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Obesity alters the ovarian glucidic homeostasis disrupting the reproductive outcome of female rats

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Abstract

Obesity constitutes a health problem of increasing worldwide prevalence related to many reproductive problems such as infertility, ovulation dysfunction, preterm delivery, fetal growth disorders, *etc.* The mechanisms linking obesity to these pathologies are not fully understood. Cafeteria diet (CAF) is the animal model used for the study of obesity that more closely reflects western diet habits. Previously we described that CAF induces obesity associated to hyperglycemia, reduced ovarian reserve, presence of follicular cysts and ovulatory impairments. The aim of the present study was to contribute in the understanding of the physiological mechanisms altered as consequence of obesity. For that purpose, female Wistar rats were fed *ad libitum* with a standard diet (*control group*) or CAF (*Obese group*). We found that CAF fed-rats developed obesity, glucose intolerance and insulin resistance. Ovaries from obese rats showed decreased glucose uptake and became insulin resistant, showing decreased ovarian expression of glucotransporter type 4 and insulin receptor gene expression respect to controls. These animals showed an increased follicular nitric oxyde synthase expression that may be responsible for the ovulatory disruptions and for inflammation, a common feature in obesity. Obese rats resulted subfertile and their pups were macrosomic. We conclude that obesity alters the systemic and the ovarian glucidic homeostasis impairing the reproductive outcome. Since macrosomia is a risk factor for metabolic and obstetric disorders in adult life, we suggest that obesity is impacting not only on health and reproduction but it is also impacting on health and reproduction of the offspring.
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Keywords: Obesity; Ovary; Insulin; Glucose; Glut-4; Fertility; Reproductive outcome

1. Introduction

The global obesity epidemic is currently one of the most serious health concerns in the developed world and is an emergent concern in the developing world [1]. The World Health Organization has recognized obesity as an epidemic of the 21st century. As this epidemic of metabolic disorders continues, the associated medical comorbidities, including those affecting reproduction, increase as well [2]. In particular, women with obesity or poorly controlled diabetes have an increased risk of infertility, miscarriage, obstetric complications, neonatal morbidity and mortality and birth defects in their offspring [3].

Nutrition is one of the key environmental factors that lead to subfertility or infertility not only in clinical medicine but also in animal production [4]. Under-nutrition, over-nutrition and obesity are associated with poor reproductive performance [5–9]. However, the physiological mechanisms that underlie these effects are poorly understood. It has been described that obesity produce insulin resistance in the classic target tissues of insulin action, such as the liver and muscle [10,11]. However, it is controversial whether the reproductive axis remains insulin sensitive in the setting of peripheral insulin resistance. Brothers et al. have described a direct role of insulin signaling in the gonadotroph during the genesis of obesity-induced infertility. There is little literature regarding the action of insulin in the reproductive axis downstream pituitary, however, it has been shown that the ovary is insulin sensitive [12,13]. In this regards, data from clinical and experimental studies supports that estrogens contribute to glucose homeostasis, besides their pivotal role in sexual development and reproduction [14]. It is well recognized that the menopause favors visceral fat deposition and insulin resistance, leading to a significant increase in type 2 diabetes risk [15]. In this regard it has been shown that in postmenopausal women hormonal replacement therapy reduces the incidence of type 2 diabetes [16,17]. In concordance with that, bilateral ovariectomy of monkeys and rodents

Abbreviations: GDM, gestational diabetes mellitus; GLUT, glucotransporter; IR, insulin receptor; IGF, insulin growth factor; IGF1R, insulin-like growth factor 1 receptor; GTT, glucose tolerance test; ITT, insulin tolerance test; AUC, area under the curve.

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was shown to impair insulin sensitivity and glucose metabolism, a deleterious effect that was reversed by the chronic administration of estrogens [18,19]. All these evidences show a pivotal role of estrogens in regulating glucose homeostasis. However, the mechanisms by which estrogens influence insulin sensitivity and glucose metabolism remain poorly understood.

To prevent obesity the classical strategy is based on physical activity and reduced calorie intake. However, changing eating behavior and maintenance of ideal weight is difficult and hard to achieve not only *per se* but also because many of the infertile patients who attend infertility clinics at an age >30 years may not have much time to wait until they can lose weight because age itself is the major factor of declining fertility [3]. Thus, the identification of new molecular targets that can avoid, or at least to limit, the metabolic disturbances induced by obesity such as insulin resistance actually represents one of the most important public health challenge [20].

The development of animal models with metabolic dysfunction induced by diets with high caloric densities have been widely reported in the literature, as they can be used to reproduce the etiology, course and outcomes of human metabolic diseases [21–26]. In a previous study we have described that a western-style diet (cafeteria diet) induces obesity and hyperglycemia in rats concomitantly with multiple ovarian disruptions; *e.g.* ovulatory impairments, diminished estradiol levels, reduced ovarian reserve and follicular cysts development [27].

Based on the foregoing, the aim of the present work was to examine the influence of obesity on insulin sensitivity and glucose tolerance not only at systemic level but also locally in the ovary. Moreover, we wanted to evaluate the impact of obesity on the reproductive outcome.

2. Materials and methods

2.1. Animals and study protocol

Twenty two days old female Wistar rats (*Rattus Norvergicus*) weighing 120–130 g were obtained from Bioterio Central, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires. 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 to 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.).

All rats had *ad libitum* access to water and standard rodent chow diet (ACA Nutrición Animal, Argentina) and were kept on a 12:12-h light–dark cycle at 22 °C. Rats were divided randomly into two 60-days intervention groups distinguished by dietary composition: (1) *Control Group* (n=20) was fed only standard rodent chow diet; (2) *Obese Group* (n=20) was also offered with a "cafeteria-style" diet (a varying menu of highly palatable human foods comprising sausages, cheese, snacks, peanuts, biscuits and chocolate biscuits). This animal model of obesity was adapted from previous studies [28] and has been previously successfully implemented by us [27]. Weight gain, abdominal circumference and body length were monitored twice a week.

