOD) or a sunflower-oil enriched diet (SFOD) from gestation until adulthood

Results are expressed as the mean ± s.e.m. ($n = 8$ in each group). Within rows, different superscript letters indicate significant differences ($P < 0.05$)

	CD	SOD	SFOD
Bodyweight (g)	33.49 ± 1.12^{a}	35.54 ± 1.06^{ab}	37.62 ± 0.73^{b}
Body length (cm)	7.79 ± 0.11	7.59 ± 0.06	7.16 ± 0.16
Abdominal circumference (cm)	8.15 ± 0.10	8.10 ± 0.09	8.17 ± 0.11
Adipose depots			
Epididymal pad weight (g)	0.30 ± 0.02	0.35 ± 0.03	0.37 ± 0.02
Retroperitoneal pad weight (g)	0.09 ± 0.01	0.11 ± 0.01	0.13 ± 0.01
Subcutaneous pad weight (g)	0.17 ± 0.01	0.18 ± 0.02	0.21 ± 0.01
Organs			
Liver weight (g)	1.69 ± 0.08	1.61 ± 0.09	1.64 ± 0.20
Seminal vesicles weight (g)	0.34 ± 0.03	0.35 ± 0.02	0.36 ± 0.02
Testes weight (g)	0.19 ± 0.01^{ab}	0.21 ± 0.01^{a}	0.18 ± 0.01^{b}
Relative liver weight (%)	5.05 ± 0.16	4.57 ± 0.28	4.36 ± 0.51
Relative seminal vesicle weight (%)	1.00 ± 0.05	0.99 ± 0.03	0.96 ± 0.03
Relative testes weight (%)	0.57 ± 0.03^{a}	0.59 ± 0.02^{a}	0.49 ± 0.01^{b}

in the CD group. Supplementation with soy or sunflower oil had no effect on body length, abdominal circumference, fat pad weight or the absolute or relative weight of the liver or seminal vesicles. However, SFOD feeding resulted in a significant decrease in the absolute weight of the testes compared with feeding of SOD. Relative testicular weight was significantly decreased in the SFOD compared with CD and SOD groups (Table 3).

Sperm functional activity

Sperm smears from the SFOD group showed a significantly reduced percentage of viable spermatozoa compared with the CD and SOD groups and a significant increase in the percentage of immature spermatozoa, not only with a bent shape but also with c.d. (Fig. 1). Concordantly, the percentage of total immature spermatozoa was significantly higher in the SFOD group. Sperm membrane integrity, evaluated using the HOST, was significantly diminished in both the SFOD and SOD groups. There were no significant differences among the three groups in terms of sperm concentration, the percentage of spermatozoa showing progressive motility, the number of spermatozoa with an intact acrosome or DHE-positive spermatozoa (Table 4).

FA profile of the epididymis and spermatozoa

When analysing epididymal lipid content, there was a significant reduction in the saturated FA content of both HFD-fed groups comparing with the CD-fed group. There was a trend for a higher ω6 FA content in the SFOD group, with a reduction in ω3, and the DBI was significantly higher in the SFOD and SOD groups compared with the CD group (Table 5).

Table 6 summarises the lipid content of pooled spermatozoa in each group. These results are not sufficient to describe the composition of the sperm membranes with any certainty. However, the data do indicate that the ω6 : ω3 ratio is higher in both HFD-fed groups than in the CD group.

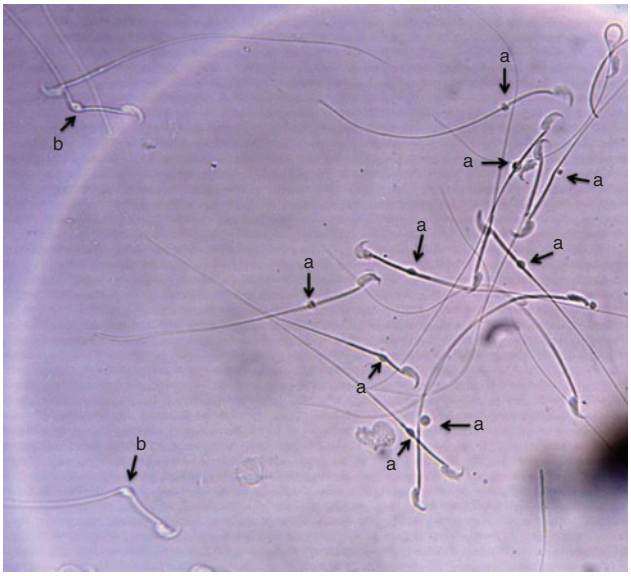

Fig. 1. Immature gametes. Image showing spermatozoa with a persistent cytoplasmic drop (a) and flagellar angularity with a cytoplasmic drop (b).

