Maternal obesity disturbs the postnatal development of gonocytes in the rat without impairment of testis structure at prepubertal age

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Abstract

In this study, we evaluated whether maternal obesity (MO) affects testis development and gonocyte differentiation in the rat from 0.5 to 14.5 postnatal days. Male Wistar rats were used at 0.5, 4.5, 7.5, and 14.5 days *post partum* (dpp). These rats were born from obese mothers, previously fed with a high-fat diet (20% saturated fat), for 15 weeks, or normal mothers that had received a balanced murine diet (4% lipids). MO did not affect testis weight or histology at birth but changed the migratory behavior of gonocytes. The density of relocated cells was higher in MO pups at 0.5 dpp, decreased at 4.5 dpp, and differed from those of control pups, where density increased exponentially from 0.5 to 7.5 dpp. The numerical density of gonocytes within seminiferous cords did not vary in MO, in relation to control neonates, for any age considered, but the testis weight was 50% lower at 4.5 dpp. A wide variation in plasmatic testosterone and estrogen levels was observed among the groups during the first week of age and MO pups exhibited higher steroid concentrations at 4.5 dpp, in comparison with controls. At this age, higher estrogen levels of MO pups impaired the gonocyte proliferation. At 7.5 dpp, the testicular size and other parameters of gonocyte development are retrieved. In conclusion, MO and saturated lipid diets disturb gonocyte development and sexual steroid levels during the first days of life, with recovery at prepubertal age.

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Introduction

Owing to the increasing incidence and the organic complications, obesity has been considered a serious public health problem not only in high-income countries worldwide but also in low- and middle-income populations (Forrester 2013). A review concerning the genetics of obesity, with an emphasis on established obesity susceptibility loci identified through candidate gene and genome-wide studies, indicated that the contribution of genetic loci to body weight increase is <2% (Loos 2009). Therefore, environmental factors, such as high saturated fat in diets, are thought to have pivotal roles in overweight and obesity (Cascio *et al.* 2012).

Epidemiological data show that over 64% of women of childbearing age were overweight or obese in the USA, in 2008, and about one-third of pregnant women were obese (King 2006, Sullivan *et al.* 2011). As both maternal nutrition and obesity influence the development of mammals during the intrauterine or breastfeeding phases (Sullivan *et al.* 2010), scientific interest was

directed to elucidate the influence of maternal obesity (MO) on the physiology of organs and health during childhood and adult life. Epidemiological and animal model studies indicated that MO at conception alters gestational metabolic adjustments and affects placental, embryonic, and fetal growth and also postnatal development (Catalano & Ehrenberg 2006, Catalano *et al.* 2009, Symonds & Budge 2009, Sullivan *et al.* 2011). Several lines of evidence show that acute or chronic stimuli during gestation can induce a permanent response in fetus and impair the physiology of several organic systems and this phenomena was designed fetal programming (Dabelea *et al.* 2000, Boney *et al.* 2005, Sewell *et al.* 2006, Catalano *et al.* 2009, Symonds & Budge 2009). The effects of MO on fetal programming of energy balance and adiposity (Sullivan *et al.* 2010) and cardiovascular and renal systems (Nistala *et al.* 2011) had been described. However, the knowledge about consequences of MO on fetal programming and histophysiology of the genital system is incipient, and

there is no information about the potential interference of MO on testis development at neonatal life.

The perinatal development of testis involves the proliferation and differentiation of three cell populations of adult organ – Sertoli cells, adult Leydig cells, and germ cells (Orth 1982, Boulogne *et al.* 1999, Mendi-Handagama & Ariyaratne 2001). The germ cells in fetal and neonatal testis, named gonocytes (Clermont & Perey 1957), are derived from the migratory primordial germ cells that colonize the genital ridge, between 13.5 and 14.5 days postcoitum (dpc), in the rat (Magre & Jost 1980). The gonocytes have been characterized a long time ago, but the regulatory mechanisms that control their differentiation is still little understood, compared with other cell types of testis (Culty 2009). The differentiation of gonocytes, in rodents, involves a proliferative period in fetal development (from 13.5 to 17.5 dpc in the rat), a quiescent period during perinatal life, followed by resumption of mitosis in the first days of age (from 1 to 4 days *post partum* (dpp) for the rat) (Magre & Jost 1980, Orth 1982, McGuinness & Orth 1992a, 1992b, Prépin *et al.* 1994, De Miguel *et al.* 1996, Boulogne *et al.* 1999). Indeed, the relocation of gonocytes to the periphery of seminiferous cords is crucial for their posterior differentiation into spermatogonia (Roosen-Runge & Leik 1968, Clark & Eddy 1975, McGuinness & Orth 1992a) and the non-relocated gonocytes die by apoptosis in the majority of rodents (Miething 1992, Boulogne *et al.* 1999, Pinto *et al.* 2010). There is solid evidence about the inhibitory action of androgens on the proliferation of fetal gonocytes (Merlet *et al.* 2007), whereas estrogen, via estrogen receptor type B (ERB), inhibits apoptosis and enhances gonocyte development (Delbès *et al.* 2004, 2007, Vigueras-Villaseñor *et al.* 2006).