The day of the first estrus phase after the 60thintervention day, animals were separated for 2 different studies: (1) Pre-gestational ovarian status studies: For that purpose, 10 animals (5 control and 5 obese) were subjected to a glucose tolerance test (GTT). Afterwards, these rats were sacrificed and ovaries were removed to perform PCRs, IHQs and to analyze the ovarian glucose intake. (2) Reproductive outcome studies: From a total of 30 animals (15 control and 15 obese), 10 were subjected to an insulin tolerance test (ITT). All rats were fed standard rodent chow from this moment and their reproductive outcome was evaluated.

2.2. Glucose tolerance test

For the glucose tolerance test (GTT), 6 h fasted control and obese rats were intraperitoneally injected with a bolus of glucose (2 g/kg) and blood glucose levels were determined at 0, 15, 30, 60, and 120 min after glucose challenge. Glycemia was measured in tail blood using glucose strips on an Accu-Chek Performa II instrument (Roche, Buenos Aires, Argentina). Data was collected for each individual animal and expressed as mean blood glucose concentration over time. The area under the curve (AUC) for glucose was calculated to evaluate glucose tolerance in control and obese animals [29].

2.3. Insulin tolerance test

The insulin tolerance test (ITT) was performed in 2 h fasted rats administrating a single intraperitoneal insulin injection (0.5 U/kg diluted in PBS) and blood glucose was sampled at times 0, 15, 30, 45, 60, 90, 120 and 150 min after insulin injection. Glycemia was measured in tail blood using glucose strips on an Accu-Chek Performa II instrument (Roche, Buenos Aires, Argentina). Insulin sensitivity for control and obese animals was estimated during ITT by the first-order rate constant of glucose disappearance (K_{ITT}) computed as the slope of the regression line of blood glucose against time during the first 60 min [30].

2.4. Anesthesia and pre-gestational tissues collection

The first estrus phase after the 60thintervention day, 10 animals (5 from control and 5 from obese group) were subjected to euthanasia after performing anesthesia with a 50 mg/kg solution of ketamine (Brouwer, Buenos Aires, Argentina) associated with 10 mg/kg xylazine (Alfasan, Woerden, Holland) that were injected intramuscularly into the inner side of one of the hind legs. Afterwards, ovaries were removed. One ovary from each animal was used fresh for uptake glucose analysis. Half of the remaining ovary was frozen for subsequent RNA extraction and the other half was fixed in 4% (w/v) formaldehyde for 24 h, dehydrated, embedded in paraffin and cut into seven-micron sections. Ovarian sections were mounted on gelatin-coated glass slides and subsequent used for immunohistochemical studies.

2.5. Glucose uptake by ovaries

The ovarian glucose uptake measurement was adapted from previous works [31]. Briefly, one ovary from each animal was isolated and divided into two halves. Both halves were incubated in Krebs-Ringer bicarbonate (KRB) buffer (117 mMNaCl, 4.7 mMKCl, 2.5 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 24.6 mM NaHCO3, pH 7.4) containing 2 mM pyruvate for 50 min at 37 °C. Ovaries were transferred to KRB containing 1 mM 2-deoxy-D-[1,2–3 H(N)]glucose (3 mCi) and incubated for an additional 10 min with or without insulin (0.1 mU/ml) at 30 °C. Incubation and transport buffers were continuously gassed with 95% O_2–5% CO_2. Transport was terminated by immersion in ice-cold KRB containing 80 mM cytochalasin B. Ovaries were frozen in liquid nitrogen and processed as previously described [32]. Aliquots of the lysate were used for protein measurement using Bradford and radioactivity in the solubilized tissue was measured in a liquid scintillation spectrometer.

2.6. Ovarian RNA extraction and polymerase chain reaction (PCR)

Total RNA was extracted from the ovaries using Trizol reagent (Invitrogen, *CA*, USA) according to the manufacturer's protocol. cDNA was synthesized by incubating 2 μg of extracted RNA in a buffer containing, 3 U AMV Reverse transcriptase (Promega, Madison, WI, USA), 1 μM oligo d(T)15 Primer (Dongsheng Biotech, Guangdong, China) and 1 mM Mix dNTPS (Dongsheng Biotech). The reaction mixture was incubated for 60 min at 42 °C followed by 15 min at 70 °C. cDNA (2 μl, selected to work within the linear range) was amplified by PCR in a buffer containing 0.5 U Taq-DNA polymerase (Invitrogen), 0.2 mM of each primer (Invitrogen), 0.2 mM of each dNTP, 1.5 mM MgCl2 and each specific primer. The primer sets used are detailed in Table 1 where the specific annealing temperature used for each pair of primers is included as well as the number of cycles used. The optimum cycle number was determined for each primer pair, so that signals were always in the exponential portion of the amplification curve. Each cycle consisted of: denaturation at 95 °C for 15 s, primer annealing at the specific temperature for 30 s and extension at 72 °C for 15 s. PCR products were electrophoresed on 2%

Table 1
Details of primers used for PCR

Gene	Primer sequence (5″-3″)	Fragment size (bp)	Annealing temperature (°c)	Cycles no.	GenBank accession no.
Glut-4	F: ACTGGCGCTTTCACTGAACT R: CGAGGCAAGGCTAGATTTTG	106	55	40	NM_012751
InsR	F: ATCCGTCGCTCCTATGCTCTGGTGT R: GTT GGT CTT CAG GGC AAT GTC GTT C	279	64,5	40	NM_017071
GAPDH	F: CCATCAACGACCCCTTCATT R: GACCAGCTTCCCATTCTCAG	110	57	35	NM_017008

agarose (Biodynamics, Buenos Aires, Argentina) gels. Gel images were taken with the ImageQuant RT ECL (General Electric, Amersham Bioscience, Argentina) and software and quantified with Image J software (version 1.42q, National Institute of Health, USA). Data were normalized to GAPDH mRNA in each sample. Negative controls were performed without reverse transcriptase or RNA.

