A high-fat diet fed during different periods of life impairs steroidogenesis of rat Leydig cells

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Abstract

This study evaluated the impact of a high-fat diet (HFD) during different stages of rat life, associated or not with maternal obesity, on the content of sex steroid hormones and morphophysiology of Leydig cells. The following periods of development were examined: gestation (O1), gestation and lactation (O2), from weaning to adulthood (O3), from lactation to adulthood (O4), gestation to adulthood (O5), and after sexual maturation (O6). The HFD contained 20% unsaturated fat, whereas the control diet had 4% fat. Maternal obesity was induced by feeding HFD 15 weeks before mating. All HFD groups presented increased body weight, hyperinsulinemia and reduced insulin sensitivity. Except for O1, all HFD groups exhibited a higher adiposity index, hyperleptinemia, reduced testosterone and estradiol testicular levels, and decreased testicular 17β-HSD enzyme. Morphometrical analyses indicated atrophy of Leydig cells in the O2 group. Myelin vesicles were observed in the mitochondrial matrix of Leydig cells in O3, O4, O5 and O6, and autophagosomes containing mitochondria were found in O5 and O6. In conclusion, HFD feeding, before or after sexual maturation, reduces the functional capacity of rat Leydig cells. Maternal obesity associated with HFD during pregnancy/lactation prejudices Leydig cell steroidogenesis and induces its atrophy in adulthood, even if it is replaced by a conventional diet at later stages of life. Regardless of the life period of exposure to HFD, deregulation of leptin is the main factor related to steroidogenic impairment of Leydig cells, and, in groups exposed for longer periods (O3, O4, O5 and O6), this is worsened by structural damage and mitochondrial degeneration of these cells.

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Introduction

Overweight and obesity in adulthood impairs gonadal steroidogenesis and fertility in both genders (Pasquali et al. 2007, Cano et al. 2008, Hammoud et al. 2008, MacDonald et al. 2010, Fernandez et al. 2011). In males, obesity alters the hypothalamic–pituitary–gonadal axis, reducing testosterone production, which in turn impairs sperm production (Pasquali et al. 2007, Hammoud et al. 2008, MacDonald et al. 2010, Reame et al. 2014, Mohammadi Roushandeh et al. 2015). Experimental studies of obesity induction by high-fat diet (HFD) feeding have shown that reduction in circulating testosterone levels was associated with a decreased amount and quality of sperm (MacDonald et al. 2010, Fernandez et al. 2011, Reame et al. 2014). McVey et al. (2007) compared the consequences of different sources of dietary lipid during sexual maturation of rats and observed that soybean oil increased serum testosterone levels in adulthood, whereas lard and polyunsaturated fat docosahexaenoic acid had no effect. Ingestion of lard in a normolipidic diet, during sexual maturation, resulted in a marked increase in circulating testosterone levels and prostate growth in adult rats (Escobar et al. 2009). It is thus known that both obesity and certain dietary fats can modulate the metabolism of testosterone; however, the mechanisms underlying these alterations seem to be variable depending on the life period and lipid type.

Both acute and chronic insults during gestation may result in permanent adaptive responses of various system organs of the offspring, at structural and physiological levels, and favor the development of diseases in adult life, a phenomenon known as developmental programming (Armitage et al. 2005, Symonds 2007, White et al. 2009). Studies on both humans and experimental animals have indicated that excessive maternal diet may induce the developmental programming of offspring and result in a spectrum of metabolic alterations that closely resemble...
the metabolic syndrome or obesity (Armitage et al. 2005, Catalano & Ehrenberg 2006). Moreover, laboratory models of maternal fat overnutrition have been widely used in the investigation of offspring metabolic outcomes in adult life (Armitage et al. 2005, Symonds 2007, 2009, White et al. 2009), but there is a paucity of information on the effects of maternal obesity or excessive maternal fat on the testicular development and reproductive functions of the adult offspring (Ramlau-Hansen et al. 2007, Christante et al. 2013). We have shown that maternal obesity and HFD consumption during gestation temporarily alter the development of gonocytes and also disturb neonatal steroidogenesis in the first days of life in rats (Christante et al. 2013). Although hormonal changes and germ cell development apparently recovered by the age of 14 days in this study, it is possible that they caused permanent adaptations in testicular function. Indeed, in a recent study comparing the effects of an obesogenic environment caused by HFD consumption at different stages of development, our group showed that the sperm damage was proportional to the metabolic impairment and the decline in circulating testosterone levels (Reame et al. 2014). In addition, we also demonstrated that HFD exposure during different periods of ontogenetic development leads to prostate hypertrophy (Pytlowanciv et al. 2016). It is thus relevant to compare the mechanisms underlying this steroidalogenic impairment during different life periods as a result of exposure to excessive dietary fat.

