REPRODUCTION
RESEARCH

Obesity modifies the implantation window and disrupts intrauterine embryo positioning in rats

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Abstract

Obesity is a chronic disease that impairs female reproduction. When gestation is achieved, maternal obesity can cause offspring's health complications. We intended to evaluate the effects of maternal pre-conceptional obesity on uterine contractile activity, embryo implantation and offspring development. Using cafeteria diet-induced obesity as an animal model, we found that maternal obesity delays embryo transport from the oviduct to the uterus and alters the intrauterine embryo positioning. Adrenergic receptor (AR) signaling is involved in embryo positioning, so all AR isoforms were screened in the pre-implantation uteri. We found that the β2AR is the dominant isoform in the rat uteri and that obesity causes its upregulation. Although β2AR activation is known to induce uterine relaxation, higher spontaneous contractile activity was detected in obese dams. Uteri from obese dams showed a higher sensitivity to salbutamol (a selective agonist of β2AR) than controls, consistent with the higher β2AR levels detected in those animals. Despite this, in obese dams, some embryos were still in the oviduct at the predicted time of initial embryo attachment, embryo implantation is successfully carried out since the total number of fetuses on gd 18.5 were similar between control and obese dams. These findings show that obesity is modifying the implantation window. Moreover, we found that maternal obesity resulted in macrosomia in the offspring, which is an important predictor of fetal programming of postnatal health. Hence, our results show that maternal obesity prior to pregnancy not only disturbs the implantation process, but also affects offspring development.

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Introduction

The rising prevalence of overweight (BMI ≥ 25 kg/m^2) and obesity (BMI ≥ 30 kg/m^2) over recent decades has been identified as a global pandemic, which is producing a negative impact on population health (WHO 2020). While traditionally associated with high-income westernized countries, obesity is also a growing problem in low and middle-income countries, particularly in urban settings (Antoniotti *et al.* 2018). The rapid rise in obesity is being driven, not only by genetic heritability and environmental risk factors in adult life, but is also linked to perinatal dietary factors (Levin 2006). It is known that suboptimal conditions during pregnancy adversely impact offspring phenotype, predisposing offspring to the development of later-life diseases, including obesity. So, of special worry is the alarming rise in the number of obese reproductive-aged women, which entails maternal and neonatal pregnancy complications, as well as the perpetuation of the cycle of obesity in future generations (Catalano & Ehrenberg 2006). Hence, maternal obesity represents a critical challenge to health care.

Several experimental models have been described for studying obesity, among which the cafeteria diet-induced obesity developed in rodents is the one that more closely reflects Western diet habits (Sclafani & Springer 1976, Rothwell & Stock 1979*a*). It provides a useful alternative to the feeding of purified high-fat diets to induce obesity since it avoids the high intakes of a particular type or source of fat and it can induce persistent hyperphagia and increased energy intake (Rothwell & Stock 1979*b*, Shafat *et al.* 2009) since it is a more palatable diet. Although it produces some variation in foods and nutrients consumed between animals in the same group, this approach has been selected as it is more closely aligned with dietary patterns observed in human subjects than conventional purified high-fat diets. It has been shown that a cafeteria

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diet can impact metabolic and reproductive functions (Akyol *et al.* 2012, Bazzano *et al.* 2015,2017,2018).

Obesity is a known risk factor for subfertility due to anovulation, but it also affects the spontaneous pregnancy chances in sub-fertile, ovulatory women. In a study of 3029 sub-fertile couples, spontaneous conception rate was 26% lower for ovulatory women with BMI=35 than the reference group (BMI 21–29) and 43% lower for BMI > 40 (Van Der Steeg *et al.* 2008). In this regard, endometrial alterations have been shown to have a role in obesity-related subfertility. A study of 9587 cycles of ovum donation from normal weight donors revealed a negative impact of recipient obesity on implantation rate, clinical pregnancy and live birth rate (Bellver *et al.* 2013), observations that were confirmed in a larger cohort later (Provost *et al.* 2016). Implantation rates are also decreased in a stepwise manner with increasing frequency of uterine contractions not only in assisted reproduction cycles at the time of embryo transfer (Fanchin *et al.* 1998) but also in natural cycles (IJland *et al.* 1997). Concerning this, we have previously shown that the uterine contractile response to a relaxation stimulus (salbutamol) is lower in obese rats than controls during the estrus cycle (Bazzano *et al.* 2018). In view of all the above, the aim was to evaluate the effects of maternal pre-conceptional obesity on uterine contractile activity, embryo implantation and offspring development. Our hypothesis is that maternal obesity alters offspring development due to altering the implantation process by altering the uterine contractile activity.

