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ARTICLE

Obesity induced by cafeteria diet disrupts fertility in the rat by affecting multiple ovarian targets

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Abstract Obesity constitutes a health problem of increasing worldwide prevalence. Among the health detriments caused by obesity, reproduction is disrupted. However, the mechanisms involved in this disruption are not fully understood. Animals fed a cafeteria diet constitute the model for the study of obesity that most closely reflects Western diet habits. The aims of this study were to evaluate whether a cafeteria diet affects ovarian function and to contribute to the understanding of the mechanisms involved. For that purpose, 22-day-old female Wistar rats were fed *ad libitum* with a standard diet (control group; $n = 20$) or cafeteria diet (CAF group; $n = 20$). The cafeteria diet induced obesity and hyperglycaemia, without altering serum triglycerides, cholesterol or C-reactive protein concentrations. This diet also altered ovarian function: the rats showed prolonged dioestrous phases, decreased serum oestradiol concentrations and increased number of antral atretic follicles. Moreover, follicular cysts were detected in the CAF group, concomitantly with a decrease in the number of anti-Müllerian hormone immunoreactive pre-antral follicles and COX-2-positive antral and pre-ovulatory follicles. The authors conclude that a cafeteria diet reduces ovarian reserve, induces the presence of follicular cysts and disturbs the ovulatory process, leading to the delayed pregnancy observed in these animals.

KEYWORDS: anti-Müllerian hormone, fertility, follicular cyst, folliculogenesis

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Introduction

Obesity presents a major public health concern since it constitutes a health problem of increasing worldwide prevalence. Obesity is a disorder of energy imbalance that develops when energy intake exceeds energy expenditure (Spiegelman and Flier, 2001). Reproduction is one of the health detriments caused by obesity (Hall and Neubert, 2005). Although most obese women are not infertile, obesity impacts negatively upon fecundity and fertility (Brewer and Balen, 2010). Obese women are three times more likely to suffer infertility than women with a normal body mass index (BMI) (Rich-Edwards et al., 1994) and to experience impaired fecundity both in natural and assisted conception cycles (Zaadstra et al., 1993). In humans, obesity induces anovulatory cycles and irregular menses (Douchi et al., 2002), reduces implantation and pregnancy rates (Hall and Neubert, 2005) and can be associated with polycystic ovarian syndrome (Rittmaster et al., 1993). However, the mechanisms by which excess body fat interferes with reproductive function are still not fully understood. Significant evidence suggests that excess body fat negatively affects female reproductive functions not only in humans but also in many models of obesity (Tortoriello et al., 2004).

Several animal models for studying obesity have been described, especially rodents fed on the 'cafeteria diet', which most closely reflects Western diet habits. In this model obesity is induced by feeding rats an assortment of highly palatable supermarket foods in addition to standard laboratory chow (Sclafani and Springer, 1976). Since it is considered a more palatable diet, the cafeteria diet has been associated with increased adiposity and insulin resistance (Akyol et al., 2012). Cafeteria feeding provides a useful alternative to feeding on conventional purified high-fat diets to induce obesity. It avoids the use of very high intakes of a particular type or source of fat while inducing persistent hyperphagia and increased energy intake (Rothwell and Stock, 1979; Shafat et al., 2009) thereby resembling more closely human dietary patterns. It has been shown that the cafeteria diet can impact on metabolic function, with changes in glucose homeostasis (Akyol et al., 2012; Higa et al., 2014; Sampey et al., 2011), but little is specifically known about ovarian glucose metabolism, as few researchers have studied its effects on the ovaries in animal models. It has been described that a high-fat diet can induce obesity and accelerate the development of ovarian follicles and the rate of follicle loss, leading to premature ovarian failure (Wang et al., 2014). Sagae *et al*. found that the cafeteria diet negatively affects female reproduction by reducing the number of oocytes and the thickness of the follicular layer. These authors also found that obese female rats showed no preovulatory progesterone or LH surges but that sexual receptiveness was not altered (Sagae et al., 2011). The aims of the present study were to evaluate whether obesity induced by the cafeteria diet affects ovarian function in female rats and to contribute to an understanding of the mechanisms involved.

Materials and methods

Animals and study protocol

Twenty-two-day-old female Wistar rats (*Rattus norvegicus*) weighing 120–130 g were obtained from the animal facilities of the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina. All research animals were treated in compliance with the guidelines for the care and use of animals approved by the Comité Institucional de Cuidado y Uso de Animales de Experimentación (CICUAL, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires) in accordance with the principles of laboratory animal care (NIH Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, Washington, D.C., USA).

Rats were divided randomly into two groups: (i) control group ($n = 20$) fed a standard rodent chow diet (ACA Nutrición Animal, Argentina) containing 11% fat, 23% protein and 66% carbohydrate; energy value 3.3 kcal/g; and (ii) cafeteria diet (CAF) group ($n = 20$), fed a varying menu of highly palatable human food comprising sausages, cheese, snacks, peanuts, biscuits and chocolate biscuits, adapted from previous studies (Akyol et al., 2009). In this group, the animals also had access to standard rodent chow. The highly palatable foods provided were altered daily to maintain variety, and the rats ate relatively large amounts of it.

Both the standard chow and cafeteria diet foods were individually weighed in and out of the cage between 10:00 h and 11:00 h daily. Daily intakes of energy, macronutrients and micronutrients in the CAF group were calculated from the manufacturers' data. All rats had ad-libitum access to all diet components as well as to water and were kept on a 12:12 h light:dark cycle at 22 °C. Food consumption was monitored daily for 60 days and weight gain was monitored twice a week.

Oestrous cycle staging

Beginning on day 50, vaginal smears were collected by lavage with 0.9% saline between 09:00 h and 11:00 h from each animal. The fluid was spotted thinly on a microscope slide, and the dried slides were stained with 0.1% Trypan Blue in deionized water. The oestrous cycle stage was determined by microscopic examination (Westwood, 2008). Vaginal cytology was examined until every rat passed through the oestrous phase after day 60. At that moment, when rats were 82–86 days old (to assure that animals had reached reproductive maturity), they were killed. Results are expressed as the time that animals spent in each phase of the oestrous cycle.

Anaesthesia and tissue collection

Twenty animals (10 from the control group and 10 from the CAF group) were killed in the first oestrous phase after day 60. After weighing animals, anaesthesia was performed with a 50 mg/kg solution of ketamine (Brouwer, Argentina) associated with 10 mg/kg xylazine (Alfasan, Holland) injected intramuscularly into the inner side of one of the hind legs.