The differentiation of the adult Leydig cell (ALC) population in rodents (Mendis-Handagama & Ariyaratne 2001, Wu et al. 2007, Teerds & Huhtaniemi 2015) and primates (Prince 2001, Svechnikov et al. 2010, Teerds & Huhtaniemi 2015) starts during the prepubertal period and involves the progressive differentiation of progenitors and immature and mature Leydig cells. The steps of ALC differentiation are regulated by luteinizing hormone and follicle-stimulating hormone, and also by locally produced growth factors such as transforming growth factors α and β, insulin-like growth factor 1 and platelet-derived growth factor-A (Mendis-Handagama & Ariyaratne 2001, Haider 2004). As regards the sexual steroids, some investigations indicate that testosterone and estrogen are inhibitory stimuli in some stages of Leydig cell development (Mendis-Handagama & Ariyaratne 2001, Haider 2004). In adult testis, the constant number of Leydig cells is maintained by the inhibitory action of these hormones on the differentiation of precursors to Leydig cells. On the other hand, androgens are required by Leydig cell progenitors to differentiate into mature ALC (Mendis-Handagama & Ariyaratne 2001, Haider 2004). Due to the complexity of these regulatory pathways, there is no conclusive evidence about how maternal obesity or excessive dietary fat at different stages of postnatal life could affect the development and function of ALC population. Considering the increasing incidence of overweight/obesity in women at reproductive age, as well as excessive fat consumption during pregnancy in worldwide population, experimental studies are of great relevance in understanding the intergenerational effects of excessive dietary fat on the steroidalogenic capacity of Leydig cells in adulthood.

This study therefore comparatively examined the effects of consuming excessive dietary fat during different stages of rat development, whether or not associated with maternal obesity, on the cellular organization of testis interstitial tissue and the steroidalogenic capacity of Leydig cells. The morphological and steroidalogenic changes observed in Leydig cells were associated with the metabolic conditions related to obesity, such as adiposity level, hyperleptinemia, hyperinsulinemia, insulin sensitivity, hyperglycemia and dyslipidemia.

### Materials and methods

#### Animals and experimental design

Wistar rats were purchased from the Animal Breeding Center of the University of Campinas. The animals were kept in the animal house of the Institute of Biosciences, Letters and Exact Sciences of UNESP, São José do Rio Preto (SP), in polyethylene boxes with wood shaving substrate under 12 h light:12 h darkness cycle (lights on at 06:00 h) at an average temperature of 23 ± 2°C and fed with a chow (as detailed below) and filtered water *ad libitum*. Animal care and experiments were performed in accordance with the Ethical Principles in Animal Experimentation adopted by the National Council for Control in Animal Experimentation (CONCEA) and approved by the local Ethics Committee in Animal Use (CEUA/IBILCE protocol no. 22/2009).

Induction of obesity was achieved by feeding rats with a HFD, which was standardized by the Experimental Laboratory of Clinical Medicine of Botucatu Medical School – UNESP and purchased from Agroceres (Rio Claro, SP, Brazil). This diet contains 20% unsaturated lipids in relation to the total fat amount and its composition has been described previously (Nascimento et al. 2008). According to the information from the suppliers, confirmed in previous experiments (Nascimento et al. 2008), the time required for excessive body weight gain and increased adiposity is around 15 weeks of feeding with this diet.

Two experiments were performed, with a total of eight experimental groups (Fig. 1). In Experiment 1, 30-day-old females were randomly divided into two groups and fed with a balanced diet or a HFD for 15 consecutive weeks in order to induce obesity. The normal and obese females were then mated with normal male rats of equivalent age, in a proportion 1:1, at nighttime. Day zero of gestation was determined with a vaginal smear to confirm the presence of sperm. During gestation, the normal and obese females were kept in individual boxes and continued to be fed with the same diet described previously, the balanced diet or the HFD. The litter kept with obese mothers was adjusted to the number of five pups (predominantly males) per...
mother at birth to induce overfeeding during lactation. The remaining pups were killed by direct decapitation. The following contexts defined the experimental groups (n=15 per group, one animal per family) (Fig. 1): C1 group – control (offspring from normal mothers, fed with a balanced diet); O1 group – indirect exposure to HFD during gestation (offspring from obese mothers, group obtained through cross fostering: Chernoff et al. 2009); O2 group – indirect exposure to HFD during gestation and lactation periods (offspring from obese mothers, subjected to overfeeding during lactation period); O3 group – fed HFD from weaning to sexual maturity (offspring from normal mothers); O4 group – indirect exposure to HFD during lactation and fed HFD from weaning to sexual maturity (offspring from normal mothers, subjected to overfeeding during lactation period, group obtained through cross fostering: Chernoff et al. 2009); and O5 group – indirect exposure to HFD during gestation and lactation periods and fed HFD from weaning to sexual maturity (offspring from obese mothers, subjected to overfeeding during lactation period). In Experiment 2, adult rats (12 weeks old) were fed a HFD for 15 consecutive weeks (O6 group) or remained with a balanced diet (C2 group – control). The rats in Experiments 1 and 2 were killed at 18 and 27 weeks of age respectively.