Testosterone assay

Supplementation with soy or sunflower oil did not affect the serum testosterone concentrations, which were 3.70 ± 0.80, 4.20 ± 1.36 and 2.84 ± 1.28 ng mL^{-1} in the CD, SOD and SFOD groups respectively.

Fertilisation rate

The percentage of untreated female mice that had a viable litter (F_2 generation) as a result of normal mating with CD- or HFD-fed males was significantly lower in the SFOD group (Fig. 2). When analysing litter size, there were no significant differences

Table 4. Functional activity of spermatozoa from male mice fed a commercial diet (CD), a soy-oil enriched diet (SOD) or a sunflower-oil enriched diet (SFOD) from gestation until adulthood

Data show the mean ± s.e.m. ($n = 8$ in each group). Within rows, different superscript letters indicate significant differences ($P < 0.05$). c.d., cytoplasmic droplet; HOST, hypo-osmotic swelling test; DHE, dihydroethidium.

	CD	SOD	SFOD
Concentration ($\times 10^6$ spermatozoa mL^{-1})	19.93 ± 1.46	18.20 ± 3.01	18.43 ± 3.34
Progressive motility (%)	86.25 ± 1.60	88.63 ± 0.96	84.63 ± 1.43
Viability (%)	82.50 ± 1.45[a]	80.63 ± 1.00[a]	76.00 ± 1.35[b]
Bending (%)	16.38 ± 1.28[a]	10.25 ± 1.57[a]	20.00 ± 2.02[b]
c.d. (%)	22.75 ± 1.29[a]	27.38 ± 2.28[a]	36.88 ± 1.89[b]
Bending + c.d.	12.25 ± 3.49	10.37 ± 2.58	15.00 ± 3.88
Total immature spermatozoa (%)	51.38 ± 5.87[a]	48.00 ± 5.72[a]	71.88 ± 7.17[b]
HOST (%)	76.63 ± 3.63[a]	68.63 ± 1.83[b]	68.50 ± 4.64[b]
Intact acrosome (%)	72.50 ± 3.76	70.88 ± 4.41	77.88 ± 5.08
DHE (%)	21.22 ± 7.54	22.89 ± 4.33	23.63 ± 7.61

Table 5. Fatty acid (FA) profile of the epididymides of mice exposed to a commercial diet (CD), a soy-oil enriched diet (SOD) or a sunflower-oil enriched diet (SFOD) from gestation until adulthood

Data show the mean ± s.e.m. ($n = 4$ in each group) of the percentage of total FAs. Within rows, different superscript letters indicate significant differences ($P < 0.05$). Only FAs accounting for more than 0.8% of total FAs are shown. SFA, saturated FA; MUFA, monounsaturated FA; PUFA, polyunsaturated FA. DBI, double bond index (indicating the degree of tissue FA unsaturation)

	CD	SOD	SFOD
16:0	14.74 ± 1.94	14.30 ± 1.94	15.06 ± 1.85
18:0	15.28 ± 2.79	5.18 ± 1.36	1.16 ± 0.15
18:1 ω9	20.64 ± 7.64	29.63 ± 4.70	28.03 ± 3.84
18:2 ω6	27.00 ± 1.67	31.02 ± 1.11	37.51 ± 6.98
18:3 ω3	2.71 ± 0.48	3.89 ± 0.87	3.27 ± 0.98
20:4 ω6	2.45 ± 0.62	2.00 ± 0.58	3.98 ± 1.66
20:5 ω3	2.08 ± 0.13	3.81 ± 2.02	0.84 ± 0.08
Total SFA	32.84 ± 7.13[a]	19.06 ± 1.41[b]	17.26 ± 3.21[b]
Total MUFA	28.50 ± 7.77	35.29 ± 3.96	35.07 ± 3.21
Total PUFA	37.74 ± 2.27	43.11 ± 3.33	46.53 ± 2.14
Total ω6	31.18 ± 1.88	35.42 ± 2.46	41.87 ± 3.36
Total ω3	6.56 ± 0.39	7.69 ± 1.49	4.66 ± 0.53
ω6 : ω3 ratio	4.75 ± 0.05[a]	5.11 ± 1.11[a]	8.89 ± 0.84[b]
DBI	4.09 ± 0.89[a]	7.31 ± 0.43[b]	8.53 ± 1.05[b]