In this study, we evaluated whether a high-fat diet and MO affect the neonatal testicular development of Wistar rats with emphasis on processes involved in gonocyte differentiation.

Materials and methods

Animals and experimental design

Male and female Wistar rats (5 weeks old) were obtained from Anilab Animais de Laboratório e Comércio Ltda (Paulínia, SP, Brazil). The animals were kept in the Animal Breeding Center of São Paulo State University – UNESP, Institute of Biosciences, Humanities and Exact Sciences – IBILCE, at controlled temperature (23–25 °C), humidity (40–60%), under 12 h light:12 h darkness cycle, and treated with filtered water '*ad libitum*'. The experimentation procedures were performed in accordance with the rules of the Ethics Committee on Animal Experimentation (CEUA/UNESP, protocol 22/2009).

MO was induced by treatment for 15 weeks with a high-fat diet (20% fat; 4.8 kcal/g), acquired from Agrocere (Campinas, SP, Brazil). This model of obesity and diet was previously standardized by researchers at the Laboratory of Experimental

Clinical Medicine, Faculty of Medicine of Botucatu, UNESP (Nascimento *et al.* 2008). Females of the control group and the males used for breeding received the standard normal calorie (normocaloric) diet (4% fat; 3.2 kcal/g) produced by the same manufacturer. Obese females (20 weeks old) were mated with thin adult males (12 weeks old) and lean females of the same age were mated with adult rats. The pregnancy was confirmed by the presence of vaginal plug. The birth date of pups was defined as 0.5 dpp and the identification of gender was based on anogenital distance, which was measured with a digital caliper (King Tools, digital caliper, 0–150 mm). A total of 48 male offspring of normal and obese mothers aged 0.5, 4.5, 7.5, and 14.5 dpp ($n=6$), each one born from different families, were used. The pups were killed by CO₂ inhalation and their weight was determined before death. Then, the pups were decapitated for blood collection, and testes were dissected and weighed. The gonadosomatic index (GSI) was obtained from the formula $GSI = (\text{testicular weight/body weight}) \times 100$.

Light microscopy

The right testes were fixed by immersion for 6 h in Bouin fluid, washed in 70% alcohol for the removal of picric acid, and processed for inclusion in Paraplast (Histosec, Merck). Serial sections of whole testes (5 µm thick) were made, part of which was used for routine histological analysis and part was subjected to immunocytochemistry.

The left testes were immersed in 2.5% glutaraldehyde, 1% tannic acid, 3.5% sucrose, and 5 mM calcium chloride in 0.1 M cacodylate buffer (pH 7.4) at 4 °C. Small incisions were made in the tunica albuginea of the gonads of animals at 4.5 and 7.5 dpp to improve fixative penetration. After 1 h in this solution, testes were cut into smaller cubes (1–2 mm thickness) and fixed for 4 h in the same solution, following washing in buffer, and postfixation in osmium tetroxide 1%. Specimens were dehydrated in acetone and embedded in Araldite 502 (Electron Microscopy Sciences, Hatfield, PA, USA). One-micrometer-thick sections were stained with a 1% solution of toluidine blue and 1% borax in water for light microscopy analysis.