Blood was obtained by cardiac puncture after anaesthesia and drawn into tubes with no anticoagulant. Blood glucose concentrations were determined immediately and blood was then centrifuged at 2000*g* to obtain serum for C-reactive protein (CRP), cholesterol, triglyceride determinations and hormone assays. All serum samples were frozen at −70 °C and

thawed on the day of the tests. After the animals were killed, the ovaries were removed, weighed and fixed in 4% (w/v) formaldehyde for 24 h. Afterwards, ovaries were dehydrated, embedded in paraffin, cut into 7-μm sections and mounted on gelatin-coated glass slides.

Reproductive outcome

After 60 days of diet administration, 20 animals (10 from the control group and 10 from the CAF group) were caged overnight with healthy males of proven fertility. Thereafter, every morning, vaginal smears were continued for oestrous cycle staging, and mating was confirmed by the presence of sperm in the vaginal smears. Results are expressed as time that the animals passed through the oestrous phase of the cycle until becoming pregnant.

Assays for glucose, triglycerides, cholesterol and CRP quantification

Food was withdrawn 12–14 h before the animals were killed and blood was collected for determination of glucose concentrations and serum concentrations of triglycerides, total cholesterol and CRP. All these determinations were performed according to the manufacturer's instructions.

Fasting blood glucose was determined using the glucose oxidase–peroxidase enzymatic colorimetric assay (GOD/PAP) (GT Laboratories, Rosario, Argentina) and its results are expressed as mg of glucose per dl. Triglycerides were determined by using the glycerol-3-phosphate oxidase enzymatic colourimetric assay (GPO-PAP) (GT Laboratories). Results are expressed as mg of triglycerides per dl. Total cholesterol was measured using the AA enzymatic method (Wiener Lab, Rosario, Argentina); its results are expressed as g of cholesterol per litre. CRP concentrations were determined using the direct latex method, in which CRP agglutinates with latex particles coated with monoclonal anti-CRP antibodies (GT Laboratories). Results are expressed as mg of CRP per dl.

Oestradiol and progesterone determination

Blood was allowed to clot and the serum removed and frozen until oestradiol and progesterone determinations. Progesterone concentrations were determined by immunochemiluminescence performed using ADVIA Centaur Immunoassay System (BAYER, Argentina). Oestradiol concentrations were determined using electrochemiluminescence using an automatic analyser Elecys 2010 (ELE) (Roche Diagnostics, Argentina). Results are expressed as ng of progesterone per serum ml and as pg of oestradiol per serum ml.

Ovarian histology and follicle counting

Tissue sections, prepared as described above, were stained with Masson trichrome stain, which was used to show the smooth muscle according to standard protocols (Britt et al., 2002), and then analysed using an Olympus light microscope. Seven-micrometer step sections were mounted at 50-μm intervals onto microscope slides to prevent counting the same structure twice, according to the method described by Woodruff et al. (1988). To prevent multiple counts of the same follicle, only follicles with a visible oocyte nucleus were included (Barreiro et al., 2011; Bernal et al., 2010). Follicles were counted and classified as described previously (Myers et al., 2004). Follicles were classified as either pre-antral or antral, according to the presence or absence of an antrum, or as pre-ovulatory. In the pre-antral group all the follicles that had no antrum were gathered, including primordial, primary and pre-antral follicles. Primordial follicles were characterized as oocytes surrounded by a single layer of flattened granulosa cells. Primary follicles were characterized as oocytes surrounded by a single layer of cuboidal granulosa cells. Pre-antral follicles were characterized as oocytes surrounded by two or more layers of cuboidal granulosa cells with no visible antrum. Antral folllicles were classified according to the presence of a small antrum and pre-ovulatory follicles according to the presence of a big central antrum showing an eccentric oocyte. In addition, the number of corpora lutea (CL) was counted in each section analysed. A cystic follicle was considered as a large follicle containing four or five plicated layers of granulosa cells surrounding a very large antrum (Convery et al., 1990; Lara et al., 2000) or a large fluid-filled structure with an attenuated granulosa cell layer and thickened theca interna cell layer (Lara et al., 2000) (**Figure S1**). The abundance of each type of follicle or corpora lutea was normalized by the total ovarian area in the section, as reported previously (Hu et al., 2004). The ovarian area was measured with Image J (version 1.42q, National Institute of Health, USA) and expressed per 10 mm^2.

Apoptosis

Apoptosis was detected using Tdt (terminal deoxynucleotidyl transferase)-mediated dUTP nick-end labelling (TUNEL) analysis. For that purpose, the ApopTag kit (Millipore, Temecula, CA, USA) was used in ovarian sections according to the manufacturer's instructions, and afterwards sections were counterstained with haematoxylin.

TUNEL-positive follicles were regarded as 'atretic' (Rosairo et al., 2008). Follicles were counted and categorized according to stage and TUNEL anlysis. The abundance of atretic follicles and CL were normalized in reference to the total number of the same structures detected in the same section.

Immunohistochemistry

The expressions of anti-Müllerian hormone (AMH) and cyclooxygenase type 2 (COX-2) were detected by immunohistochemistry as previously (Elia et al., 2013). Briefly, ovarian sections were subjected to an antigen retrieval technique by heat. Tissue slides were placed in a solution containing 0.01 mol/l citrate buffer, pH 6.2 for 5 min in a microwave oven at 100°C at 600 W. Endogenous peroxidase activity was blocked by incubation in 3% (v/v) hydrogen peroxide at room

temperature for 15 min. Background blocking was achieved by incubating with 5% (w/v) non-fat milk at room temperature for 30 min. The tissue sections were then incubated at 4°C overnight with mouse monoclonal anti-AMH (AbD Serotec, Oxford, UK) diluted 1:30 or with 1:50 rabbit polyclonal anti-COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as primary antibodies. The sections were incubated with biotinylated goat anti-mouse diluted 1:300 (Dako Cytomation, Carpinteria, CA, USA) and with 1:200 biotinylated goat anti-rabbit, respectively (Chemicon, Millipore, Temecula, CA, USA) at room temperature for 40 min, followed by 40-min incubation with streptavidin–biotin horseradish peroxidase complex reagent (Dako). Colour development was performed with a solution containing 3,3′-diaminobenzidine (Dako), and then the sections were counterstained with haematoxylin. Finally, the sections were dehydrated, mounted with Entellan New (Merck, Darmstadt, Germany) and observed with an FV-300 Olympus light microscope. Control sections were obtained by omitting the primary antibody.

Follicles immunoreactive for COX-2 or AMH were classified according to the stage of development. Afterwards, the abundance of immunoreactive follicles or CL was normalized by reference to the total number of the same structures detected in the same ovarian section.

Statistical analysis

Statistical analyses were carried out using the Instat program (GraphPAD software, San Diego, CA, USA). All data are expressed as mean ± SEM. The *t*-test was used to compare groups. Differences were considered significant when $P < 0.05$.