2.7. Immunohistochemistry

The ovarian expression of glucose transporter type 4 (GLUT-4) and Nitric Oxide Synthase (NOS) were analyzed by immunohistochemistry according to Elia et al. [33]. Tissue slides were placed in a solution containing 0.01 M 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. Tissue sections were then incubated at 4 ° C overnight with rabbit polyclonal anti-GLUT-4 (1:300; Santa Cruz Biotechnology, *CA*, USA; sc-7938) or anti-NOS (1:50; Abcam, Cambridge, Mass, USA; ab 15,203) as primary antibodies. Controls were performed by omitting the primary antibody. Sections were, then, incubated with biotinylated goat anti rabbit (Chemicon Millipore, Temecula, CA, USA) diluted 1:1000 at room temperature for 40 min; and, afterwards, incubation for 40 min with streptavidin–biotin horseradish peroxidase complex reagent (DakoCytomation, Carpinteria, CA, USA) was done. Color development was performed with a solution containing 3,3′-diaminobenzidine (DakoCytomation) and sections were counterstained with hematoxylin. Finally, the sections were dehydrated, mounted and observed with an FV-300 Olympus light microscope and photographed.

Follicles were classified according to the stage of development as previously described [34]. Briefly, in the preantral group we gathered all the follicles that had no antrum, including primordial, primary and secondary 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. Secondary 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 preovulatory follicles according to the presence of a big central antrum showing an eccentric oocyte. In addition, the number of corpora lutea was counted in each section analyzed.

Afterwards, each structure was defined as immunoreactives when specific label was observed compared to the appropriate negative controls. Densitometric analysis was performed using Image-Pro Plus software (Media Cybernetics) using the mean integrated optical density (IOD) of the label for each class of immunoreactive structure (follicles in different stage of development and corpora lutea) as previously described by us [33].

2.8. Reproductive performance

After diet protocols, obese and control rats were transferred to a mating cage and cohabited with proven fertility male rats (1:1). Mating was confirmed by the presence of a vaginal plug and/or sperm in the vaginal smear taken each morning during cohabitation. The day on which evidence of copulation was identified was termed day 0.5 of gestation. On day 18.5 of pregnancy, rats were laparatomized and the total number of implantations was counted and fetuses were removed by the uterine opening. The number of live and dead pups and their body weights were recorded.

Mating index (number of sperm-positive females/number of cohabitated females×100), fertility index (number of pregnant females/number of cohabitated females×100) fecundity index (number of pregnant females/number of sperm-positive females×100), and post-implantation loss (difference between the number of implantations and the number of live fetuses expressed as per number of implantations×100) were calculated as previously described [35]. In addition, the conception time: number of days after initiation of cohabitation required for each pair to mate (detected by the presence of sperm in vaginal smears) was recorded for each female.

2.9. Statistical analysis

Experimental data are presented as the mean ± S.E. M unless otherwise is indicated and the number of animals used for each determination is indicated in the figure legends as n. Statistical analyses were carried out by using the Instat program (GraphPAD software, San Diego, *CA*, USA) and $P<.05$ was considered statistically significant.

For GTT and ITT, the significance of differences between control and obese rats were determined by two-way ANOVA with repeated measures followed by the Newman–Keuls test. For the glucose uptake measurement, comparisons between groups were performed using one-way analysis of variance (ANOVA) and Tukey's was used for the *Post Hoc* multiple-comparison ANOVA analyses. Mating, fecundity and fertility index were analyzed by Fisher's exact test. The significance of the remaining results was determined using Student's *t*-test.

3. Results

3.1. Cafeteria diet induces obesity altering body fat distribution

Body weight gain was significantly increased after 60 days of cafeteria diet administration in comparison with control fed animals ($P<.05$; Fig. 1), supporting the use of cafeteria diet for inducing obesity in rats as previously described. Moreover, the relation between the abdominal circumference and the total body length was also increased in cafeteria diet fed animals ($P<.05$; Fig. 1), pointing out that cafeteria diet is modifying the body fat distribution. Moreover, body weight gain was significantly higher in obese rats than controls throughout pregnancy ($P<.001$, Fig. 1) despite all animals were fed standard chow during this period, indicating the importance of the maternal body weight at conception time.

3.2. Obesity induces glucose intolerance in rats

Glucose tolerance was evaluated in control and obese rats on the first estrus after the 60th intervention day. As shown in the GTT curve (Fig. 2), control animals showed the expected rapid increase in the glycemia followed by rapid clearance of glucose to basal concentrations within 2 h of glucose challenge. Obese rats had higher blood glucose levels compared to the controls in all the times analyzed: 0, 15, 30 and 60 min ($P<.001$) and 120 min ($P<.05$). It resulted in a significant rise in the AUC corresponding to obese animals compared to controls ($P<.001$). All this data suggest that cafeteria diet induced obesity associated to glucose intolerance in rats.

3.3. Obesity is associated to insulin resistance in the cafeteria diet model

Insulin tolerance was evaluated on the first estrus after the 60-thintervention day to accurately determine insulin sensitivity in control and obese animals (Fig. 3). Control rats showed the expected decrease of glycemia followed by clearance of glucose to basal concentrations within 2 h of insulin challenge. So, the slope of the kinetic curve (K_{ITT}) was negative in control rats, describing the glucose disappearance in response to insulin. Meanwhile, obese rats showed higher glycemia than control animals in all the analyzed times after insulin injection: 0 ($P<.01$), 15, 30, 60, 120 ($P<.001$) and 150 min ($P<.05$). It is worth noting that in obese animals the K_{ITT} was not only different from that in controls ($P<.001$) but it was close to zero, indicating that obese animals are insulin resistant.