Immunocytochemistry

Immunocytochemical reactions were performed for anti-Müllerian hormone (AMH), androgen receptor (AR), ERs (ERA and ERB), and activated caspase 3. Briefly, paraffin sections were immersed in citrate buffer (pH 6), at 92 °C, for 45 min, for antigen retrieval. The blocking of endogenous peroxidase was achieved by incubation with 3% H₂O₂ in methanol, for 20 min. Then, the tissue sections were incubated with 5% non-fat milk in PBS, for 30 min, to block non-specific protein linkage. Incubations with primary antibodies were performed overnight at 4 °C in 1% BSA using the following antibodies and dilutions: 1:75 rabbit IgG anti-human AR (Santa Cruz Biotechnology, sc-816), 1:100 goat IgG anti-human AMH (Santa Cruz Biotechnology, sc-6886), 1:50 rabbit IgG anti-human ERα (Santa Cruz Biotechnology, sc-8974), 1:50 rabbit IgG anti-human ERβ (Santa Cruz Biotechnology, sc-542),

and 1:200 rabbit IgG anti-human activated caspase 3 (Abcam, Cambridge, MA, USA; ab-13847).

The above-mentioned reactions were then incubated with the biotinylated secondary antibody followed by ABC avidin–biotin complex Kit (Santa Cruz Biotechnology), for 45 min, at 37 °C. Sections were revealed with diaminobenzidine (DAB) for ~1 min and counterstained with hematoxylin.

Also, double immunocytochemical reactions for proliferating cell nuclear antigen protein (PCNA) protein and VASA were performed. In this case, the blockages were used as described above, but the sections were incubated overnight at 4 °C with 1:50 primary antibody rabbit IgG anti-human VASA (Santa Cruz Biotechnology, sc-67185), followed by incubation with 1:50 primary antibody mouse IgG anti-human PCNA (Santa Cruz Biotechnology, SC-56), for 1 h at 37 °C. The incubation with Rat and Mouse Double Stain Kit (Biocare, Wayne, PA, USA) proceeded for 1 h. PCNA was revealed with DAB as described above and VASA with Vulcan Fast Red Chromogen (PAP). The negative control was obtained by omission of the primary antibody.

Numerical density of gonocytes

The numerical density (Nv) of gonocytes, or the number of these cells in a given tissue volume, was estimated for the seminiferous cord. This parameter was estimated in order to examine whether the number of gonocytes in the same volume of seminiferous cord varied among the different experimental groups. These analyses were conducted based on the procedures of Roosen-Runge & Leik (1968) and Zogbi *et al.* (2012).

For this purpose, we primarily determined testicular volume (mm^3). Testicular volume for the pups at 0.5 and 4.5 dpp was determined by the Cavalieri method (Gundersen *et al.* 1988). One of the 20 histological sections had its area determined using the Image Pro-Plus Software (Media Cybernetics, version 4.5, Rockville, MD, USA), and testicular volume was calculated by multiplying the sum of the areas by 100, a value that is related to the distance between two successive sections ($20 \times 5 \mu\text{m}$). The testicular volume of 7.5 and 14.5 dpp rats was equal to fresh testis weight (g), without correction for density. Subsequently, the seminiferous cord volume density in the testis was determined, based on Weibel (1963). For each animal, we used two different histological sections and ten random fields per section, examined with $20\times$ objective. After application of the M132 reticle, the volume density was determined by the percentage of dots that covered the seminiferous cords. Based on seminiferous cord volume density, the absolute volume of seminiferous cord was calculated for each organ. Then, the crude counting (CC) of gonocytes was estimated using five histological sections, from approximately equidistant regions of the testis, for each animal. The tissue sections were previously submitted to immunocytochemistry for AMH and gonocytes appeared unmarked against AMH-positive cytoplasm of Sertoli cells. For estimation of CC, only the gonocytes with evident nuclei were counted. The Nv was calculated as the ratio between CC and seminiferous cord volume.

Density of relocated gonocytes, cell proliferation, and apoptosis

The density of relocated gonocytes and mitotic cells in seminiferous epithelium was determined for 0.5, 4.5, and 7.5 dpp pups. We used three different histological sections per testis for each animal subjected to immunocytochemistry for AMH, which were analyzed at $40\times$ objective, in all of its extension. For each tissue section, the gonocytes at the base of seminiferous cord (relocated gonocytes) were counted, using the Image Pro-Plus Software (Media Cybernetics, version 4.5). We considered the cells with typical morphology situated at the periphery of the seminiferous cords, without AMH-positive cytoplasmic processes of the Sertoli cells involving their basis, as relocated gonocytes. The other gonocytes were considered non-relocated.

The cell proliferation in seminiferous epithelium was estimated following the same tissue samples and procedures described above in which the mitotic figures were counted. The values obtained were expressed as number of mitotic figures per cubic millimeter of seminiferous cords, previously determined as described for gonocyte count.