The significance of differences between the body weights of each group throughout the 60 days of the protocol was determined by two-way ANOVA with repeated measures followed by the Newman–Keuls test.

Results

Body weight and metabolic markers

Prior to the start of the study, there were no differences in body weight between animals assigned to the experimental diet groups, and all rats gained weight during the protocol (Figure 1). The caloric intake was significantly higher and continually increasing in the CAF group compared with controls (week 8: 85.80 ± 13.20 versus 322.96 ± 14.98 kcal/day; $P < 0.001$). This higher caloric intake led to a significantly increased weight gain after 60 days of diet administration in the CAF group in comparison with the control group (262 ± 9.03 g versus 310 ± 10.12 g, respectively; $P < 0.05$; Figure 1). Although animals became overweight, cafeteria diet-fed rats had little evidence of metabolic disturbances since they showed no alterations in the serum CRP, cholesterol or triglyceride concentrations (Table 1). In contrast, serum glucose concentrations were higher in the CAF group than in the control group (191.51 ± 9.85 mg/dl versus 324.72 ± 31.67 mg/dl, respectively; $P < 0.001$), suggesting that the cafeteria diet induced hyperglycaemia (Table 1).

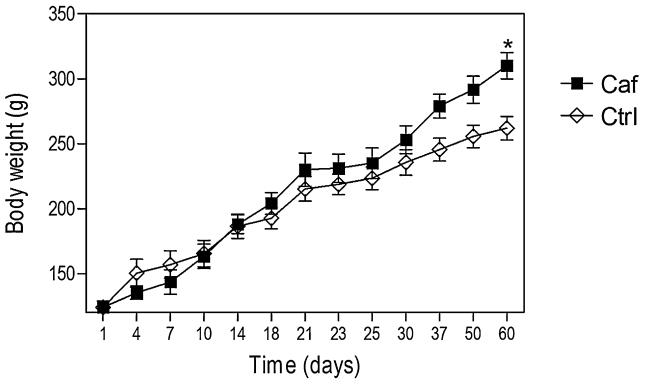

Figure 1 Body weight changes in rats fed: (◊) Ctrl = standard control diet and (■) Caf = cafeteria diet. Each time point represents the mean ± SEM, for $n = 20$. * $P < 0.05$ with respect to controls.

Table 1 Metabolic marker concentrations before (day 1) and after 60 days of diet administration.

Metabolic parameter	*Day*	*Group*	
		Control	*CAF*
Glucose (mg/dl)	1	170.85 ± 7.80	172.80 ± 8.36
	60	191.51 ± 9.85	324.72 ± 31.67[a,b]
Triglycerides (mg/dl)	1	72.50 ± 10.56	48.09 ± 20.39
	60	70.44 ± 12.74	51.09 ± 25.56
Cholesterol (g/l)	1	0.99 ± 0.28	1.22 ± 0.04
	60	0.97 ± 0.20	1.20 ± 0.18
CRP (mg/dl)	1	<8	<8
	60	<8	<8

$n = 10$ in each group.
CAF = cafeteria diet; CRP = C-reactive protein.
[a] $P < 0.001$ with respect to the control group.
[b] $P < 0.001$ with respect to day 1.

Reproductive cycles of rats

As a first approach to analyse whether a cafeteria diet alters reproductive function in the rat, the reproductive cycles were studied. Daily inspection of vaginal cytology in the CAF group revealed absence of oestrous cyclicity compared with the control group, with a prolonged period of persistent mixed cells (metoestrus), which lasted for a few days, followed by a period of persistent leukocytes (dioestrus) (Figure 2).

Serum progesterone and oestradiol determinations

Since an alteration in the oestrous cycle of the animals was found, the next aim was to analyse whether the ovarian function was affected by the cafeteria diet. For that purpose, the serum concentrations of progesterone and oestradiol, the principal steroids produced by the ovaries, were evaluated. It was found that serum progesterone concentrations in CAF-fed animals were similar to those in control rats. In contrast, serum oestradiol concentrations in cafeteria diet-fed animals were

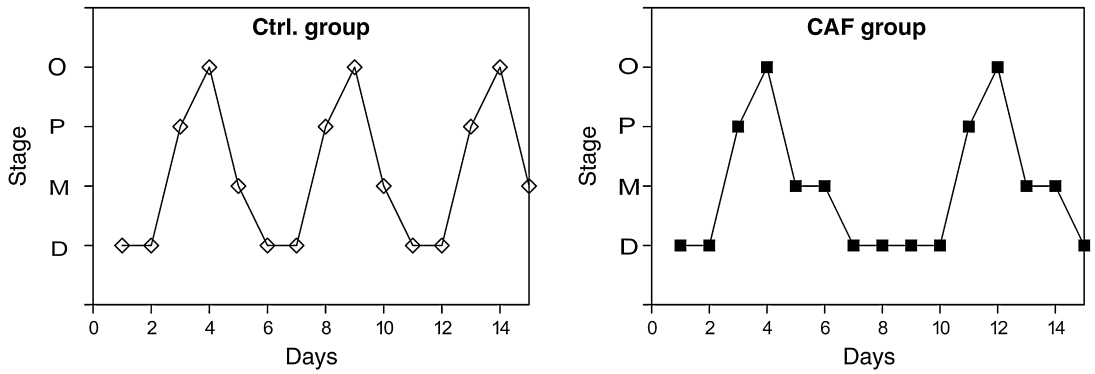

Figure 2 Effect of diet administration on the ooestrous cyclicity of rats ($n = 10$). Each graph corresponds to one animal, since the cycles were similar in the 10 animals. D = dioestrous-stage leukocytes; M = metoestrusstage nucleated, cornified cells and leukocytes; O = oestrous-stage cornified cells; P = proestrous-stage nucleated epithelial cells.