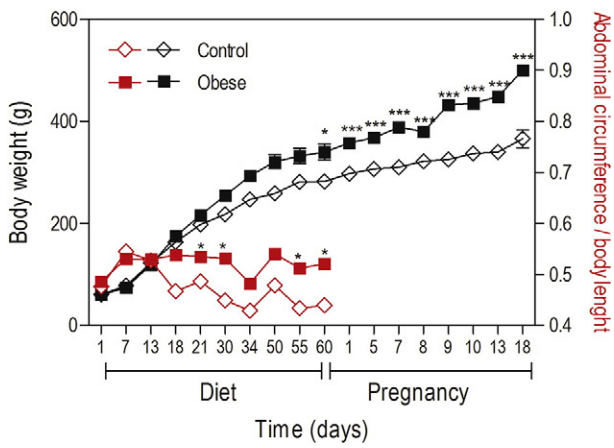

Fig. 1. Body weight and ratio between abdominal circumference and body length changes in rats fed control diet (Control, open diamonds) and cafeteria diet (Obese, filled squares) during 60 days. Body weight gain was also determined throughout pregnancy. Each time point represents the mean ± S.E.M. ($n=20$). * $P<.05$ and. *** $P<.001$ respect to controls.

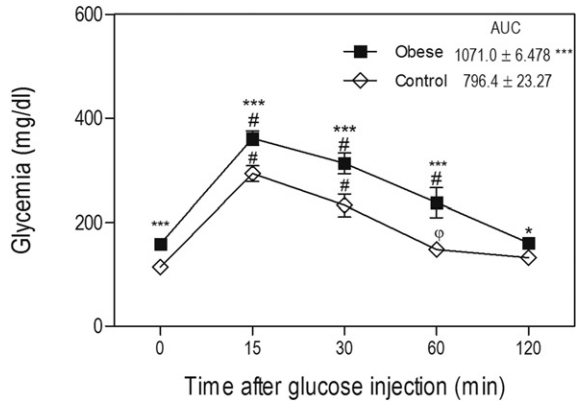

Fig. 2. Blood glucose levels during the glucose tolerance test in control (open diamonds) and obese animals (filled squares). Values in the insert show the area under the curve (AUC) during the glucose tolerance test. The data are given as the means±S.E.M. (n=5). * P<.05 and *** P<.001 respect to control group; # P<.001 and φ P<.01 respect to the time 0.

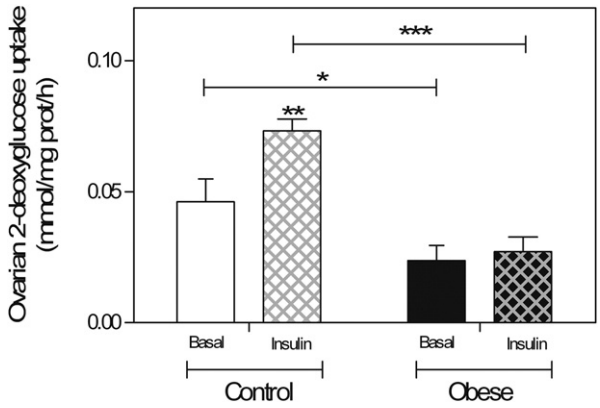

Fig. 4. Basal (plain bars) and insulin stimulated (squared bars) 2-deoxyglucose uptake in ovaries from control (open bars) and obese (filled bars) animals. The data are given as the means ± S.E.M. (n=5). * P<.05, ** P<.01 and *** P<.001 respect to the basal condition or between the indicated groups.

3.4. Obesity induces ovarian insulin resistance

The results of the glucose uptake by ovaries from control and obese rats are shown in Fig. 4. The basal glucose uptake was~50% lower in ovaries from obese rats compared to controls (P<.05). Insulin stimulated glucose uptake by nearly 1.5-fold in ovaries from control rats (P<.01), whereas the insulin response was almost completely blunted in ovaries from obese rats. These results shows that ovaries from obese rats have lower glucose uptake under normal conditions but they also develop insulin resistance.

3.5. Obesity decreases the ovarian insulin receptor gene expression

Since obese animals showed ovarian insulin resistance, the gene expression of the insulin receptor (InsR) was evaluated in these ovaries. A decrease in the InsR mRNA levels was detected in ovaries from obese animals when compared to controls (P<.05, Fig. 5 A). This result shows that the ovarian insulin resistance induced by obesity is, at least in part, due to a change in the transcriptional regulation of the *InsR* gene.

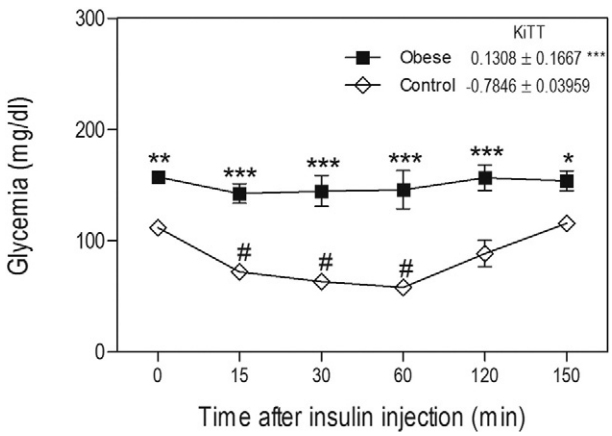

Fig. 3. Blood glucose levels during the insulin tolerance test in control (open diamonds) and obese animals (filled squares). The slopes of the kinetic curves (K_{ITT}) are shown in the insert. The data are given as the means±S.E.M. (n=5). * P<.05, ** P<.01 and *** P<.001 respect to control group and # P<.001 respect to the time 0.