The estimation of apoptosis in seminiferous epithelium was made for four animals in groups from 4.5 to 14.5 dpp, as only after 4.5 dpp were apoptotic cells observed. We used three histological sections for each animal subjected to immunocytochemistry for activated caspase 3. The total area of the histological section was estimated from images digitized at $40\times$ magnification, using the above program, excluding the region corresponding to the tunica albuginea. The total number of apoptotic cells per histological section was divided by the corresponding area.

Grade of testicular maturation

The grade of testicular maturation and onset of spermatogenesis were evaluated for animals at 14.5 dpp. One testis from each animal ($n=6$ per group) was scanned under a microscope and the seminiferous cords staged according to the most advanced cell type of spermatogenic lineage, as follows: A spermatogonia – both undifferentiated (A_{single} , A_{paired} , and A_{aligned}) and differentiated (A1–A4) were considered; B spermatogonia; pre-leptotene or ‘resting’ – the final period of DNA replication; and leptotene primary spermatocytes and zygotene primary spermatocytes. These cells were identified as described by Clermont & Perey (1957), with $20\times$ objective, in histological sections stained with HE. At least 150 tubules were evaluated for each animal and the data were expressed as percentages of tubules according to the most advanced cell type present.

Hormone dosage

The blood samples were collected immediately after decapitation, centrifuged at 1200 g for plasma separation, and frozen at $-20\text{ }^{\circ}\text{C}$ for the subsequent analysis of testosterone and estrogen levels. The measurements were performed by capture/sandwich ELISA (antibody–antigen–antibody), using specific commercial kits (Enzo Life Sciences International, Inc.,

Plymouth Meeting, PA, USA), with high sensitivity (5.67 and 14.0 pg/ml respectively) and coefficients of variation for inter-assay of 11.3 pg/ml (for testosterone) and 8.3 pg/ml (for estrogen). The readings were taken in reader Epoch Multi-Volume Spectrophotometer System (BioTek Instruments, VT, USA).

Statistical analyses

Statistical analysis of the collected data was performed in Statistica 7.0 Software (StatSoft, Inc., 1984–2004, Tulsa, OK, USA) and *t*-test, $P \leq 0.05$ was considered statistically significant.

Results

Body weight, testicular weight, and GSI

MO decreased the mean body weight of pups at birth in 11% ($P=0.018$; Table 1) but did not alter the anogenital distance ($C=2.05 \pm 0.21$ mm and $MO=2.05 \pm 0.14$ mm). The testicular weight (Table 1) did not change at birth but was ~20% lower for pups from obese mother at 4.5 dpp. The GSI of these pups was also lower at this age (Table 1). The body weight, testicular weight, and GSI of male offspring subjected to MO were recovered from 7.5 dpp onward (Table 1).

Morphological analysis and repositioning of gonocytes

The analysis of paraffin (not shown) and semi-thin (Fig. 1A and B) sections showed no marked changes in testis histology or gonocyte morphology for the pups subjected to MO. At birth, the majority of the gonocytes were located in the center of the seminiferous cords (Fig. 1A, B, C, and D). The density of relocated gonocytes increased exponentially in control rats from 0.5 to 7.5 dpp (Fig. 1C, D, E, F, and H). By contrast, the pups subjected to MO had a higher density of relocated gonocytes at 0.5 dpp, compared with the control group, with a reduction at 4.5 dpp and a posterior recovery at 7.5 dpp (Fig. 1H). The stereological analysis of testes indicated an increase in volume density of seminiferous cords and a decrease in volume density of the interstitial tissues during

postnatal testis development, neither of which was affected by MO (Table 2).

Nv of gonocytes

As expected, the Nv of gonocytes per volume of seminiferous cord dramatically decreased during the first days of age, being very small in animals at 7.5 dpp (Fig. 1I). These results reflect the differentiation of gonocytes into spermatogonia. No difference in Nv of gonocytes was observed for the pups subjected to MO, compared with the controls, for any of the ages studied (Fig. 1I). As previously mentioned, the volume density of seminiferous cords did not vary between the groups subjected to MO and their respective controls (Table 2); however, the testicular volume was significantly lower ($P=0.03$) in the MO group at 4.5 dpp compared with control animals (Fig. 1J). Therefore, the total number of gonocytes per testis decreased in the MO group at this age compared with controls.