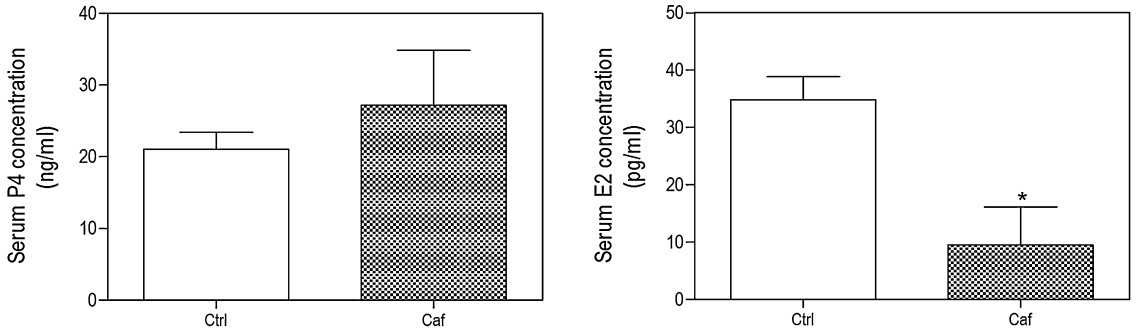

Figure 3 Effect of cafeteria diet administration on serum progesterone and oestradiol concentrations in rats. Each bar represents the mean ± SEM, $n = 10$. * $P < 0.05$ with respect to the control group. Caf = cafeteria diet; Ctrl = control; E2 = oestradiol; P4 = progesterone.

decreased with respect to controls (9.55 ± 6.55 pg/ml versus 34.83 ± 4.01 ml, respectively; $P < 0.05$; Figure 3).

Ovarian morphology

The lower serum oestradiol concentrations detected in the obese animals led to a study of whether the cafeteria diet affected the number of follicles in each stage of development, since oestradiol is produced by granulose cells in the developing follicles. For that purpose, a quantitative morphometric analysis was done by classifying follicles according to the stage of development. No differences were detected in the number of follicles in each stage of development (primordial, primary, secondary, antral and pre-ovulatory) between obese animals and controls. Moreover, the number of CL was similar in the CAF group compared with the control group (Figure 4A). However, cysts were found in the ovaries from cafeteria diet-fed animals (0.71 ± 0.18 cysts per 10 mm^2 of ovarian area) but not in control animals ($P < 0.05$, Figure 4A and B).

Apoptosis

The presence of follicles in the ovaries is not sufficient evidence to guarantee that they are functional, since they can, for example, be going through atresia and consequently not producing oestradiol. So, the next objective was to analyse whether the atretic process was altered in the ovaries from obese rats by performing a TUNEL analysis of follicles and classification of them as atretic if they had five or more apoptotic granulosa cells. Apoptosis was detected in pre-antral, antral and pre-ovulatory follicles as well as in CL in ovaries from both control and overweight animals (Figure 5A). When the number of atretic follicles and CL were analysed relative to the total number of the structures detected in the ovaries, it was found that the cafeteria diet had induced an increase in the number of atretic antral follicles in the CAF group compared with controls (12.88 ± 1.09 apoptotic antral follicles/total number of antral follicles versus 4.50 ± 0.87 apoptotic antral follicles/total number of antral follicles, respectively; $P < 0.001$), whereas the proportion of atretic CL, pre-antral or pre-ovulatory follicles did not differ between the two groups (Figure 5B).

Ovarian localization of COX-2

It is well documented that prostaglandins, produced via cyclooxygenase-2 (COX-2) within the peri-ovulatory follicle, are required for successful ovulation. The fact that there was an increase in the number of atretic antral follicles led us to speculate that their subsequent development may be

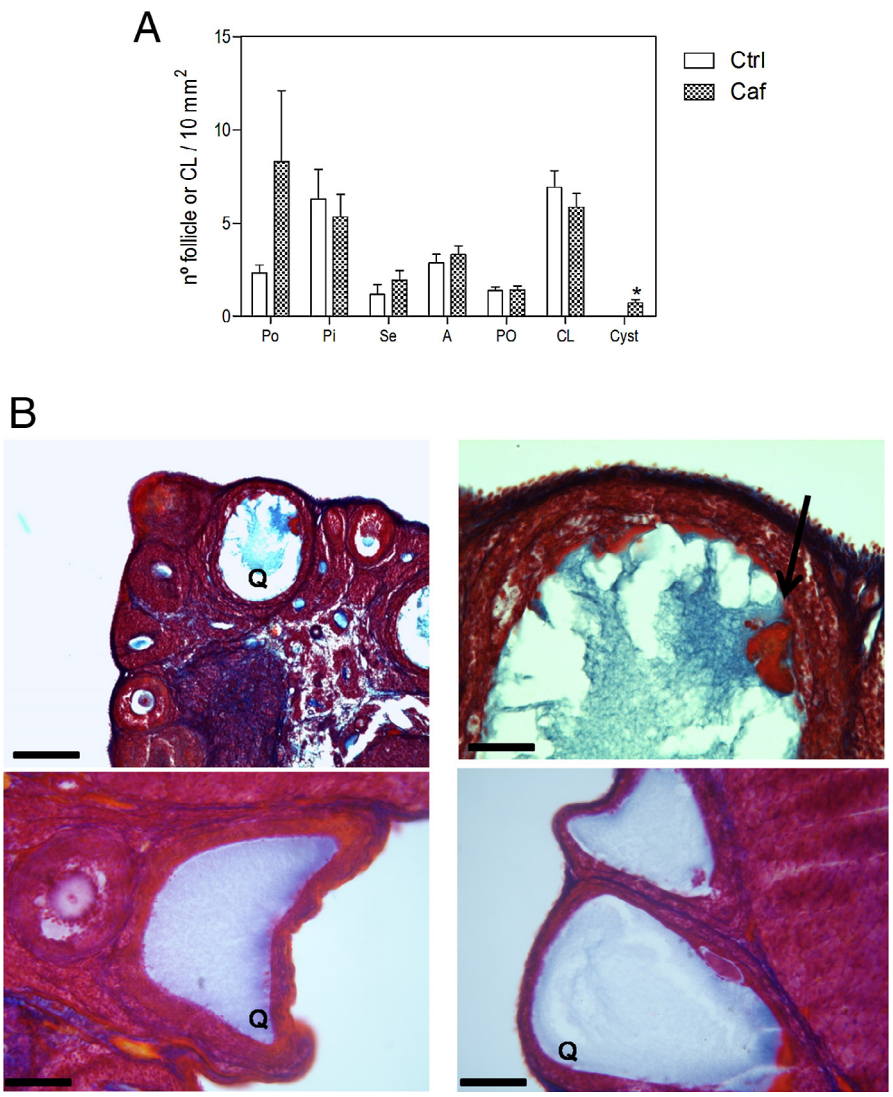

Figure 4 Effect of CAF administration on ovarian rat morphology. After Masson trichrome staining, a quantitative morphometric analysis was done. **(A)** Follicles were classified as primordial (Po), primary (Pi), secondary (Se), antral (A), and pre-ovulatory (PO) and corpora lutea (CL), as indicated in Materials and methods. Values are expressed as the number of the structure per 10 mm^2. Data represent the mean ± SEM, $n = 10$, and each value represents the mean of 10 sections from the same ovary. * $P < 0.05$ with respect to the control group (Ctrl). **(B)** Representative ovarian sections showing the cyst (Q) in the CAF group. In the *upper panel* a haemorrhagic cyst is shown (left: 100 ×, bar = 500 μm, right: 100 ×, bar = 100 μm, the arrow is showing bleeding in the haemorrhagic cyst. *Lower panel*: 100 ×, bar = 100 μm.