Table 6. Sperm fatty acid (FA) profile of mice exposed to commercial diet (CD), a soy-oil enriched diet (SOD) or a sunflower-oil enriched diet (SFOD) from gestation until adulthood

Values show the percentage of total FAs obtained from a pool of two samples (two animals for each sample). Only FAs accounting for more than 0.5% of total FAs are shown. SFA, saturated FA; MUFA, monounsaturated FA; EFA, essential FA; LA, linoleic acid; ALA, α-linolenic acid; PUFA, polyunsaturated FA. DBI, double bond index (indicating the degree of membrane FA unsaturation)

	CD	SOD	SFOD
16:0	14.86	24.24	23.59
18:0	14.26	26.97	21.85
18:1 ω9	9.33	16.97	11.47
18:2 ω6 (LA)	7.82	12.05	16.85
18:3 ω3 (ALA)	4.28	2.97	5.26
20:4 ω6	5.79	6.76	6.23
20:5 ω3	2.82	1.10	2.85
22:5 ω6	6.11	0.57	0.79
22:6 ω3	3.87	1.24	1.38
Total SFA	43.34	51.93	48.55
Total MUFA	23.01	23.16	17.31
Total EFA (LA + ALA)	12.1	15.02	22.11
Total PUFA	33.65	24.91	34.14
Total ω6	19.72	19.78	24.64
Total ω3	10.97	5.31	9.49
ω6 : ω3 ratio	1.79	3.72	2.59
EFA/PUFA	0.35	0.60	0.64
DBI	2.97	1.78	2.31

between groups (litter size 13.27 ± 0.67, 14.25 ± 0.40 and 14.10 ± 0.38 in the CD, SOD and SFOD groups respectively).

Discussion

The purpose of this study was to evaluate the effect of different HFD enriched with two of the vegetable oils most frequently used in Western diets and animal chows: sunflower and soybean oils. The ω6 : ω3 ratios of the diets supplemented with these oils are quite different: SFOD has the highest ratio (51.55), whereas the ratio in the SOD is more balanced (9.62). When the absolute amount of LA is considered, both SFOD and SOD contain excessive amounts. In terms of ALA, according to Reeves *et al.* (1993), the amount in the SOD is sufficient, whereas the SFOD and CD are extremely poor in ALA. All these factors are important because there are close links among energy metabolism, nutritional status and reproductive physiology (Wathes *et al.* 2007).

In the present study, dams (F_0 generation) and their offspring (F_1 generation) were exposed to HFD (SFOD or SOD) with PUFA concentrations >50% of total FAs. The main results indicate that although both enriched diets are high in fat, only the

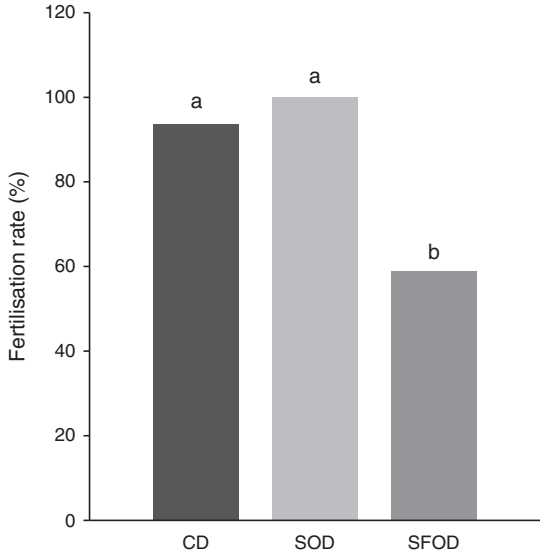

Fig. 2. Fertilisation rate expressed as percentage of males that gave rise to a viable litter after mating ($n = 16$ dams in each group). CD, control diet; SOD, soy oil-enriched diet; SFOD, sunflower oil-enriched diet. Different letters above the columns indicate significant differences ($P < 0.05$).