Cell proliferation and apoptosis

There were no statistical differences in mitotic density figures in the seminiferous epithelium among controls and pups subjected to MO at 7.5 dpp (Fig. 2A). However, the numerical values show a peak of cell proliferation at 4.5 dpp in controls, whereas the cell proliferation is higher at birth and decays at 4.5 and 7.5 dpp in the group subjected to MO (Fig. 2A).

The immunocytochemistry showed that the non-relocated gonocytes were PCNA negative, but became PCNA positive after relocation (Fig. 2B, C, D, E and F). The Sertoli cells were PCNA positive from 0.5 to 7.5 dpp and lacked PCNA at 14.5 dpp (Fig. 2B, C, D, E and F).

All neonatal gonocytes at 0.5 dpp showed cytoplasmic staining for activated caspase 3 (Fig. 3B and C). These gonocytes exhibited normal morphology and were not considered in apoptosis. Also, for animals at 0.5 dpp, apoptotic cells were not detected in either the control group or those subjected to MO (Fig. 3B and C). Apoptotic cells/bodies that were intensely labeled by activated caspase 3, corresponding to advanced apoptosis stages, were detected at 4.5 dpp (Fig. 3A). The density of apoptotic cells/bodies increased slightly from 4.5 to 7.5 dpp and dramatically from 7.5 to 14.5 dpp (Fig. 3A). MO did not alter the pattern of apoptosis in seminiferous cords (Fig. 3A, B, C, D, and E).

Immunocytochemical analyses

The immunocytochemical analyses of neonatal rat testes indicated that postnatal gonocytes did not show immunoreactivity for AR (Fig. 4A, B, and C). No remarkable changes could be observed between the controls and those pups born from obese mothers, with

Table 1 Body weight, testis weight, and GSI of control (C) or MO rats at 0.5, 4.5, 7.5, and 14.5 dpp.

Age (dpp)	Groups (C and MO)	Body weight (g)	Testis weight (mg)	GSI
0.5	C	7.33 ± 0.12	2.62 ± 0.27	0.29 ± 0.03
	MO	$6.51 \pm 0.28^*$	2.41 ± 0.28	0.26 ± 0.04
4.5	C	12.7 ± 0.75	6.85 ± 0.41	0.77 ± 0.06
	MO	11.7 ± 0.55	$5.6 \pm 0.36^*$	$0.6 \pm 0.04^*$
7.5	C	16.44 ± 1.02	12.06 ± 0.50	1.26 ± 0.07
	MO	16.92 ± 0.66	12.68 ± 0.46	1.33 ± 0.06
14.5	C	26.63 ± 0.70	39.57 ± 1.76	4.13 ± 0.29
	MO	26.93 ± 0.88	37.28 ± 1.59	3.92 ± 0.22

Values are mean and S.E.M. Statistical differences between groups C and MO are indicated by $*P \leq 0.05$.

Figure 1 (A and B) Semi-thin sections, stained with toluidine blue, of testes from control (Control) or MO rats. (C, D, E, and F) Histological sections of testes of rats born from control (Control) or obese mothers (MO) subjected to immunocytochemistry for AMH. The sections were counterstained with hematoxylin. The age of the animal is shown on the left. The negative control of reaction is shown in (G). G, gonocytes; arrowheads, nuclei of Sertoli cells; and arrows, repositioned gonocytes. Bars=10 μ m (A and B) and 20 μ m (C, D, E, F, and G). (H) Repositioned gonocyte density in testes of control (Control) or MO rats. (I) Density number of gonocytes (Nv) in control (Control) and MO groups. We observed a linear decrease in the numerical density of gonocytes from 0.5 to 7.5 dpp. (J) Testicular volume of neonatal rats subjected to MO compared with controls. The values are expressed per cubic millimeter of seminiferous tubules. * $P \leq 0.05$.

respect to the location of AR in gonocytes, at any age considered, using immunocytochemistry. Immunocytochemistry for ERs revealed that gonocytes of rats at 4.5 dpp exhibited cytoplasmic localization for ERA (Fig. 4I and J) and ERB with nuclear localization (Fig. 4F, G, and H). Apparently, there was an increase in immunostaining for ERB in gonocytes of animals subjected to MO.