3.6. Obesity decreases the number of follicles expressing the glucose transporter type 4

Glucose transporter type 4 (GLUT-4) is the insulin-responsive transporter, so, we evaluated the GLUT-4 expression by immunohistochemistry. The results of the immunohistochemical analysis (Fig. 6 k) revealed that GLUT-4 protein expression was not detected in preantral follicles (PA), that includes: primordial (Fig. 6 a and c), primary and secondary (Fig. 6 b and d) follicles, neither in obese nor control animals. Antral (A) follicles from control animals (Fig. 6 e) showed GLUT-4 immunoreactivity in the oocytes and in granulose cells, however GLUT-4 immunoreactivity was not detected in granulose cells of A follicles in obese animals (Fig. 6 f). Preovulatory (PO) follicles from control animals (Fig. 6 g and i) were positive for GLUT-4 in granulose cells however that immunoreactivity was not detected in obese animals (Fig. 6 h and j). So, GLUT-4 follicular expression is development dependent and obesity inhibits this differential expression pattern.

3.7. The ovarian glucose transporter type 4 mRNA levels are not modified by obesity

Since ovaries from obese rats are insulin resistant and show a decreased GLUT-4 expression and given that insulin regulates trafficking of vesicles that contain GLUT-4, we were interested in analyzing if obesity modifies glut-4 gene expression or if obesity impacts on GLUT-4 trafficking as insulin does. Glut–4 mRNA abundance in ovaries from control and obese rats were determined by PCR. The results shows that cafeteria diet did not altered Glut-4 mRNA level in the ovaries (Fig. 5 B), indicating that the regulation of ovarian Glut-4 expression by obesity is not at transcriptional level.

3.8. The number of follicles and corpora lutea expressing the nitric oxide synthase is increased by obesity

Insulin resistance has been associated with impaired production/release of nitric oxide (NO), one of the main factors responsible for ovulation. Since NO is produced by the action of Nitric Oxide Synthase enzyme (NOS) we aimed to analyze whether obesity alters the ovarian NOS expression in association to insulin resistance development. The results of the immunohistochemical analysis (Fig. 7 g) revealed that NOS was not detected in preantral (PA: primordial, primary and secondary follicles) and small A follicles neither in control nor obese animals (Fig. 7 a and b). Large A and PO follicles from control animals

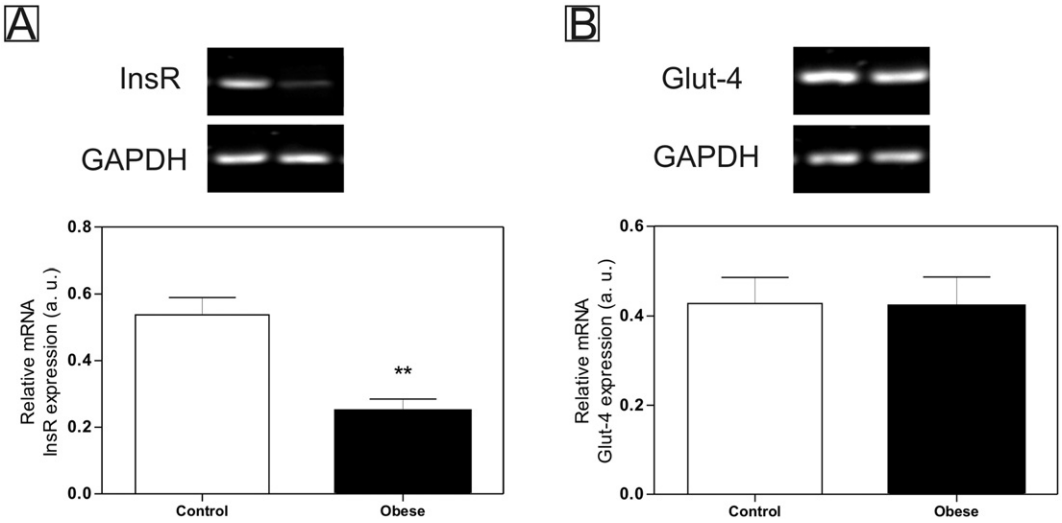

Fig. 5. Ovarian gene expression of (A) Insulin Receptor (InsR) and (B) Glucotransporter type 4 (Glut-4) in control (open bars) and obese (filled bars) animals evaluated by RT-PCR. Representative gels are shown in the upper panel. In the lower panel the densitometric analysis are shown. Values are expressed in arbitrary units (a.u.) as means ± S.E.M. of optical density of InsR or Glut-4 mRNA relative to the optical density of GAPDH mRNA as the housekeeping (n=5). ** P<.01 respect to the control group.

did not show NOS immunoreactivity (Fig. 7 c). Conversely, in obese rats, NOS immunoreactivity was detected in theca cells and to a lesser extent in granulose cells of large A and PO follicles (Fig. 7 d). NOS was detected in corpora lutea (CL) from both control and obese animals (Fig. 7 e and f). NOS immunoreactivity was higher in CL from obese animals rather than controls (Fig. 7 g). Thus, the ovarian NOS expression is physiologically modified during the follicular development and obesity alters this pattern by increasing NOS expression.

3.9. Obesity disrupts the rat reproductive outcome and impacts on the fetal health

The reproductive performance of obese rats was determined by considering the end points such as mating, fertility and fecundity index, conception time and number of implantations, total and live fetuses per rat and post-implantation losses (Table 2). The effect of maternal obesity on fetal body weight was also evaluated.

It was found that the mating index in both control and obese rats was 100%, however conception time was higher in obese rats when compared to controls (P<.001), indicating that obesity delayed conception. Moreover, obese rats showed lower fecundity and fertility rates than controls (P<.005), suggesting that obesity is associated to sub-fecundity and sub-fertility. The number of implantations as well as the number of live fetuses was not altered by obesity, therefore the post-implantation loss rates were similar between obese and control rats. The evaluation of pups reveled that body weight were higher in fetuses from obese mothers than from control rats (P<.005), indicating that obesity is affecting not only the mother's reproductive outcome but it is also impacting on fetal health.