At the end of each experimental period, rats whose testicles were used for morphological analyses (n=8 per group) were perfused as described previously by Sprando (1977). Briefly, rats were anesthetized with ketamine (800μL/kg body mass (b.m.)) and xylazine (200μL/kg b.m.), then received via the aorta, heparinized saline with Bovin’s fixative or 2.5% glutaraldehyde solution, 1% tannic acid, 3.5% sucrose and 5mM calcium chloride in cacodylate buffer 0.1 M, pH 7.4. The remaining rats were killed by CO₂ inhalation followed by decapitation for blood collection. The testes of these animals were used for other techniques (n=7 per group). The wet weight of the testis was determined and the gonadosomatic index (GSI) was calculated, based on the body and testicular masses, using the following formula: GSI = ((testicular weight/body weight) × 100).

Figure 1 Schematic representation of experimental design used in the study. The upper part of the scheme exhibits the groups related to Experiment 1 (E1) – C1, O1, O2, O3, O4 and O5 (groups exposed to high-fat diet during different periods before sexual maturation), and the lower part of the scheme shows the groups belonging to Experiment 2 (E2) – C2 and O6 (groups exposed to high-fat diet after sexual maturity). Groups were divided according to the period of exposure to high-fat diet. *groups exposed to overfeeding during lactation; †end of the experiment and death of the animals.

Characterization of the metabolic state

For the characterization of overweight/obesity, the following parameters were assessed: body weight and abdominal circumference, measured to determine the abdominal fat deposition inference; adiposity index, obtained by the formula (sum of retroperitoneal, epididymal and visceral fat/body weight) × 100), according to Taylor and Phillips (1996); and serum leptin levels, quantified by ELISA Capture/Sandwich (antibody-antigen-antibody) using specific commercial kits – Enzo Life Sciences International (Farmingdale, NY, USA), with a minimal sensitivity of 67.2 pg/mL. Absorbance readings were taken using a SpectraMax Plus 384 reader (Molecular Devices, Sunnyvale, CA, USA).

The following glucose and lipid metabolism-related parameters were also evaluated: blood glucose levels, quantified by a glucometer (AccuChek Active; Roche Diagnostics); serum insulin levels, quantified by radioimmunoassay using specific commercial kits (Little Chalfont, Buckinghamshire, UK); insulin sensitivity, determined by the constant rate of glucose decay after an intraperitoneal insulin tolerance test (ipITT) according to Rafacho et al. (2008); serum lipid profile, evaluated by the quantification of total cholesterol, high-density lipoproteins (HDL), low-density lipoprotein and serum triglyceride levels using commercial kits (In vitro Diagnostica Ltda, Itabira, MG, Brazil).
Serum and intratesticular sex steroid hormone contents

Blood samples were collected from cervical vessels, centrifuged at 1200g for serum separation and stored at −80°C for subsequent analysis. Fragments of testicular parenchyma without the capsule were removed, weighed and stored at −80°C. They were subsequently processed for steroid extraction as follows: the fragments were homogenized in PBS and incubated with diethyl ether for 10 min at room temperature. Soon afterward, the upper-phase solution was transferred to another container and stored. Again, the fragments of homogenate were incubated with diethyl ether for 10 min in dry ice. In the same way, the upper-phase solution was transferred to the same tube with the previously stored solution. The tubes containing this...
solution were left overnight in the linked-flow chamber. After the complete evaporation of the diethyl ether, the material was resuspended in PBS and stored at –80°C for subsequent analysis of intratubular testosterone and estradiol content. Both circulating and intratubular testosterone and estradiol levels were quantified by ELISA capture/sandwich (antibody-antigen-antibody) using specific commercial kits (R&D Systems). The testosterone kit has a minimal sensitivity of 0.03 ng/mL and the estradiol one 4.84 pg/mL. Readings were taken as described above. From each experimental group, seven animals were used, and each sample was analyzed in triplicate.

Table 1 Relative (%) and absolute (mm³) volume of the seminiferous tubules and interstitial components (Leydig cells, macrophages, peritubular cells, blood vessels and lymphatic space) of the C1, O1, O2, O3, O4 and O5 groups (Experiment 1) and the C2 and O6 groups (Experiment 2).

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Values represent mean ± s.d.

*Mandatory difference among the groups C1, O2, O3, O4 and O5; *significant difference between the groups C2 and O6. (P ≤ 0.05).

Morphological analyses

**Light microscopy processing**

After perfusion, the testes were removed, fragmented and immersed in Bouin’s solution for 6 h. These fragments were washed for several days in 70% alcohol to remove picric acid and then processed for histochemistry including Leica Historesin embedding kit (Nussloch, Germany) and ParaPlast (Merck). This was followed by a controlled routine of histological processing to minimize the possible variations of immunohistochemistry reactions. The histochemistry sections (3 μm thick) were stained with hematoxylin–eosin for general morphological studies and quantitative analyses. The histological paraffin sections (4 μm thick) were submitted to immunohistochemistry. The slides were analyzed and photomicrographed in a Leica-Jenaval light microscope, coupled with a CCD digital camera and a system of acquisition and images.