Testicular maturation in 14.5 dpp rats

The evaluation of testis maturation in 14.5 dpp control rats indicated that the majority of seminiferous cords, at this age, contained spermatocytes in primary leptotene, a lower percentage contained B spermatogonia and pre-leptotene, and only a small percentage of tubules contained spermatocytes in zygotene (Fig. 5A, B, C, D, E, and F). An increase in the percentage of tubules containing A spermatogonia ($P=0.99$) and a reduction of those containing leptotene and zygotene primary spermatocytes ($P=0.053$) in the testis of rats subjected to

MO (Fig. 5G) were observed, but these alterations were not statistically significant.

Testosterone and estrogen levels

Testosterone and estrogen plasma levels were very variable among animals, with no statistical differences between the control and the MO groups (Fig. 6). Control

Table 2 Relative proportion of seminiferous cords (SC) and interstitial tissue (IT) in testis of control (C) or MO rats at the ages of 0.5, 4.5, and 7.5 dpp.

Age (dpp)	Groups (C and MO)	SC (%)	IT (%)
0.5	C	44.34 $\pm$ 1.7	55.65 $\pm$ 1.7
	MO	43.31 $\pm$ 2.17	56.69 $\pm$ 2.17
4.5	C	51.81 $\pm$ 9.02	48.19 $\pm$ 9.02
	MO	54.34 $\pm$ 2.44	45.65 $\pm$ 2.44
7.5	C	62.83 $\pm$ 2.55	37.17 $\pm$ 2.55
	MO	60.17 $\pm$ 2.19	39.82 $\pm$ 2.19

Values are mean and s.d.

Figure 2 (A) Density of apoptotic cells/bodies in the seminiferous epithelium expressed by the total area of the testis. We observed a linear increase in apoptotic cells from 4.5 to 14.5 dpp. There were no significant differences between the control group (Control) and those subjected to MO. (B, C, D, and E) Histological sections of testes of control (Control) or MO groups subjected to immunocytochemistry for activated caspase 3. The age of the animal is shown on the left. The sections were counterstained with hematoxylin. Note the diffuse labeling for activated caspase 3 in the cytoplasm of the gonocytes in 0.5 dpp rats (B and C). (F) Negative control. G, gonocytes and arrowheads, cells or apoptotic bodies. Bars = 20 μ m.

pups exhibited a decrease in plasmatic testosterone levels at 4.5 dpp, whereas estrogen levels increased from birth to 14.5 dpp (Fig. 6). On the other hand, pups subjected to MO exhibited lower plasmatic testosterone levels at birth and higher levels at 4.5 dpp (Fig. 6A). The estrogen levels of MO pups at 4.5 dpp were also higher in comparison with control pups (Fig. 6B). In addition, comparing the MO and the control groups, the mean estrogen levels were similar from 7.5 dpp onward (Fig. 6B) and the testosterone levels at 14.5 dpp (Fig. 6A).

Discussion

In this study, we used a model of obesity based on a high-fat diet to evaluate the consequences of MO on neonatal

testis development and on male germ cell differentiation in Wistar rats. MO did not affect testis weight or the Nv of gonocytes at birth, but it caused perturbations in development of rat gonocytes and steroidogenesis in the first days of life. The quantification of relocated gonocytes indicates that offspring of obese mothers do not follow the same pattern of gonocyte migration observed in control pups and previously described in the literature (Roosen-Runge & Leik 1968, Clark & Eddy 1975, McGuinness & Orth 1992a). Whereas the number of relocated gonocytes increased in control pups in the first week of age, the pups from obese mothers exhibited higher number of relocated gonocytes at 0.5 dpp and a decrease at 4.5 dpp. Therefore, it can be assumed that MO affects the migratory behavior and relocation of gonocytes in the first days of life.

Figure 3 (A) Density of mitotic figures in testes of control (Control) and MO rats at 0.5, 4.5, and 7.5 dpp. There were no statistically significant differences. (B, C, D, E, and F) Histological sections of testes of control (Control) or MO groups subjected to double immunocytochemical reaction for PCNA and VASA protein. The age of the animal is shown on the left. The sections were counterstained with hematoxylin. G, gonocytes; S, labeling nuclei of Sertoli cells; arrowhead, unlabeled nuclei of Sertoli cell; and arrows, labeling nuclei of gonocytes. Bars = 10 μ m.