4. Discussion

The current rise in obesity and associated disorders such as metabolic syndrome, polycystic ovarian syndrome and type 2 diabetes has drawn attention to the effect of these diseases on the reproductive system. Nutrition is one of the key environmental factors that lead to sub-fertility or infertility [4] and hyperinsulinemia is also associated with infertility [36] but the underlying mechanisms remain poorly understood. Moreover, there is considerable evidence indicating that the excessive accumulation of white adipose tissue is strongly correlated with the development of insulin resistance and type 2 diabetes [37,38]. To examine the influence of obesity on insulin sensitivity and glucose tolerance not only at systemic level but also locally in the ovaries, we worked with an animal model of obesity induced by cafeteria diet, evaluating the impact of obesity on their reproductive outcome as well.

Here, we found that cafeteria diet induced obesity associated to glucose intolerance and insulin resistance in rats. Our data corroborate previous studies that demonstrated higher body weight, hyperglycemia and insulin resistance as a consequence of cafeteria diet administration in rodents [38–40]. Furthermore, our data demonstrate that ovaries are insulin sensitive under normal conditions becoming insulin resistant as consequence of obesity.

The little literature regarding the action of insulin on the ovary showed that in insulin resistant women insulin acts in the ovary by binding to the insulin-like growth factor 1 receptor (IGF1R) and not to its own receptor (InsR); and in those cases it is common to find morphological ovarian changes, such as polycystic changes, that are produced by continuous stimulation of the ovary by insulin over a long period of time [13]. In this regards, we have previously described, using the same animal model, that obese rats showed follicular cyst [27]. The fact of finding that cafeteria diet induces ovarian insulin resistance and the development of follicular cysts led us to suggest that in obese animals insulin may be inducing follicular cyst development acting through IGF1R. This suggestion is also supported by the fact that here we found a reduced ovarian InsR gene expression in obese animals when compared to controls.

Glucose is an essential metabolic substrate that is important controlling ovarian function and activity [41]. It is taken up into cells mediated by glucose transporter proteins (GLUT) [42]. GLUT-1–4 are engaged to transport enough glucose to ovarian cells that results necessary for follicular development, terminal follicular maturation and the resumption of meiosis [43]. In particular, GLUT-4 is the insulin-responsive transporter and probably the most studied protein among glucose transporter isoforms since it plays an important role in whole body glucose homeostasis and in the pathogenesis of type II diabetes mellitus [44–46]. Regarding GLUT-4, our data also shows that ovaries from obese rats have a reduced ovarian glucose uptake and a decreased number of follicles expressing GLUT-4 despite Glut-4 gene expression is normal in these animals. Moreover, these ovaries did not modify the glucose uptake after insulin- stimulation, for sure as a consequence of the detected reduction in the ovarian InsR levels;