disrupted and therefore that oocyte release may be altered. For this purpose, ovarian COX-2 expression was analysed both in control and overweight animals. Representative ovarian sections showing COX-2 immunoreactivity are shown in Figure 6A. The results of the immunohistochemical analysis revealed no COX-2 immunostaining in pre-antral follicles (primordial, primary and secondary). However, moderate COX-2 immunoreactivity was found in the oocyte and granulosa cells in antral and pre-ovulatory follicles. Moreover, COX-2 immunostaining was also detected in CL. With respect to controls, ovaries from obese animals showed a lower proportion of (+)COX-2 antral (0.53 ± 0.07 versus 0.17 ± 0.12, respectively; $P < 0.05$) and pre-ovulatory follicles (0.87 ± 0.09 versus 0.17 ± 0.06, respectively; $P < 0.001$), although COX-2 localization was not modified. However, COX-2 expression was not altered by the cafeteria diet in CL (Figure 6B).

Ovarian localization of AMH

AMH is produced by granulosa cells in early developing follicles and inhibits the transition from the primordial to the primary follicular stage. AMH concentrations can be measured in serum and have been shown to be proportional to the number of small antral follicles (La Marca et al., 2009). To ask whether the cafeteria diet altered the number of follicles selected for developing, AMH distribution in the ovaries was analysed by immunohistochemistry. In ovaries from control and CAF animals, positive immunostaining for AMH was

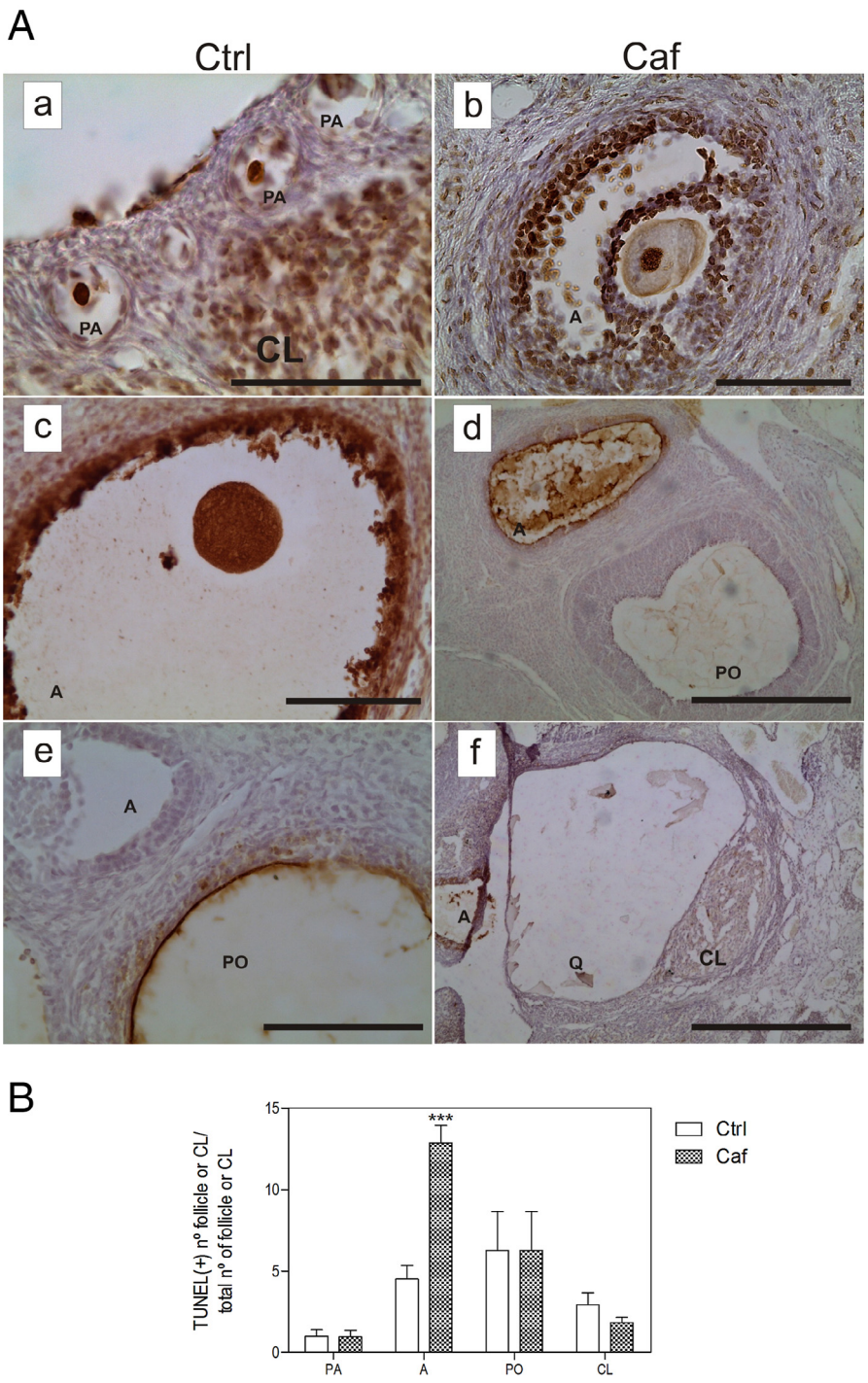

Figure 5 Effect of cafeteria diet administration on the number of apoptotic follicles and corpora lutea. Apoptosis was detected in pre-antral (PA), antral (A) and pre-ovulatory (PO) follicles and corpora lutea (CL) in ovaries from control and obese animals. **(A)** Representative ovarian sections showing: (a) TUNEL(+) PA follicles, CL and (b and c) A follicles. (d) An apoptotic A follicle and a TUNEL(−) PO follicle. *(e)* TUNEL(−) A follicle and an apoptotic PO follicle. (f) A TUNEL(−) cyst (Q) detected in the obese group. a: 1000 ×, bar = 50 μm; b, c, d, e and f: 400 ×, bars = 100 μm. **(B)** Analyses of the number of apoptotic PA, A, PO follicles and CL relative to the total number of the structures detected in the ovaries. Each bar represents the mean ± SEM, $n = 8$, and each value represents the mean of 10 sections from the same ovary. *** $P < 0.001$ with respect to the control group (Ctrl).

detected in pre-antral (primordial, primary, secondary) and antral follicles but not in pre-ovulatory follicles (Figure 7A). Moreover, ovaries from the CAF group showed a decreased number of primordial (2.75 ± 0.48 versus 0.50 ± 0.29, respectively, $P < 0.05$), primary (6.25 ± 0.48 versus 2.75 ± 0.85, respectively, $P < 0.01$) and secondary follicles (3.75 ± 0.63 versus 1.25 ± 0.25, respectively, $P < 0.05$) immunoreactive for AMH with respect to controls, but no difference was found in the proportion of AMH+ve antral follicles between the two groups.