Figure 4 Histological sections of rat testes at 4.5 dpp of control (Control) or MO groups subjected to immunocytochemistry for AR (A, B, C, and D), ERB (F, G, and H), and ERA (I and J). The sections were counterstained with hematoxylin. (E and K) Negative controls. F, unlabeled nuclei of fetal Leydig cells; G, gonocytes; R, relocated gonocyte; arrowheads, labeling nuclei of Sertoli cells; arrows, aggregates of fetal Leydig cells; and *labeling nuclei of fetal Leydig cells. Bars = 10 μ m (B, C, D, G, H, I, and J) and 20 μ m (A, E, F, and K).

The migration from the center to the periphery of the seminiferous cords during neonatal development is a crucial event for the cell cycle and mitosis resumption and differentiation of gonocytes into spermatogonial lineage (Roosen-Runge & Leik 1968, McGuinness & Orth 1992a, Tres & Kierszenbaum 2005). Therefore, as

expected, both control and MO pups showed an inverse relation between density of relocated gonocyte and cell proliferation; they also exhibited different patterns of cell proliferation at seminiferous epithelium in the first week of age, with progressive decay in MO and a peak at 4.5 dpp in control pups, parallel to gonocyte relocation. Additionally, only relocated gonocytes were PCNA positive.

The Nv of gonocytes in control rats decreases exponentially during the first week of life, due to their transition into spermatogonia, and it was not altered by MO. However, testicular volume was $\sim$ 50% lower in the MO group at 4.5 dpp compared with the control group, indicating a reduction in the total number of gonocytes at 4.5 dpp. Such reduction might be explained by low proliferative activity verified to seminiferous epithelium of MO pups at this age.

The increasing levels of apoptosis observed in the testes of control rats during the first week of age corroborate previous studies that have shown about 25–30% of such cells die at 7 dpp in this rodent (Boulogne *et al.* 1999). Boulogne *et al.* (1999) also demonstrated that there is no apoptosis of Sertoli cells in the perinatal period (Boulogne *et al.* 1999). Already high apoptosis levels observed at 14.5 dpp relate to the cells in the first wave of spermatogenesis. It should be mentioned that the immunocytochemistry for activated caspase 3 indicated a diffuse cytoplasmic labeling in rat gonocytes at 0.5 dpp, as observed by Zogbi *et al.* (2012). The cytoplasm of all gonocytes at this age was labeled and a slight cytoplasmic staining could be seen until 7.5 dpp. This diffuse cytoplasmic staining for activated caspase 3 was observed in normal gonocytes and was not considered an apoptosis indicator, unlike that for apoptotic cells/bodies detected from 4.5 to 14.5 dpp, which represented advanced apoptosis stages (Fig. 3D and E). The presence of activated caspase 3 in these cells can be explained by the fact that it is involved not only in the cell death system but also in the self-renewal and

Figure 5 Testicular maturation degree analysis of control (Control) or MO groups at 14.5 dpp. (A, B, C, D, E, and F) Seminiferous cords in different stages of maturation. (G) Percentage of seminiferous cords in each phase of the cycle, from A spermatogonia to spermatocyte in zygotene at 14.5 dpp. There were no statistical differences between the control group (Control) and the MO group. A, A spermatogonia; B, B spermatogonia; L, leptotene primary spermatocytes; pL, pre-leptotene primary spermatocytes; and arrowheads, zygotene primary spermatocytes. Bars = 20 μ m.

Figure 6 Testosterone (A) and estrogen (B) plasmatic levels of control (Control) or MO rats from 0.5 to 14.5 dpp. There were no statistically significant differences.

differentiation processes of germ cells (Fujita *et al.* 2008, Zogbi *et al.* 2012). According to Dejosez *et al.* (2008), caspase 3 also recognizes critical pluripotency factors for embryonic stem cell function.

The hormonal dosage of five animals per group, using high-sensitivity ELISA, indicated wide variations in plasma testosterone levels, especially at ages below 14.5 dpp, with no statistical differences observed for any age. However, most MO pups showed testosterone levels below 75 ng/dl at 0.5 dpp, whereas most control pups exhibited testosterone above this value at this age. In addition, MO pups showed higher plasma testosterone and estrogen levels at 4.5 dpp, in comparison with control pups. Thus, the numerical values obtained for each animal and the means for each group suggest that MO interferes in neonatal steroidogenesis. Male rats normally experience a surge in plasma testosterone during 18 and 19 dpc (Ward & Weisz 1984) and another peak is observed during the first few hours following birth (Corbier *et al.* 1992). Several reports indicate that stress-induced hormonal changes in pregnant mother and/or male fetuses may affect plasma testosterone surges in late stages of pregnancy or *post partum* (Ward