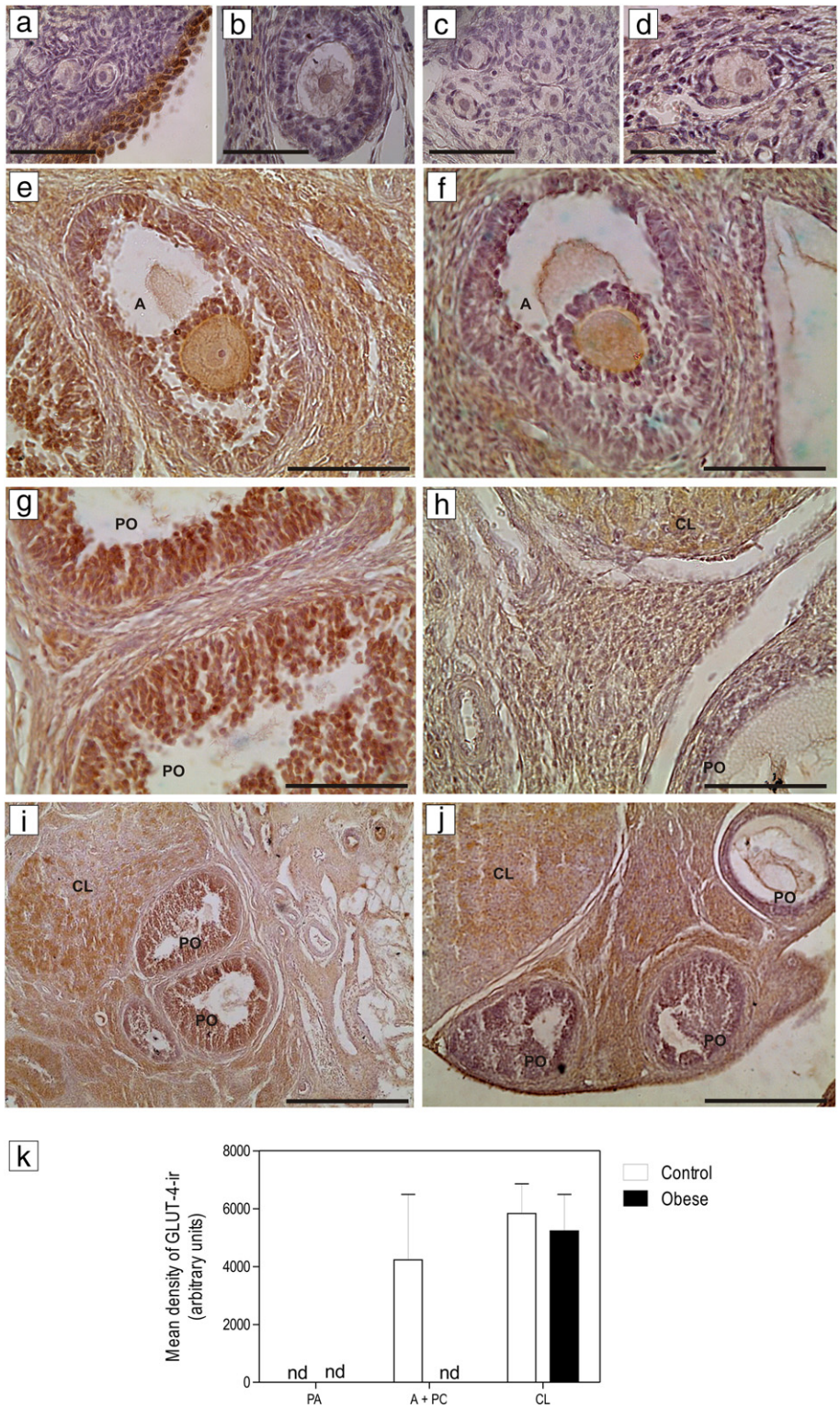

Fig. 6. Ovarian glucose transporter type 4 (GLUT-4) immunohistochemical staining in control (left column, open bars) and obese (right column, filled bars) rats. GLUT-4 was not detected in preantral follicles (PA) that includes primordial (a and c), primary and secondary (b and d) follicles. GLUT-4 was detected in the oocyte and granulose cells in antral (A) and preovulatory (PO) follicles in ovaries from control animals (e and g). A and PO follicles in obese animals did not express GLUT-4 (f and h). GLUT-4 immunoreactivity (ir) was detected in corpora lutea in both control (i) and obese animals (j). Bars=50 μm in a, b, c and d; 100 μm in e, f, g and h and 400 μm in i and j. The densitometric analyses is shown is k. Each bar represents the mean±S.E. M (n=5) and the P values were determined by Student's t-test for each stage of structure.

showing that ovaries from obese rats are insulin resistant. All these findings allow us to conclude that, in our animal model, obesity decreases the basal ovarian insulin uptake and induces ovarian insulin resistance. Insulin mediates glucose transport through controlling GLUT4 trafficking; thus, the insulin resistance developed in obese animals may be leading to the reduction in the follicular GLUT-4

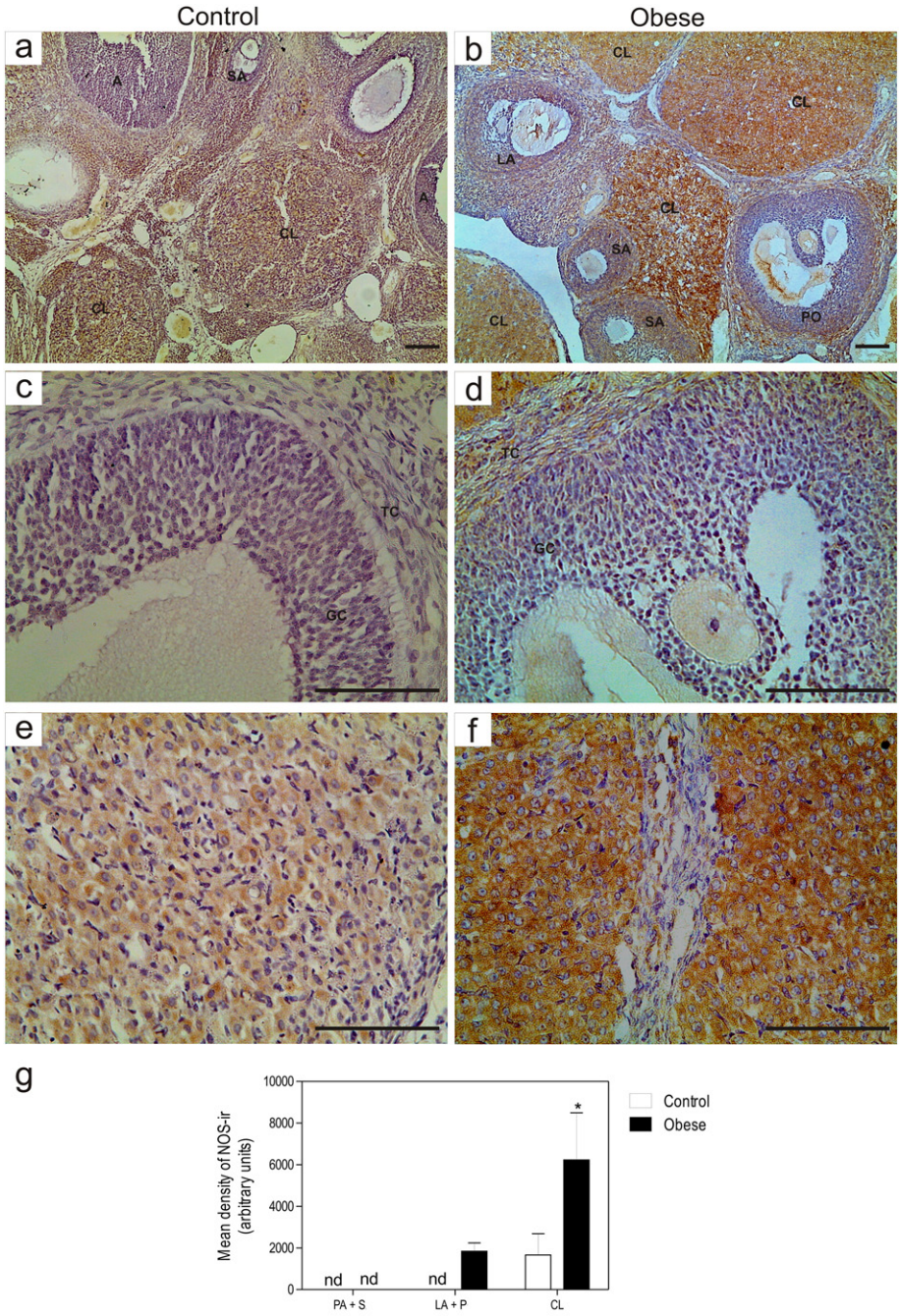

Fig. 7. Ovarian Nitric Oxide Synthase (NOS) immunohistochemical staining in control (left column, open bars) and obese (right column, filled bars) rats. NOS was not detected in preantral follicles (PA: primordial + primary + secondary) and small antral follicles (SA) neither in control nor obese animals (a and b). Large antral (LA) and pre-ovulatory (PO) follicles from control animals did not show NOS immunoreactivity (ir) (c). In obese rats, NOS label was detected in theca cells (TC) and to a lesser extent in granulose cells (GC) of LA and PO (d). NOS was detected in corpora lutea (CL) from both control (e)and obese animals (f). CL from obese animals showed a higher intensity in the NOS label respect to controls. Bars: 100um. The densitometric analysis is displayed in g. Each bar represents the mean±S.E. M (n=5) and the P values were determined by Student's t-test for each stage of structure. *P<.05 respect to the control group.

trafficking and consequently to the reduction in the ovarian glucose uptake. Finally, the reduction in the glucose uptake by the ovaries (among a number of tissues with reduced glucose uptake) may be responsible, at least in part, for the hyperglycemia detected in those animals.