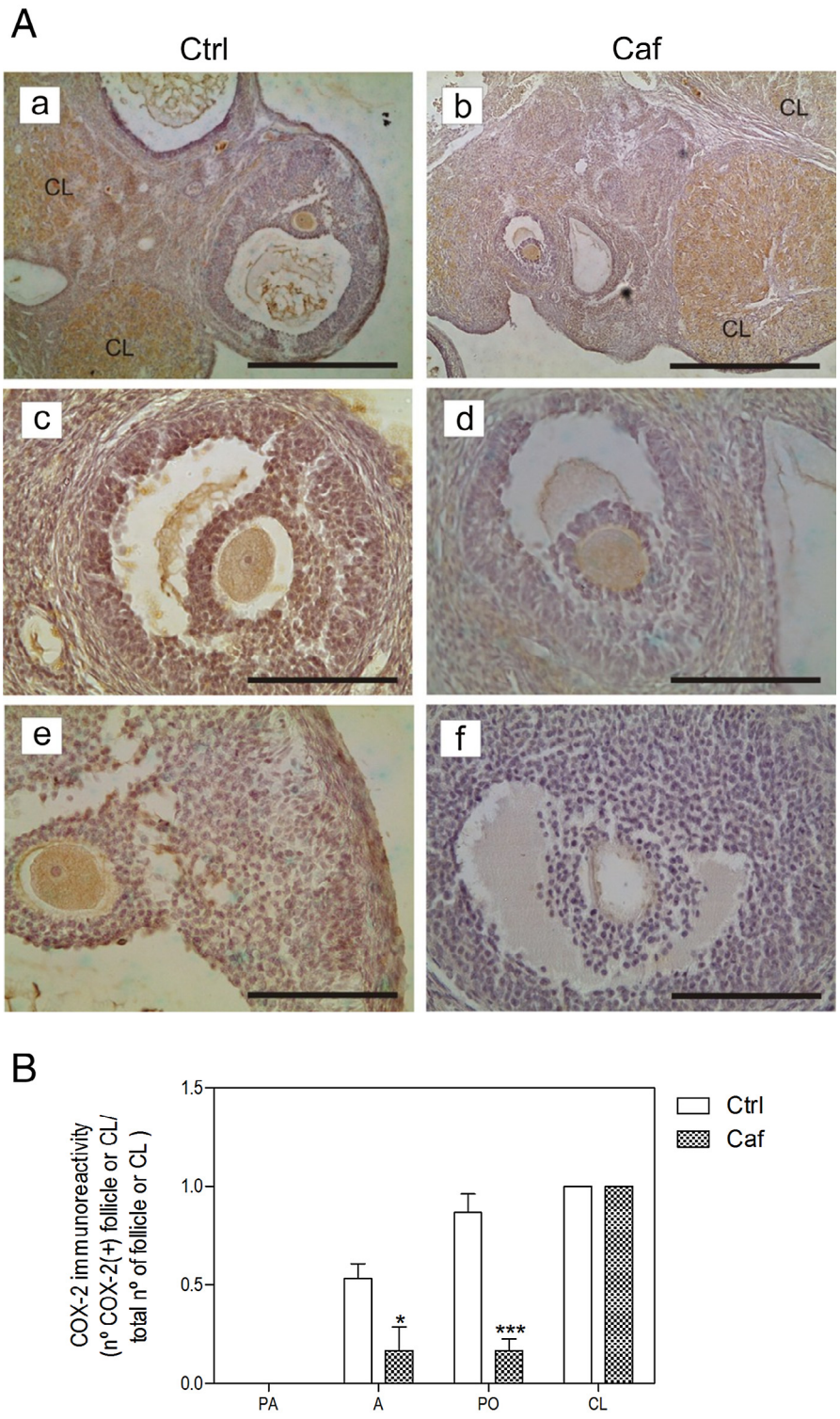

Figure 6 Effect of cafeteria diet administration on Cyclooxygenase 2 (COX-2) immunoreactivity. COX-2 was detected in antral (A) and pre-ovulatory (PO) follicles and corpora lutea (CL) in ovaries from control and obese animals. No immunoreactivity was detected in pre-antral follicles (PA). **(A)** Representative ovarian sections showing COX-2 immunoreactivity in CL (a and b) and in granulosa cells from A (c and d) *and* PO follicles (e and f) from control (Ctrl) and obese (CAF) animals. a and b: 100 × , bars = 400 μm; c, d, e and f: 400 × , bars = 100 μm. **(B)** Analyses of the number of COX-2(+) follicles or CL relative to the total number of the structure detected in the ovaries. Each bar represents the mean ± SEM $n = 8$, and each value represents the mean of 10 sections from the same ovary. * $P < 0.05$ and *** $P < 0.001$ with respect to the control group.