Materials and methods

Animal husbandry

Wistar rats (Rattus Norvergicus) were obtained from Bioterio Central, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires. All rats had *ad libitum* access to water and standard rodent chow diet (ACA Nutrición Animal, Argentina) and were kept on a 12 h light:12 h darkness cycle at 22°C. 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) from Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires (No 23/2015) in accordance with 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.).

Animal diets

Twenty-two days old female Wistar rats were divided randomly into two 60 days intervention groups. Random number calculators (GraphPAD software) were used to randomly assign subjects to both groups. Groups we fed with different diets: (1) Control Group was fed only standard rodent chow diet (ACA Nutrición Animal, Argentina; containing 11% fat, 23% protein and 66% carbohydrate) and (2) Obese Group 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; that contains 16% fat, 15% protein and 69% carbohydrate). This animal model of obesity was adapted from previous studies (Akyol *et al.* 2009) and has been previously successfully implemented by us (Bazzano *et al.* 2015). Weight gain was monitored twice a week and rats were classified as obese when their body weight was significantly higher than those of control rats.

Matings

After diet protocols, obese and control rats were transferred to a mating cage and cohabited with proven fertility male rats (1:1). From then on, all animals were fed standard rodent chow to establish how the pre-conception weight affects maternal weight gain during pregnancy. Every morning vaginal smears were performed and the day when spermatozoa were found in the smears was defined as gd 0.5. If no mating occurred, rats were placed again with a fertile male in the subsequent proestrus stage for two more times. If rats were not pregnant after the third attempt, they were classified as 'unsuccessful pregnancies' and were excluded from the foregoing determinations.

Rats on gd 4.5 (pre-implantation), gd 5.5 (post-implantation) and gd 18.5 (advanced pregnancy) were anesthetized with a 50 mg/kg solution of ketamine (Brouwer, Buenos Aires, Argentina) associated with 10 mg/kg xylazine (Alfasan, Woerden, Holland) and euthanasia was performed by cardiac exsanguination. Sample size calculation was performed using GPower. Considering data on literature and our previous experience, we knew the expected mean, and the S.E. of body weight of control and cafeteria diet-fed rats. So, we used a priori analysis considering one tail and an error prob (a)=0.5. The accepted standard power (b) is 0.8; using this b value, the indicated sample size for both groups was 5. If we increased the sensitivity to a strongly high b=0.95, the indicated sample size for both groups was 7. Therefore, the acceptable sample size ranges from 5 to 7 animals/group.

We used $n = 5$ for 4.5 and 5.5 gd studies and $n = 7$ for 18.5 gd studies. We increased the sensitivity for 18.5 gd studies since it could imply a higher risk of miscarriage, and we had to guarantee to have enough pregnant rats that reach up to 18.5 gd.

Ovaries from all dams were collected to determine the ovulation rate by counting the number of corpora lutea in each ovary.

Pre-implantation analysis

Embryos were flushed from oviducts and uteri from gd 4.5 dams using Krebs Ringer buffer (KRB). Embryos were retrieved and classified according to their: (1) origin: oviductal or uterine horn, right or left; (2) quality: viable or non-viable (when presented a partitioned blastocelic cavity and/or fragmentation in trophoblast or in the inner cell mass); and (3) developmental stage of those viable: morulae (uncompacted

and/or compacted), initial blastocyst (with blastocele cavity in formation, occupying less than 50% of the embryo volume) or expanded blastocyst (with a fully expanded blastocele cavity).