**Transmission electron microscopy**

After perfusion, the testicular fragments were fixed by immersion in 2.5% glutaraldehyde solution, 1% tannic acid, 3.5% sucrose and 3 mM calcium chloride in cacodylate buffer 0.1 M pH 7.4 at 4°C. After 1 h in this solution, they were cut into smaller fragments and fixed for a further 1 h in the same solution. The fragments were washed and post-fixed in aqueous solution of 1% osmium tetroxide, dehydrated in acetone and embedded in Araldite (Electron Microscopy Sciences, Hatfield, PA, USA). The ultrathin sections were collected in copper grids, contrasted with uranyl acetate and lead citrate, and examined in the transmission electron microscope, LEO 906 (Zeiss). Considering previous studies showing discrepancies in Leydig cells near seminiferous tubules at different stages of pregnancy to adult life, the seminiferous epithelium was followed by a controlled routine of histological processing was followed by a controlled routine of histological processing.
Testicular volume was considered.

**Quantitative analyses**

Stereologic analyses were conducted in order to determine changes in the relative and absolute volume of Leydig cells. The relative volume (%) of Leydig cells and other components of the interstitial tissue (macrophages, peritubular cells, blood vessels and lymphatic space) was obtained by the method of point counting (Weibel 1979) on the scanned images at 400× magnification, resulting from historesin sections. A total of 30 fields were used per group (three blocks/rat, one cut/block and two fields/section). The relative volume, expressed as a percentage, was calculated from the number of points on the Leydig cells in relation to the total number of points in the analyzed area (Ariyaratne & Mendis-Handagama 2000). The absolute volume (mm³) of Leydig cells and other components of the interstitial tissue (macrophages, peritubular cells, blood vessels and lymphatic space) was obtained by the product of relative volume and the testicular volume (Ariyaratne & Mendis-Handagama 2000). Testicular volume was considered equal to its wet weight (Weibel 1979).

The cytoplasmic and nuclear area (µm²) and the nucleus:cytoplasm ratio of Leydig cells were determined from digitized ultrastructural images using Image ProPlus software (Media Cybernetics, Rockville, MD, USA). A total of 30 images were used per group.

**Immunohistochemistry analyses**

Histological sections of rat testes were subjected to immunohistochemistry reactions with specific antibodies to detect the presence and distribution of 17ß-hydroxysteroid dehydrogenase (17ß-HSD) and aromatase (CYP19) enzymes. The sections were immersed in citrate buffer pH 6.0 and heated at 98°C for 15 min for antigen retrieval. The blocking of endogenous peroxidase activity was performed by treating the sections with 3% H2O2 in methanol for 20 min. The blocking of nonspecific protein interactions was then performed using 4% bovine albumin in PBS and normal horse serum. Next, the sections were incubated overnight with the following polyclonal rabbit primary antibodies, from Santa Cruz Biotechnology: 17ß-HSD (sc-32872) and CYP19 (sc-30086), at 4°C in 1:100 dilution in PBS containing 1% BSA. After being washed in PBS and incubated with appropriate secondary antibodies (Santa Cruz Biotechnology), the sections were incubated with avidin/biotin conjugated to peroxidase (ABC Staining System; Santa Cruz Biotechnology) for 45 min. The reaction was visualized after diaminobenzidine incubation for 3 min and counterstaining with Harris hematoxylin.

The negative controls for all the immunohistochemistry reactions with specific antibodies to detect the presence and distribution of 17ß-HSD and aromatase was quantified by Western blotting (n=4). The tissue was homogenized in cold buffer (10mmol/L Tris–HCL buffer pH 7.6 containing 

**Analysis of protein content by Western blotting**

The protein content of 17ß-HSD and aromatase was quantified by Western blotting (n=4). The tissue was homogenized in cold buffer (10mmol/L Tris–HCL buffer pH 7.6 containing
Leydig cells after exposure to high-fat diet

5 mmol/L EDTA, 3 mmol/L EGTA, 250 mmol/L sucrose and protease inhibitor cocktail). The homogenate was centrifuged at 18,000 g for 20 min at 4°C to precipitate membranes. Aliquots of protein were denatured under 95°C for 10 min with Laemli and β-mercaptoethanol (Bio-Rad). Proteins weighing 50 μg were separated on 10% SDS-PAGE and, after electrophoresis, transferred to a nitrocellulose membrane. After blocking nonspecific protein binding in 3% skimmed milk in a Tris buffer containing 0.2% Tween 20 (TBST) for 30 min at room temperature, membranes were subsequently incubated overnight with the primary antibodies, purchased from Santa Cruz Biotechnology: 17β-HSD (sc-32872) and CYP19 (sc-30086) at a dilution of 1:400 in TBST. After washing, membranes were incubated with specific secondary antibodies at a dilution of 1:4000 in TBST for 1 h. The immunoreactive components were revealed with an ECL chemiluminescent detection kit (GE Healthcare). The membranes were then exposed to radiographic films and the density of the bands was analyzed by densitometry program – ImageJ software. As an internal control, each membrane was recovered from the first antibody binding and incubated with β-actin.