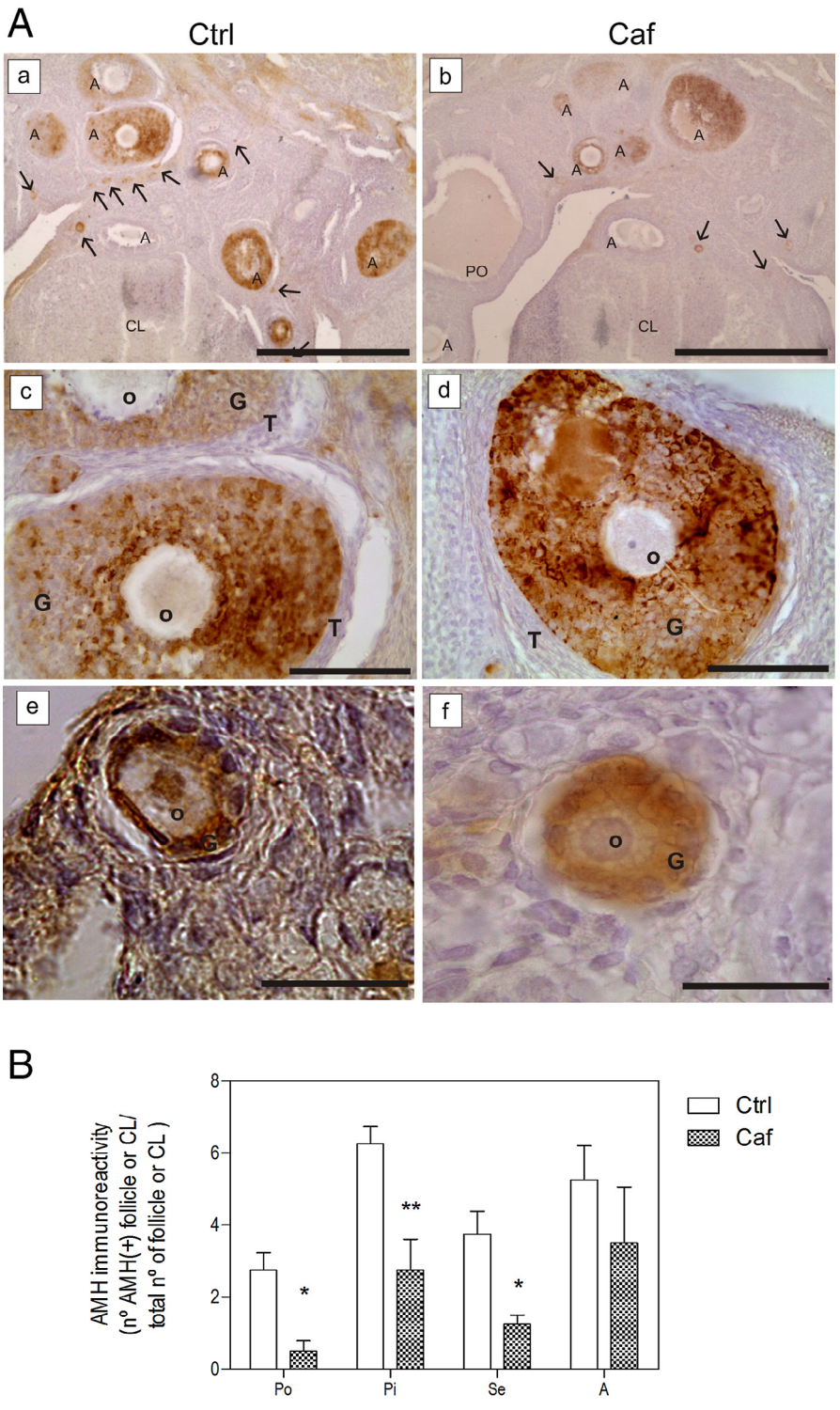

Figure 7 Effect of cafeteria diet administration on anti-Müllerian hormone (AMH) immunoreactivity. AMH was detected in primordial (Po), primary (Pi), secondary (Se) and antral (A) follicles in ovaries from control and obese animals. No immunoreactivity was detected in pre-ovulatory (PO) follicles or corpora lutea (CL). **(A)** Representative ovarian sections showing AMH immunoreactivity in: PA (arrow) and A follicles from control (a) and obese (b) animals. AMH was not expressed in oocytes (O) or theca cells (T) but it was found in granulosa (G) from antral (c and d) and pre-antral follicles (e and f). a and b: 100× , bars = 400 μm; c and d: 400 × , bars = 100 μm; e and f: 1000× , bars = 50 μm. **(B)** Analyses of the number of AMH(+) follicles detected in the ovaries. Each bar represents the mean ± SEM $n = 8$, and each value represents the mean of 10 sections from the same ovary. * $P < 0.05$ and ** $P < 0.01$ with respect to the control group.

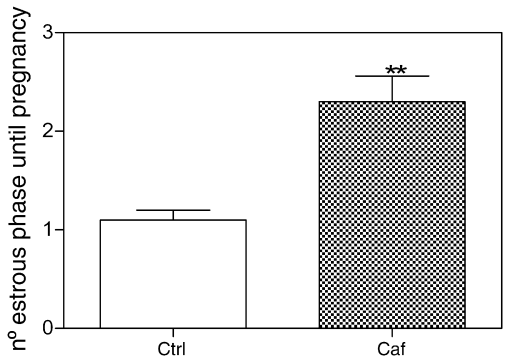

Figure 8 Effect of diet administration on the reproductive outcome analysed as the number of times that animals passed through the oestrous phase of the cycle until becoming pregnant ($n = 10$ for each group). Each bar represents the mean ± SEM and ** $P < 0.01$ with respect to the control group.

Reproductive outcome

To assess whether the multiple ovarian alterations described above had any impact on the reproductive success of the rats, reproductive outcome after the diets was evaluated. Most animals in the CAF group (9 out of 10) became pregnant. However, animals from the CAF group became pregnant later than rats from the control group. Animals in the CAF group passed through the oestrous phase of the cycle two to three times without becoming pregnant while control animals became pregnant in the first oestrous phase ($P < 0.01$; **Figure 8**).

Discussion

The results of the present study show that the cafeteria diet induced obesity and affected reproduction by disrupting several ovarian mechanisms. Previous reports have suggested that obesity is strongly associated with an imbalance in glucose homeostasis (Hotamisligil et al., 1993), which is frequently associated with a decreased capacity of insulin to regulate glucose metabolism in peripheral tissues (Boden, 1997), and these imbalances are also present in CAF-induced obesity. Thus, the cafeteria diet has been previously reported to increase energy intake and cause obesity in humans (Bull, 1988) as well as in animals (Rothwell et al., 1983). It induces hyperphagia in rats (Naim et al., 1985), which results in higher fat stores resulting in increased body weight (Barr and McCraken, 1984). The cafeteria diet is more efficient at inducing hyperphagia, adipocyte hypertrophy, hyperglycaemia, earlier glucose intolerance and insulin resistance than a high-fat diet (Higa et al., 2014).

The fact that CAF-fed rats not only became obese but also showed abnormally increased serum glucose concentrations supports the view that this diet is reducing the capacity of insulin to regulate glucose metabolism.

Obesity is associated with an inflammatory status (Zagotta et al., 2015) and the amount of dietary fat influences inflammation (Aoun et al., 2012; Westerbacka et al., 2005). To test whether the cafeteria diet induced inflammation, serum CRP concentrations were determined, but no alterations were found in obese animals, suggesting that these rats were not developing an inflammatory process. One possible explanation for this result is that cafeteria diet contains less fat than high-fat diets but is higher in sugar and salt, and therefore did not provide enough fat for the activation of the inflammatory pathway (Carillon et al., 2013). In addition, it has been described that the initial stage of diet-induced insulin resistance is independent of inflammation, which seems to appear later in the more chronic state (Lee et al., 2011). So, the short duration of the diet (60 days) could also explain why animals became obese without developing an inflammatory process.