After flushing, uteri were: (1) frozen for subsequent RNA extraction, (2) lysed for Western blotting, (3) used fresh for myometrial contractile studies, or (4) fixed in 4% (w/v) formaldehyde for 24 h for immunohistochemical studies. The later were then dehydrated, embedded in paraffin, and cut into 7 µm sections that were mounted on gelatin-coated glass slides.

Implantation sites analysis

Dams on gd 5.5 were injected in the tail vein with 1% Evan's blue dye (Sigma) after anesthesia and prior to performing euthanasia. Implantation sites (IS) appear as blue bands by the blue dye method. So, the number and position of IS were recorded and analyzed. If no implantation sites were detected on gd 5.5, the uterine horns were flushed with KRB to determine the presence of delayed embryos and thus the status of pregnancy.

Advanced pregnancy

Uteri from dams on gd 18.5 were harvested; IS were exposed by removing fetal membranes and photographed. The number of live and dead pups, their body weights and placental weights were recorded. Post-implantation loss rates were calculated as the difference between the number of IS and the number of live fetuses ×100.

Uterine homogenates and Western blotting

Pieces of uteri from gd 4.5 dams were homogenized in Tris-buffer (100 mM 100 mM NaCl, 10 mM 10 mM Tris, pH 7.4, 1 mM 1 mM EDTA, 0.5% NP40, 1% Triton, 1 mM 1 mM PMSF) containing protease inhibitor cocktail (Sigma-Aldrich). The lysate was centrifuged at 4°C for 10 min at 10,000 ***g***, and the pellet was discarded. Protein concentrations in the supernatant were measured by Bradford assay (Bio-Rad). After boiling for 5 min, 90 µg of protein from each sample was applied to an SDS-polyacrylamide gel (10%), and electrophoresis was performed at 100 V for 1.5 h. The separated proteins were transferred onto PDVF membranes in transfer buffer (20% methanol, vol/vol; 0.19 M glycine; 0.025 M Tris-Base, pH 8.3) for 1 h at 4°C. Blots were blocked for 1.5 h in TBS (4 mM 4 mM Tris–HCl, pH 7.5, 100 mM 100 mM NaCl) containing BSA (0.1%) at room temperature as previously described (Elia *et al.* 2011). The primary antibodies used were: rabbit polyclonal anti-β2 adrenergic receptor (β2 AR) (1:1000, overnight; Santa Cruz Biotechnology Inc., sc: 9042), and mouse monoclonal anti -Glyceraldehyde-3-PDH (GAPDH) (1:500, overnight; Millipore, MAB374) that was used as an internal control. The identity of the bands was established using molecular weight standards (14.3–200 kDa, Bio-Rad), which allows the identification of the bands of interest: β2 AR (56-85 kDa) and GADPH (38 kDa); which were absent in the negative control experiments.

Afterward blots were incubated for 1 h with biotin-conjugated secondary antibodies: anti-rabbit IgG (1:2000; Millipore) or anti-mouse (1:500; DakoCytomation, USA, Eo-354) followed by streptavidin–peroxidase complex (1:2000; DakoCytomation). The specific signals were visualized using ECL detection solution (Thermo Scientific), acquired in an ImageQuant RT ECL (General Electric, Amersham Bioscience, Argentina) and quantified with Image J software (version 1.42q, NIH). The density of the bands of interest was normalized to that of GADPH in each sample.

Uterine RNA extraction and retrotranscription

Total uterine RNA was extracted from uteri on gd 4.5 using Trizol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized by incubating 2 µg of extracted RNA in a buffer containing 3U AMV Reverse transcriptase (Promega), 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.

Polymerase chain reaction

The cDNA obtained by retrotranscription (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 2 mM of each primer (Invitrogen), 0.2 mM 2 mM of each dNTP, 1.5 mM 5 mM MgCl2 and each specific primer. Primer sets used are detailed in Table 1, where the specific annealing temperature and the number of cycles used for each pair of primers are also included. 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. Negative controls were performed without reverse transcriptase or RNA. Positive control consists of a mixture of cDNAs from brain, liver, and kidney, which covers the expression of all adrenergic receptor family members.