Statistical analysis

Statistical analysis was performed among groups of each experiment using Statistica 9.0 software (Statsoft Inc, Tulsa, OK, USA). After checking normalization of the data with Kolmogorov–Smirnov test, parametric data were compared by applying one-way ANOVA followed by Tukey’s post hoc analysis (Experiment 1) or Student’s t-test (Experiment 2). When homoscedasticity was not achieved, nonparametric data were compared by applying the Kruskal–Wallis test followed by Dunn’s post hoc analysis (Experiment 1) or the Mann–Whitney test (Experiment 2). In addition, a correlation test was performed, with coefficient denoted by r, for all groups using the Pearson test for parametric data and the Spearman test for nonparametric data. Data were expressed as mean ± standard deviation and P < 0.05 was considered statistically significant.

Results

Metabolic overview

As regards Experiment 1, an increase in body weight and waist circumference was found in animals of all HFD groups (O1, O2, O3, O4 and O5 groups) in relation to control (C1; Fig. 2A). Rats exposed during the gestation period alone (O1) showed no difference in adiposity index compared with control (C1); however, a progressive increase in adiposity index was noted throughout the experimental periods, and the highest index was found in the group with prolonged exposure: from gestation to adulthood (O5). Serum leptin levels were unchanged in O1 group but increased in O2 group, being even higher in O3, O4 and O5 groups (Fig. 2B). The rats in O3, O4 and O5 groups showed increased fasting blood glucose levels (Fig. 3A). Serum insulin levels were enhanced in O1, O2, O3 and O4 groups, and this increase was higher in the O5 group (Fig. 3B). The ipITT for these groups showed that, even after an acute insulin dose, there was a delay in reducing blood glucose levels in the dietary treatment animals compared with controls. Also, at the end of the experiments, these values remained higher for all the groups, showing a deficiency of insulin action in glucose normalization (Fig. 3C). As demonstrated by Kitt (Fig. 3E), the HFD resulted in reduced sensitivity to insulin in all the groups, being most evident in O5 group. Serum levels of total cholesterol increased only in the O5 group (Fig. 4A), whereas serum triglyceride levels increased in dietary treatment groups, mainly in groups with a longer exposure period (O3, O4 and O5) (Fig. 4B). Serum HDL levels decreased (Fig. 4C) and non-HDL levels increased (Fig. 4D) in all dietary treatment groups, except O1 group.

As regards Experiment 2, an increase in body weight, waist circumference and adiposity index was noted in O6 group compared with control (C2 group; Fig. 2A and B). Serum leptin and insulin levels also increased.
Morphological changes

In relation to Experiment 1, there was no relevant change in testicular weight in the HFD groups; however, with the exception of O1 and O2, all other experimental groups showed a decrease in GS1 (Fig. 5). No significant histological alterations were observed in the interstitial tissue of rats in this experiment. No change was noted in the relative volume of Leydig cells; however, there was an increase in the absolute volume of these cells in O1 and O5 groups and a decrease in the O2 and O4 groups (Table 1). An increase was also found in the relative and absolute volumes of blood vessels and macrophages in testes from groups O2, O3, O4 and O5 and a slight decrease in the relative and absolute volume of peritubular cells in testes from O4 and O5 groups (Table 1). Additionally, morphometric analysis of Leydig cells at ultrastructural level showed a reduction of cytoplasmic and nuclear areas of these cells in the O2 group and an increase in both areas in the O1 group (Table 2).

The results of Experiment 2 were similar to those of Experiment 1. There was no relevant change in testicular weight, and a decrease in GS1 was observed in the O6 group when compared with control (C2 group) (Fig. 5). No histological alterations were observed in the interstitial tissue of these rats; however, a decrease in the relative and absolute volume of macrophages and an increase of these parameters for the blood vessels were observed in the
Regarding Leydig cells, morphometric analysis at ultrastructural level showed an increase in the cytoplasmic area and decreased nucleus:cytoplasm ratio in the O6 group (Table 2).

As revealed by transmission electron microscopy, control rats (C1 and C2) exhibited similar cytoplasm ultrastructure with typical Leydig cell-type morphology including large amounts of smooth endoplasmic reticulum cisterns and abundant mitochondria (Fig. 6A). An accumulation of myelin vesicles was observed in the mitochondrial matrix of Leydig cells of the O3, O4, O5 and O6 groups (Fig. 7A, B, C, D and E), but they were not found in the O1 and O2 groups (Fig. 6B and C). These vesicles were generally related to structural disorganization, with increased mitochondrial size and signs of mitochondrial degeneration. The myelin vesicles were more prominent in the O5 and O6 groups, and almost all Leydig cells carried these vesicles (Fig. 7C, D and E). In addition, the presence of autophagosome-like structures containing degenerated mitochondria was common in the O5 and O6 groups (Fig. 7C, D and E).