Since it has been described that obese women experience impaired fecundity both in natural and assisted conception cycles (Zaadstra et al., 1993), an observation supported here for the rat, the aim of the present work was to study the ovarian mechanisms altered by obesity. AMH induces inhibition of follicular recruitment and decreases the sensitivity of follicles to FSH (Tsepelidis et al., 2007), preventing the depletion of any follicle cohort (La Marca et al., 2009). AMH is lower in obese women than in normal weight women (Su et al., 2008), although the number of ovarian follicles does not seem to be influenced by body mass in obese patients (De Pergola et al., 2006). In the present study it was also found that the number of follicles in each stage of development was not altered in obese animals compared with controls, and that ovarian AMH expression was reduced in the obese group. The fewer primordial, primary and secondary follicles positive for AMH in ovaries from cafeteria diet-fed animals compared with those in control ovaries suggests that the reduced local exposure to AMH in the obese rats would have contributed to an increased rate of primordial follicle recruitment, which may have led to early depletion of follicle cohorts. The present results are in accordance with previous works that also detected a significant negative correlation between BMI and serum AMH and the effect of BMI on the follicles retrieved (Aghadavod et al., 2015; Piouka et al., 2009).

Oestradiol is produced by the granulosa cells of the follicles during reproductive years, and the effect of obesity on its synthesis is controversial. Some authors have described obese women showing higher oestradiol concentrations than normal-weight patients (Zatko et al., 2013) while others have found that obese patients have lower oestradiol concentrations (Rehman et al., 2012; Rochester et al., 2009; Ross et al., 2014; Santoro et al., 2004), with a possible direct inhibitory effect of body mass on gonadotrophin and oestradiol production (De Pergola et al., 2006). In agreement with these latter findings, in this study it was found that obese CAF-fed animals showed decreased serum oestradiol concentrations. Oestradiol is known to inhibit food intake, water intake and body weight in several animal models, especially rats (Rao et al., 2014), as well as in women. In rodents, ovariectomy leads to the development of obesity, whereas oestradiol supplementation antagonizes these effects (Hertrampf et al., 2008; Naaz et al., 2002; Zoth et al., 2010). Taking into account all these data, we suggest that a cafeteria diet induces obesity and that the lower oestradiol concentrations may be a central feature in the development of this pathology. New experiments are being done in our laboratory to test this hypothesis.

Low concentrations of oestrogen induce a prolonged dioestrus in the rat (Gilmore and McDonald, 1969). Moreover,

it has been reported that cafeteria feeding for a period of 45 days is associated with long oestrous cycles with a long di-oestrous phase and that replacement of the cafeteria diet with purina rat chow corrects the oestrous cycle irregularities (Glick et al., 1990). Here it was found that cafeteria diet administration for 60 days produced a decrease in oestradiol serum concentrations and prolonged the duration of the dioestrous phase. Oestrogen plays a pivotal role in reproduction, affecting behaviour to folliculogenesis (Drummond and Findlay, 1999). The lack of oestrogenic actions is related to follicular atresia, which leads to the early exhaustion of follicles (Carson et al., 1981; Cheng et al., 2002). Moreover, oestrogen suppresses apoptosis in granulosa cells (Billig et al., 1993) and maintains corpus luteum function (Khan et al., 1987). Here, a decrease in oestradiol concentration was observed concomitant with an increase in the number of apoptotic antral follicles in obese rats with respect to controls, suggesting that the reduced concentrations of oestradiol induced by the cafeteria diet could be responsible for the increase in the number of atretic antral follicles.

The results discussed so far allow us to suggest that the lower serum oestradiol concentrations, the lower number of follicles expressing AMH and the higher number of atretic antral follicles are factors that may lead to an early exhaustion of follicles.

Prostaglandins (PGs) are important mediators of female reproductive processes (Algire et al., 1992), since they play important roles both in follicular development and rupture (Richards, 2001). The LH surge induces the expression of COX-2, which is a rate-limiting enzyme of PG synthesis in ovarian follicles (Joyce et al., 2001; Sirois et al., 1992). Bata et al. showed that administration of meloxicam, a partially selective COX-2 inhibitor, is associated with a significant delay in follicular rupture (Bata et al., 2006). Moreover, COX-2-deficient mice are infertile because of severely impaired ovulation (Davis et al., 1999; Lim et al., 1997). Furthermore, non-steroidal anti-inflammatory drugs such as aspirin and indomethacin have been reported to inhibit ovulation in various species, including humans, by inhibiting COX-2 (Mikuni et al., 1998; Priddy et al., 1990). It has also been described that aromatase-deficient mice are totally anovulatory due to insufficient oestrogen production but that sequential administration of high doses of 17β-oestradiol and gonadotropins induced ovulation in these mice by stimulating the mRNA expression of COX-2 (Toda et al., 2012). The authors demonstrated the requirement of oestradiol for the pre-ovulatory enhancement of prostaglandin E_2 synthesis, which in turn leads to future success in ovulation (Toda et al., 2012). The fact that, here, the ovarian COX-2 expression was decreased by the cafeteria diet, concomitantly with the low concentrations of oestradiol observed in the obese animals, suggests that this diet may be initially reducing the oestradiol concentrations and consequently, by reducing COX-2 expression, impairing follicular rupture. Although the mechanisms by which follicular cysts are formed are still unknown, it is plausible that the cysts are formed when the atretic process is disrupted. Here, the impairment in follicular rupture may have contributed to cyst formation.

Taken together, the present results suggest that a cafeteria diet administered from weaning age was able not only to induce obesity and hyperglycaemia but also to reduce the reproductive capability in adult female rats, indicating that this obesity model can be used to understand better the mechanisms underlying reproductive dysfunction in obese patients. We conclude that the cafeteria diet altered the ovarian function since it delayed pregnancy and affected multiple ovarian targets. The reduction in the number of primordial, primary and secondary follicles expressing AMH in the CAF group may be impairing the inhibition in the transition from primordial to primary follicles. So, the number of follicles initiating growth may be higher in obese rats than in controls. The fact that the total number of follicles in each stage of development was similar to that in controls, together with the increase in the number of atretic antral follicles in the obese group, leads us to suggest that a higher proportion of follicles may initiate growth but become atretic in the CAF group. Taken together, these results support the idea that obesity may lead to an early depletion of the follicle cohorts. The reduction in COX-2 expression in antral and pre-ovulatory follicles and the decreased serum oestradiol concentrations detected in obese animals suggests that follicular rupture may be impaired, an idea supported by the delay in becoming pregnant observed in obese animals. Finally, the impairment of ovulation may be responsible for the continuing presence of follicles in the ovary that are not going to atresia or ovulation, leading to follicular cyst formation, as detected in obese animals.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.rbmo.2015.08.004.

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