Insulin resistance has been associated with impaired production/release of endothelium-derived nitric oxide (NO) [47,48]. NO is produced by the action of Nitric Oxide Synthase enzyme (NOS) [49] that is one of the main factors responsible for ovulation and is known as a local inflammatory generator [50]. The fact that obese animals are insulin resistant together with the impairments in the ovulatory process that we have previously described [27] in these animals led us to analyze the ovarian NOS expression. The increase in the luteal NOS expression detected in obese animals, suggests that NO levels in ovaries from obese animals may be increased. High levels of NO may be responsible, at least in part, for the ovulatory disruptions described and may lead to inflammation, another common feature associated to obesity.

Table 2
Effect of obesity on reproductive performance of female rats and on fetal body weight

Parameter	Control	Obese
No. of cohabitated females	15	15
No. of sperm-positive females	15	15
No. of pregnant females	15	11
Mating Index (%)	100 (15/15)	100 (15/15)
Fertility index (%)	100 (15/15)	73.33 (11/15)*
Fecundity Index (%)	100 (15/15)	73.33 (11/15)*
Conception time (days)	1.40±0.40	5.80±1.00 ***
No. implantations/rat	16.25±0.48	17.50±0.50
No. live fetuses/rat	15.00±1.08	16.50±0.87
Post implantation loss (%)	7.69	5.71
No. of pups	16.00±0.58	16.50±0.87
Pups weight (g)	1.33±0.06	1.47±0.03 *

Data are expressed as the mean ± SEM (n=15); unless otherwise indicated. *P<.05 and *** P<.0001 significantly different from the control group.

Due to all the physiological impairments produced by obesity that we described, the ultimate goal of this paper was to understand whether obesity also alters the reproductive outcome. In this regards, we found that obesity reduces fertility and fecundity rates and obese rats spend more time until becoming pregnant however they all mated. In view of the above, we conclude that obesity induces sub-fertility and sub-fecundity and delays conception, without affecting mating index. Moreover, we found that the number of pups per rat and the post-implantation losses rates were similar between fertile obese animals and controls.

It has been shown that the offspring of cafeteria diet-fed pregnant dams shows increased adiposity and permanent metabolic changes [51]. In this regards, we found that maternal obesity is associated to fetal macrosomia. Historically, macrosomia was primarily associated to mothers with pregravid or gestational diabetes mellitus (GDM) and later it was also linked with pregravid overweight/obesity and excess net gestational weight gain [52–57]. Several studies have shown the possible influence of maternal birth weight on offspring birth weight [58]. Similarly, Ahlsson et al. found that women born with a high birth weight experienced a nearly two-fold increase in the risk of macrosomic birth [59]. All these observations suggest that, women who were themselves born macrosomic tend to give birth to macrosomic infants, independent of concomitant GDM. Despite the improvement in general health status over the last decades, the mean birth weight [60] and the rate of macrosomia have been increased [54] and [61]. Ogonowsky et al. have described that maternal birth weight, prior macrosomia, pre-gravid BMI and gestational weight gain are predictors of macrosomia in offspring, but GDM was not [62]. Our results are in accordance with the latter since obese pre-gravid mothers had pups with higher body weight despite the maternal birth weight was normal as well as their feeding and weight gain during gestation. Furthermore, high maternal birth weight seems to contribute to intergenerational transmission of macrosomia. It is worth noting that macrosomia is a clinically significant risk factor for obstetric complications and metabolic disorders in adult life, such as metabolic syndrome [63], type 2 diabetes [64] and obesity [65]. Here we found that pups from obese mothers had higher birth weight, so, we suggest that when these pups become adult they may suffer metabolic and/or obstetric complications and their offspring may also be macrosomic. In summary, western style diet contributes to intergenerational transmission of metabolic disorders and macrosomia.

Taking into account all the above it can be concluded that cafeteria diet fed-rats develops obesity associated to glucose intolerance, insulin resistance, sub-fertility, sub-fecundity and fetal macrosomia. Ovaries from obese animals become insulin resistant that may explain the reduction in the follicular expression of GLUT-4 and, consequently, the reduction in the ovarian glucose uptake. Regarding the reproductive problems, our model of obesity is associated to sub-fecundity, sub-fertility and to high birth weights in the offspring.

Maternal obesity in pregnancy creates a very abnormal milieu in which the embryo and fetus develop which increases the risk of the offspring developing obesity after birth [51]. The sensitivity of an organism to its environment at critical stages during the preimplantation period can influence their children's future health since preimplantation is an exquisitely vulnerable period with the potential to affect postnatal growth, glucose metabolism, fat deposition, and vascular function [66] as well as reproductive function [67]. Our findings show that obesity is impacting not only on the obese subject but it also impacts on the health of the progeny. More studies focusing on the pre-conceptional maternal health status are needed in order to understand the reproductive mechanisms altered as consequence of obesity, to be able to think about possible treatments that could prevent them. The importance of these types of studies should be highlighted since finding a therapeutic for these patients may impact not only on the mother's health but also on the health and reproduction of the future generations.

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