Steroidogenic capacity of Leydig cells

In Experiment 1, except for rats exposed to HFD during gestation alone (O1 group), intratesticular testosterone levels decreased considerably in all dietary treatment groups when compared with the controls (Fig. 8A). Serum testosterone levels followed the same pattern, being significantly decreased in the O2, O3, O4 and O5 groups (Fig. 8B). In addition, an inverse correlation was detected between serum testosterone levels and leptin for O2 ($r = −0.95$), O3 ($r = −0.80$), O4 ($r = −0.94$) and O5 ($r = −0.99$) groups. Intratesticular estradiol levels decreased in O2, O3, O4 and O5 groups (Fig. 8C), whereas the serum levels of estradiol increased only in those rats exposed to a more prolonged period of HFD (O5 group) (Fig. 8D). The testicular protein content of 17β-HSD enzyme declined (~50%) in the O2, O3 and O4 groups, and this reduction was more pronounced in the O5 group (Fig. 9C). This decrease was also observed by immunohistochemistry analysis, in which the intensity of 17β-HSD staining was reduced in the Leydig cells of the dietary treatment groups (representative image of O5 group) (Fig. 9A and B). Immunolocalization and also the
protein content of aromatase in testicular tissue were similar among groups (Fig. 10A, B and C).

In Experiment 2, intratesticular and serum testosterone levels decreased in the O6 group (Fig. 8A and B), and an inverse correlation between serum testosterone levels and leptin \( (r = -0.89) \) was also observed in the O6 group. However, intratesticular and serum estradiol levels exhibited no changes in this group (Fig. 8C and D). The content of 17β-HSD enzyme in the testes of the O6 group also decreased (Fig. 9C), but the aromatase content remained unchanged (Fig. 10C).

**Discussion**

Our experimental data revealed that HFD feeding during different life stages, whether or not associated with maternal obesity, reduced the steroidogenic capacity of Leydig cells of rats and serum testosterone levels in adulthood. This finding was found in the all investigated periods except that restricted to gestation (O1). We thus demonstrated for the first time that maternal obesity in gestation and breastfeeding, regardless of normal diet intake in posterior life, led to a functional impairment of rat Leydig cells in a similar magnitude to prolonged HFD feeding. It should be noted that as well as being obese, dams continued to consume excessive fat during gestation/lactation. It is therefore impossible to distinguish the effects of excessive dietary fat \( \text{per se} \) from other obesity-related effects in both dam and offspring. The O2, O3, O4 and O5 groups, besides being obese in adulthood, displayed other metabolic alterations, such as hyperleptinemia, insulin resistance and hyperinsulinemia. The O1 group exhibited higher body weight and abdominal circumference and presented insulin resistance associated with hyperinsulinemia, but did not show higher adiposity index and serum leptin, and thus was not considered obese. These effects are illustrated in Fig. 11.

Testosterone biosynthesis is mainly regulated by the pulsatile secretion of LH, but is also influenced by other circulating hormones or paracrine factors such as growth factors and cytokines \( (\text{Saez 1994}) \). Thus, the cause of low testosterone levels in obese individuals is multifactorial and not only due to deregulation of hypothalamic–pituitary–gonadal axis but also to other factors such as homeostasis of insulin and leptin \( (\text{Isidori et al. 1999, Couillard et al. 2000, Pitteloud et al. 2005a, Davidson et al. 2015}) \). Leptin, a hormone produced by adipocytes, regulates satiety at the hypothalamic level \( (\text{Bjorbaek & Kahn 2004}) \) and is an important marker of adipose tissue amount in both humans and rodents \( (\text{DuPlessis et al. 2010, Farooq et al. 2014}) \). This hormone also regulates sexual maturation and reproduction via actions in hypothalamus, hypophysis and gonad \( (\text{Watanobe 2002, DuPlessis et al. 2010, Hofny et al. 2010, Zhao et al. 2014}) \). The relationship linking leptin and androgens levels in men had been widely examined and leptin levels had been considered the best hormonal predictor of the lower androgen levels in male obesity \( (\text{Isidori et al. 1999, Luukkaa et al. 1998, DuPlessis et al. 2010, Farooq et al. 2014}) \). In the present investigation, it was observed that leptin levels increased in animals exposed to HFD during pregnancy/lactation (O2), and this increase was even more robust after prolonged HFD feeding. Beyond that, as indicated by correlation analysis, only the groups exhibiting higher levels of leptin presented lower levels of serum testosterone. Then, beyond confirming previous clinical studies, our experimental data indicated that regardless of the time of obesity onset, leptin deregulation is a pivotal component of steroidogenic impairment caused by this metabolic alteration, at least when induced by HFD.

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**Figure 10** Immunohistochemistry (A and B) and Western blotting (C) for aromatase enzyme in the testes of rats treated with balanced diet (C1 and C2) or high-fat diet, before (O1, O2, O3, O4 and O5) or after sexual maturation (O6). Bars: 20 μm.