SFOD promoted significant bodyweight gain and changed reproductive success, especially via alterations in sperm maturation. The deleterious effects of obesity on sperm physiology are already widely documented (Teerds *et al.* 2011; Palmer *et al.* 2012). No differences were found in maternal food intake during gestation and lactation, despite more calories from fat in both enriched diets, as reported by Santillán *et al.* (2010). Nevertheless, there was a lower food intake in pups from supplemented groups in the post-weaning period, probably due to the higher FA dietary content. Variations in plasma FA concentrations are detected by neurons in specific brain areas, such as the ventromedial hypothalamus, and act as a food intake regulator. The FA-sensing process in the hypothalamus involves the FA translocase FAT/CD36 (Moullé *et al.* 2013). It has been proved that this transporter is altered by aging in skeletal muscle (Romic *et al.* 2017); this could explain the lack of maternal satiety in response to high dietary FA levels.

In the SFOD group, bodyweight was higher than in the control group, with a slight increase in fat pad weight. A high ω6 FA intake and a high ω6 : ω3 ratio are associated with weight gain in both animals and humans, and have been identified as possible factors contributing to the increase in obesity and inflammatory diseases associated with metabolic syndrome among people in industrialised countries (Simopoulos 2008), whereas a high ω3 FA intake decreases the risk of weight gain (Muhlhausler 2011). A decrease in the LA : ALA ratio in animal diets prevents overweight and obesity (Simopoulos 2016). The fact that only SFOD produced an increase in bodyweight, despite both enriched diets being high in fat and having a similar net content of ω6 FA, highlights the importance of the ω6 : ω3 ratio, the quality of the FA that generates the excess fats, its relationship with other FAs and the type of alteration that may occur in the body, as described by Williams *et al.* (2014).

With regard to body length, we observed in a previous study that mice in the SFOD group were shorter at weaning (Santillán *et al.* 2010). However, these animals demonstrated post-weaning catch-up growth, reaching a similar length to pups in the other groups in adulthood, although they remained slightly shorter. Most studies relating HFD and body length describe an increase in body length (Lanham *et al.* 2010; Dunn and Bale 2009); this apparent discrepancy could be due to the diets used in these previous studies, which were higher in lipid content (18−45%) and rich in saturated fats.

With regard to organ weight and relative organ weight, there was a reduction in absolute and relative testes weights in the SFOD group, with the latter being significantly reduced compared with the SOD and CD groups. This means that even though bodyweight was highest in the SFOD group, it was not related to testicular growth; on the contrary, the testes in this group were the lightest. In the present study, mice were fed LA and ALA, which are subsequently converted into LC-PUFAs through a series of elongation and desaturation steps performed by distinct enzymes. Chain elongation is controlled by elongation of very long chain fatty acids (ELOVL) enzymes (Mandal *et al.* 2004). A study with *Elovl2*$^{+/-}$ and *Elovl2*$^{-/-}$ mice (Zadravec *et al.* 2011) presented evidence that ELOVL2 is essential for the formation of PUFAs in the testis and that these FAs are required for normal spermatogenesis and fertility. These knockout mice also exhibited marked hypogonadism, with testes weight reduced by 60% compared with control mice (Zadravec *et al.* 2011). This phenotype is in accordance with the reduction in testes weight and reproductive performance found in the SFOD group in the present study; for this reason, we wonder whether such high concentrations of ω6 in this group could have inhibited this enzyme.

At this point, it is necessary to consider that although in the present study the mice were fed with the diet from weaning, they received the same FAs through their mothers during pregnancy and lactation. Considering that the proportion of different PUFAs in the cell membranes reflects the amount in which they are consumed in the diet, its manipulation may affect the composition of these membranes and modify many processes involved in reproduction, not only those related to the biosynthesis of eicosanoids and steroids (Simopoulos 1991; Wathes *et al.* 2007; Kirkup *et al.* 2010) but also in spermatogenesis, spermiogenesis and fertilising ability.

The lipid composition of the sperm plasma membrane plays an important role in determining sperm membrane fluidity, as well as sperm motility and viability (Miller *et al.* 2004). Characteristics of the plasma membrane showed functional alterations in the two supplemented groups in the present study, as evidenced by the diminished response to the HOST in both these groups. As demonstrated previously by Jones (1998), substantial changes take place in the lipid composition of spermatozoa during epididymal maturation and lead to an overall increase in the proportion of unsaturated phospholipids in the sperm plasma membranes. The findings of the present study, although they are just a first approximation, show that the higher PUFA content of oil-enriched diets is reflected in the FA composition of epididymal tissue of mice in the SOD and SFOD groups, with the highest ω6 content, ω6 : ω3 ratio and DBI in the

SFOD group. Spermatozoa from this group also seem to have the highest ω6 content, and this could be related to the changes in the maturation process observed here. Chromatographic analysis of sperm FA composition shows that the proportion of essential FAs (LA + ALA) of total PUFAs is greater in supplemented groups, suggesting a lower conversion to LC-PUFAs. However, these observations are not conclusive and further studies are necessary to clarify whether the conversion rate is actually involved.