1972, Ward & Weisz 1984, Pereira *et al.* 2003). In this study, births were nocturnal and death of animals and plasma collection were always made at 0900 h. Although the analysis of prenatal testosterone levels was not the focus of this study, the lower testosterone levels observed for pups from obese mothers at 0.5 dpp strongly indicate that MO might have affected the testosterone neonatal peak, which usually occurs in the first hours after birth. Thus, present results suggest that obesity induced by a high-fat diet in Wistar rat may act as a stressor and unbalance steroidogenesis in the neonatal period. To our knowledge, this is the first study that examines the effects of MO on sex hormones and testicular development during the neonatal period in a rodent model. Considering that alterations in prenatal hormonal milieu might result in partial failure in the masculinization of behavior, this issue deserves to be examined.

Microscopic and quantitative data obtained to 0.5 dpp pups indicated that the current model of MO does not affect the processes concerning gonocyte fetal development, such as proliferation and apoptosis. On the other hand, our hormonal data suggest that higher estrogen levels of MO pups, mainly at 4.5 dpp, impaired gonocyte proliferation. Such a conclusion is supported by previous reports dealing with the inhibitory action of endogenous estrogen on male germ cell development during fetal and neonatal testis, via ERB signaling (Atanassova *et al.* 1999, Delbès *et al.* 2004, Delbès *et al.* 2007). Thus, Habert and colleagues have shown that homozygous inactivation of ERB increased the number of gonocytes by 50% in mice at 2 and 6 dpp due to an increase in the proliferation and decrease in apoptosis (Delbès *et al.* 2004). Indeed, there are few data dealing with the production of estrogen in fetal and neonatal rodent testis (Habert & Picon 1984, Greco & Payne 1994, Rouiller-Fabre *et al.* 1998, Delbès *et al.* 2004). These studies indicated that aromatase is expressed in Sertoli cells of fetal testis after the period of gonocyte proliferation (Greco & Payne 1994, Rouiller-Fabre *et al.* 1998), which may explain no alterations in the Nv of gonocytes. However, our data indicate that postnatal aromatase activity probably was affected by MO. MO and high consumption of saturated lipids reprogram adipose tissue metabolism, at postnatal life, leading to permanent changes in adipose tissue adipokines and enzyme activities (Benkalfat *et al.* 2011), and it is possible that such effects may have occurred to the testis. Besides, this obesity model results in insulin resistance (Ribeiro *et al.* 2012). As testicular development and function are influenced by the insulin signaling pathway (Nef *et al.* 2003) and leptin (Landry *et al.* 2013), they should also be implicated in the transient alterations here described. Other experiments are underway in our laboratory to evaluate the contribution of changes in aromatase expression and insulin signaling in testis development alterations induced by MO.

All parameters of gonocyte neonatal development examined here (Nv, migration, proliferation, and apoptosis), as well as the plasma levels of estrogen and testosterone indicate the changes that occur in the first days of life, especially at 4.5 dpp, are normalized at 7.5 dpp. The testicular volume and degree of maturation (early spermatogenesis) in MO pups at 14.5 dpp were similar to those of controls, suggesting an apparent recovery of germ cell development and steroidogenic capacity. The same model of MO followed by breastfeeding in non-obese mothers led to trend ~10% decline in rat sperm production and 5% in testosterone levels at adulthood (V Reame, R T Taboga, M E Pinto-Fochi & R M Góes 2012, unpublished data). The trend in sperm count reductions and hypoandrogenemia, together with the current data on alterations in testicular development in the first few days of life, suggests that MO affects testis development during the fetal period, leading to developmental reprogramming of organs and consequences for adult life. This is very worrying for male reproductive function when considering the influence of numerous other environmental factors that individuals are exposed to throughout life, as these could be exacerbated by discrete impairment induced by MO.

Taken together, our data indicate that MO, associated with saturated lipid consumption, affects the pattern of gonocyte differentiation in the first days of life, probably as a result of alterations in estrogen levels. However, testicular development recovery occurs at the end of the prepubertal period. These results show that the first postnatal days are crucial to the repair of germ cell development and steroidogenic activity in testis of rats affected by MO.

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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