**Figure 11** Summary of the results found in all HFD groups.
The action of leptin is triggered by activation of leptin receptors (Ob-R) that were previously described in the central nervous system, rat Leydig cells and mouse tumoral Leydig cell line (Hoggard et al. 1997, Caprio et al. 1999, Morris & Rui 2009). The direct inhibitory action of leptin via Ob-Rb on testosterone synthesis has been demonstrated by the different experiments (Tena-Sempere et al. 1999, Yuan et al. 2014). Tena-Sempere and coworkers (1999) evidenced that leptin action in Leydig cells is variable depending on the state of sexual maturation and nutrition, whereas Yuan and coworkers (2014) showed that testosterone secretion was stimulated by a lower concentration (10 nM) but was inhibited by a higher concentration (100 nM) of leptin. We were unsuccessful in labeling Leydig cells using commercially available antibodies to leptin receptors to properly investigate their stimuli on these cells. But in accordance with previous data described above, our data strongly suggest that the inhibitory action of high leptin concentrations on Leydig cell steroidogenesis is triggered by direct mechanisms and activation of Ob-Rb. Furthermore, it should also be mentioned that leptin stimulates GnRH release by hypothalamus, thereby stimulating the synthesis of testosterone (Hoggard et al. 1997, Michalakis et al. 2013). However, high leptin levels can trigger a leptin resistance state both in central as peripheral organs, preventing the correct action of this hormone (Isidori et al. 1999, Munzberg et al. 2005). Then, the impaired leptin signaling in the hypothalamus–pituitary unit, particularly in the O3, O4, O5 and O6 groups, may have aggravated the steroidogenic damage. In addition, Yuan and coworkers (2014) also demonstrated that high leptin concentrations induced by MSG increased the expression of suppressor of cytokine signaling 3 (SOCS3), an important member of the suppressors of cytokine signaling, identified as a mediator of central leptin resistance and also inhibited the phosphorylation of STAT3 (Yuan et al. 2014). Future analysis will be performed to find out whether these signaling pathways are also implicated in steroidogenic impairment of the HFD-induced obesity.

Another factor that may be associated with reduced androgen levels is insulin action on Leydig cells, which can contribute to impairment in the testosterone synthesis. Although previous evidence showed that insulin stimulates testosterone production in Leydig cell cultures (Lin et al. 1986, Bebakar et al. 1990), Leydig cell steroidogenesis is impaired in insulin-resistant states. Several studies have shown an inverse correlation between total testosterone and insulin levels in insulin-resistant situations, such as obesity and diabetes (Seidell et al. 1990, Couillard et al. 2000, Pitteloud et al. 2005a). Pitteloud and coworkers (2005a) evaluated the impairment of each level of the hypothalamic–pituitary–gonadal axis in a cohort study of men with mild to moderate obesity and verified that the low testosterone levels found in these patients are due to decreased testosterone secretion by Leydig cells and did not result from the effects on the hypothalamus or pituitary. The O1 group presented insulin resistance and hyperinsulinemia; however, it did not present reduced androgen levels. We therefore suggest that the reduced androgen levels observed in this study were probably not caused by changes in glucose metabolism or insulin signaling in Leydig cells.

The functional impairment of Leydig cells was, at least in part, due to the decrease in steroidogenic enzyme 17β-HSD, as indicated by Western blotting. 17β-HSD catalyzes the final stages of sex steroid production, converting the least active androgen (androstenedione) and estrogen forms (estrone) into their most biologically active forms (Saez 1994). Beyond the deregulation of leptin signaling in Leydig cells, the steroidogenic damage probably involved structural damage to these cells and also additional mechanisms dependent on the developmental period and the duration of HFD exposure. Ultrastructural analysis revealed structural alterations in the mitochondria of Leydig cells from the O3, O4, O5 and O6 groups, such as myelin vesicle accumulation in the mitochondrial matrix. The frequency and size of these vesicles progressively increased according to the time of HFD exposure, which were smaller and less frequent in the O3 and O4 groups, and prominent and more frequently found in the O5 and O6 groups, where structures similar to autophagosomes were also detected. Considering that mitochondria are involved in lipid metabolism such as fatty acid β-oxidation, prolonged HFD may have led to a functional overload and/or accumulation of lipids within this organelle, triggering mitochondrial autophagy or the mitophagia process (Tolkovsky 2009, Barth et al. 2010). The accurate characterization of autophagosomes requires the identification of some specific markers such as the modified product of microtubule-associated protein 1 light chain 3 (LC3-II) into their lipid membranes. However, as we noted, the presence of a double membrane and the remnants of organelles are strong indications of autophagosomes (Barth et al. 2010). Mitophagy has been observed in hypoxic situations, allowing the cell to reduce its mitochondrial mass to limit reactive oxygen species production at a time when oxygen is not available to accept electrons from the respiratory chain (Zhang et al. 2008). Defects in the mitochondrial function (Kelley et al. 2002, Petersen et al. 2004) of skeletal muscle, including aberrant mitochondrial morphology (Kelley et al. 2002) and reduced gene expression responsible for the oxidative metabolism (Patti et al. 2003), which leads to the accumulation of intramyocellular lipids, have been observed in obese individuals with insulin resistance. Additionally, mitochondrial dysfunction has also been found in the liver of rodents fed with a HFD (Ferramosca et al. 2015). It is noteworthy that recent studies have shown that glucose intolerance due to HFD
or experimental diabetes alters the glucose metabolism, mitochondrial bioenergetics and oxidative stress in the testis (Rato et al. 2013). In this regard, it is possible that the functional impairment in Leydig cells may also be related to mitochondrial physiology, but additional biochemical studies of this isolated organelle are necessary to confirm this assumption. Thus, metabolic alterations and mitochondrial overload, together with the accumulation of lipids, may have caused damage and driven mitochondrial autophagy in the groups exposed for prolonged periods to HFD. Furthermore, mitochondria are responsible for the early steps of androgen synthesis, i.e., where cholesterol is converted to pregnenolone and subsequently directed to SER to be converted to androgens (Saez 1994). Thus, in addition to disturbances in leptin action, degenerative alterations involving the mitochondria may also have contributed to damage in the steroidogenic function, principally for the O5 and O6 groups.