Membrane integrity is essential to maintain sperm volume. In certain cases of infertility in domestic species and in the homozygous c-ros tyrosine kinase-knockout mouse, males are infertile as a result of unopposed sperm swelling. This induces flagellar angulation, preventing the normal migration of spermatozoa in the female tract (Yeung *et al.* 2000, 2002). Cooper and Yeung (2003) have postulated that several small water-soluble components of the epididymal fluid are taken up by spermatozoa during post-testicular maturation to act as a reserve of intracellular osmolytes against the osmotic challenges that spermatozoa experience at ejaculation. The ω6-enriched diets in the present study probably generated alterations that affected the capacity of the membrane to regulate the osmotic changes that the spermatozoa undergo after ejaculation. In the SOD group, with a healthier ω6 : ω3 ratio, membrane integrity was the only parameter adversely affected.

In the SFOD group, vitality and maturity were also altered. The former can be directly related to the membrane damage described above. The signs of immaturity were evidenced by an increase in angulated flagella and cytoplasmic droplets. As a result of spermatogenesis and epididymal maturation, male germ cells differentiate to become fully functional spermatozoa. During the last maturation phase of spermiogenesis, the Sertoli cells extrude and phagocytose most of the germ cell cytoplasm as 'residual bodies', the remnants of which become the cytoplasmic droplets. Cytoplasmic extrusion, along with various other maturation steps, is essential for the zona pellucida-binding capacity and fertilisation potential of spermatozoa (Rengan *et al.* 2012). Prominent among the hallmarks of epididymal sperm maturation is the proximal–distal migration of the cytoplasmic droplets. Yeung *et al.* (1999) considered that angulation always occurs at the site of the cytoplasmic droplets, located in mature spermatozoa at the annulus, and spermatozoa exhibit pronounced angulation of the tail following ejaculation due to their inability to osmoregulate. In the present study, the HOST demonstrated failures in the osmoregulation process, and this may also be the cause of the increase in cytoplasmic droplets.

The association of higher percentages of bent spermatozoa and retention of cytoplasmic droplets in the SFOD group possibly occurred because in non-human mammalian spermatozoa these droplets can induce swelling and flagellar angulations, inhibiting progressive motility and being associated with infertility (Cooper *et al.* 2004). Yeung *et al.* (2000) also demonstrated that the presence of flagellar angulation is related to a reduction in fertility, arguing that the angulation indicates an increased cell volume. Failure to shed the cytoplasmic droplet has also been associated with male infertility (Amann *et al.* 2000; Mak *et al.* 2000; Aitken 2004). The fact that droplet loss at ejaculation is necessary for fertility is suggested by the association of subfertility or infertility in certain transgenic infertile male mouse models in which flagellar angulation occurs at the site of the retained droplet (Cooper *et al.* 2004; Hinton and Cooper 2010). The retention of cytoplasmic droplets on ejaculated spermatozoa is also associated with infertility in bulls (Amann *et al.* 2000) and pigs (Kuster *et al.* 2004). The infertility of spermatozoa with droplets has been suggested to reflect poor adherence to the zona pellucida and oviductal epithelium. Because spermatozoa with retroflected flagella swim 'backwards' (head against the direction of travel), it is equally likely that their passage through the mucus or uterotubal junction is hampered and the oocyte is never reached, as is the case for the c-ros-knockout mice (Cooper 2011).

In the present study, there were no differences in sperm concentration, motility or acrosome reaction in the SFOD compared with CD group, but the lack of vitality and aberrant sperm maturation contributed to the reduced fertility in the former group. For this reason, we propose that the high ω6 : ω3 ratio did not affect spermatogenesis, but altered epididymal sperm maturation.