Another noteworthy aspect to be considered is whether the functional impairment of Leydig cells observed here might reflect the effects of ALC differentiation. The morphological and ultrastructural data of this investigation do not indicate that HFD exposure affected the ALC number or differentiation, but do indicate an increase in the absolute volume of these cells in the O1 and O5 groups. Exposure during pregnancy (O1) corresponds to the stage where only mesenchymal cells are found, which are not yet committed to differentiation into mature ALC (Mendis-Handagama & Ariyaratne 2001, Teerds & Huhtaniemi 2015). On the other hand, the exposure of the O2 group occurred during the first differentiation step of these cells, that is, the differentiation of mesenchymal cells into progenitor cells, which occurs in rats around day 10 after birth (Mendis-Handagama & Ariyaratne 2001, Teerds & Huhtaniemi 2015). As indicated by morphological and ultrastructural analyses, the Leydig cells of the O2 group are atrophied, which explains the decreased 17β-HSD expression and steroidogenic capacity in this group. This atrophy may be the result of hormonal changes induced by maternal obesity during lactation in the differentiation process of these cells. Thus, although both the O1 and O2 groups were insulin resistant and hyperinsulinemic in adulthood, the damage in testosterone production that was significant for O2 but not for O1 indicates that the Leydig cell differentiation or secretory capacity may have been affected permanently by maternal obesity. In turn, the greater Leydig cell functional impairment detected in the O5 group is due to the effects of maternal obesity in the early stage of ALC differentiation combined with the effects of metabolic damage caused by HFD intake in postnatal life. Moreover, it is important to note that other factors may also be involved in the functional impairment of Leydig cells observed here, such as increased oxidative stress, which has been considered an inducer of apoptosis of these cells against the excess of lipids in the diet (Zhao et al. 2014).

A decrease in intratesticular estradiol concentration was noted in the O2, O3, O4 and O5 groups, with no change in aromatase testicular contents. This indicates that the decrease in intratesticular estradiol production is a consequence of testosterone synthesis reduction. On the other hand, regarding serum levels, there was an increase in estradiol only for the HFD-exposed throughout life group (O5). The increased serum levels of estradiol in obese men (Davidson et al. 2015) and rodents (Fernandez et al. 2011, Viguera-Villaseñor et al. 2011) have been explained by higher androgen aromatization in peripheral adipose tissue (Pasquali et al. 2007, Hammoud et al. 2008). However, this issue is still controversial, as some studies have shown that obese individuals have an increase in serum estrogen levels (Hammoud et al. 2008, Mah & Wittert 2010, Mammì et al. 2012, Michalakis et al. 2013), whereas others do not (MacDonald et al. 2010, Ribeiro et al. 2012). Although we could not determine when different groups became obese, our data show that the increase in estradiol does not occur in all conditions associated with obesity. They also indicate that the length of exposure to HFD and levels of adiposity/leptin may modulate hyperestrogenemia in obese individuals.

In conclusion, HFD feeding, before or after sexual maturation, reduces the functional capacity of Leydig cells in rats and induces hypoandrogenism in adulthood. Maternal obesity in association with high dietary fat during pregnancy/lactation, even when followed by a conventional diet in later stages of life, drastically affects the steroidogenic capacity of Leydig cells and induces their atrophy in adulthood. Regardless of the life period of exposure to HFD, deregulation of leptin is the main factor related to steroidogenic impairment of Leydig cells, and for groups exposed for longer periods (O3, O4, O5 and O6), this is worsened by structural damage and mitochondrial degeneration in these cells.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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