In addition to the previous analysis, it is important to consider oxidative stress. Normally, sperm mitochondria produce low levels of ROS (Aitken and De Iuliis 2010). Physiological levels of ROS trigger and modulate tyrosine phosphorylation, which elicits vital functions like capacitation and acrosome reaction. An excess of ROS impairs sperm cell function and plays a negative role in male fertility (Agarwal *et al.* 2003). The ω6 FAs are well known because of their high oxidative function and ω3 FAs are considered potentially important antioxidants (Richard *et al.* 2008). Furthermore, there is considerable evidence that HFDs also produce elevated oxidative stress in spermatozoa and increase sperm DNA damage, which leads to negative effects on reproductive performance (Bakos *et al.* 2011; Fullston *et al.* 2012). Other studies have reported a detrimental effect of PUFA supplementation on sperm parameters when they are used without the addition of an antioxidant, such as vitamin E (de Graaf *et al.* 2007). Despite all the above evidence, our evaluation of sperm ROS production showed that the processes by which dietary oil supplementation altered sperm quality did not include changes in oxidative status. Additional research to elucidate the mechanisms by which diet manipulation affects sperm membranes and subsequent sperm quality is warranted. The ω6 : ω3 ratio in the SOD group was lower than that in the SFOD group, which seems to have a safety profile to prevent ω6 damage. Safarinejad *et al.* (2010) confirmed the beneficial effects of higher concentrations of ω3 in spermatozoa on semen parameters, as well as on the antioxidant status of seminal plasma.

As mentioned above, manipulation of dietary lipids may affect processes involved in the biosynthesis of eicosanoids and steroids. Frungieri *et al.* (2015) reported that prostaglandins (PGs) are synthesised through the action of the rate-limiting enzyme cyclo-oxygenase (COX) and further specific enzymes. The two key somatic cell types in the testis, Leydig and Sertoli cells, express the inducible isoenzyme COX2 and produce PGs, which modulate steroidogenesis in Leydig cells and glucose uptake in Sertoli cells. Hence, the COX2/PG system in Leydig and Sertoli cells acts as a local modulator of testicular activity,

and consequently may regulate spermatogenic efficiency. In addition to its expression in Leydig and Sertoli cells, COX2 has been detected in the seminiferous tubule wall, as well as in testicular macrophages and mast cells of infertile patients (Frungieri *et al.* 2015). Collectively, these data indicate that the COX2/PG system plays crucial roles not only in testicular physiology (i.e. development, steroidogenesis and spermatogenesis), but also, more importantly, in the pathogenesis or maintenance of infertility status in the male gonads. LA and ALA act as precursors and compete for the same enzymes. They produce eicosanoids that have different biological functions, being affected by different LA : ALA ratios in the diet (Smith and Langenbach 2001; Wathes *et al.* 2007; Kirkup *et al.* 2010). According to these findings, the synthesis of PGs would be more markedly affected in the SFOD than SOD group because of the higher LA : ALA ratio, altering processes specially involved in maturation and fertilisation.

With regard to plasma testosterone concentrations, it is well known that in rodents high fat diets increase the androgen content, presumably acting directly at the gonad level (Meikle *et al.* 1989; Dorgan *et al.* 1996; Clinton *et al.* 1997; Gromadzka-Ostrowska 2006). It is proposed that the mechanisms involved are changes in cell membrane lipid composition, which may affect LH binding to its receptor, activation of the adenylyl cyclase system (Sebokova *et al.* 1990) and modifications in testis steroidogenic enzyme activities (Meikle *et al.* 1989). Although some authors have proposed that ω6 PUFAs stimulate testosterone synthesis in Leydig cells (Catala 2007) and 17β-hydroxysteroid dehydrogenase activity in the rat testes (Cao *et al.* 2004), others have suggested the opposite effect. Astiz *et al.* (2012) demonstrated that ω6 FAs stimulate lipid peroxidation and inhibit the steroidogenic activities of enzymes like 3β- and 17β-hydroxysteroid dehydrogenases. In the present study, we did not find significant differences in plasma testosterone concentrations in mice from the CD, SOD or SFOD groups. Because Leydig cells are very rich in PUFAs (Coniglio 1994) and arachidonic acid (ARA) is directly linked to the androgenic activity of Leydig cells (Payne and Hales 2004; Cano *et al.* 2006), the oxidative stress described previously may be an important mechanism for the ARA-dependent loss of steroidogenic ability, because this FA is essential for the biological activity of the steroidogenic acute regulatory protein (StAR; Maloberti *et al.* 2007).

The mechanisms underlying HFD-induced male spermatogenesis deficiency remain unclear. The results of the present study showed that the higher dietary fat content itself is not enough to produce an increase in bodyweight and impaired fertility, and these changes are observed when the former is combined with an excessive ω6 : ω3 ratio.

Conflict of interest

The authors declare no conflicts of interest.

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