PCR products were electrophoresed on 2% agarose (Biodynamics, Buenos Aires, Argentina) gels. Gel images were taken with the ImageQuant RT ECL (General Electric) and software and quantified using Image J software (version 1.42q, NIHA). Density of the bands of interest was normalized to that of GADPH in each sample.

Real-time PCR (qPCR)

Quantitative real-time PCR (qPCR) was performed in a DNA Engine Opticon 2 Real-Time Cycler (Roche Applied Science) using cDNA samples and following the standard curve using FastStart Universal SYBR Green Master Mix (Roche) and primers at a final concentration of 0.9 µM. β2 AR mRNA expression was analyzed and normalized to those of L30 that were used as housekeeping. Primer sequences are shown in Table 1.

Table 1 Details of primers used for PCR.

Gene	Primer sequence (5'–3')		Fragment size (bp)	AT (°C)	No. of cycles	GenBank accession no
	Forward	Reverse				
α1A AR	TCTTCCTAGTGATGCCCATTG	GCTTTCTTGAACTCCTGGCTG	145	55.35	40	NM_017191
α1B AR	CCTGTTCTCCACCCTAAAGC	ACCCAAGGATACGCATGAAG	140	55.05	40	NM_016991
α1D AR	AAAAGGCTGCCAAGACGT	AAGATGACCTTGAAGACACCC	133	55	40	NM_024483
α2A AR	GCGAGATCTACTTGGCCCTC	CGTTAATCTTGCAGCTCGGC	258	57	40	NM_012739.3
α2B AR	GTCTTCAACCAGGACTTCCG	AGAGACTGTGGAGGTGGG	147	55.6	40	NM_138505.2
α2C AR	TTCAAGCACATCCTCTTCCG	GAACTCTGGAGAAGCCACAC	143	55.3	40	NM_138506.1
β1 AR	CTGCTACAACGACCCCAAG	TCTTCACCTGTTTCTGGGC	146	54.2	40	NM_012701.1
β2 AR	GTACTGTGCCTAGCCTTAGC	GGTTAGTGTCCTGTCAGGGAGG	118	58	40	NM_012492.2
β3 AR	AGAACTCACCGCTCAACAG	CATGGACGTTGCTTGTCTTTC	137	54.65	40	NM_013108.2
GAPDH	CCATCAACGACCCCTTCATT	GACCAGCTTCCCATTCTCAG	110	57	35	NM_017008.4
L30	CCATCTTGGCGTCTGATCTT	GGCGAGGATAACCAATTTC	200	58	35	NM_022699.3

AT, annealing temperature.

Immunofluorescence

Localization of β2 AR in uteri on gd 4.5 was analyzed by immunofluorescence as previously described (Chen *et al.* 2011). For this purpose, uterine 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 for antigenic recovery, followed by background blocking that 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-β2 AR (H-73) (1:200, overnight; Santa Cruz Biotechnology sc: 9042) as primary antibody. Controls were performed by omitting the primary antibody. Sections were, then, incubated with the biotinylated secondary antibodies: goat anti-rabbit (1:1000; Millipore) at room temperature for 40 min; and, afterward, incubation for 40 min with streptavidin–biotin complex reagents were done. Streptavidin–biotin Alexa Fluo 488 conjugate (1:500; Molecular Probes) was used and nuclei were stained with propidium iodide (Invitrogen). Finally, sections were mounted in 50:50 PBS-glycerol and observed and photographed with an Olympus Bx-61 microscope for laser confocal microscopy attached to an Olympus FV-300 camera.

Myometrial contractile analysis

Small strips (~10 × 5 mm) of longitudinal myometrium were dissected from each animal on gd 4.5 and suspended in a separate 20 mL organ bath filled with KRB warmed at 37°C and gassed with 95% 02–5% CO_2. Each strip was set to a resting tension of 9.8 mN (1gF); its contractile activity was recorded using isometric force transducers (Harvard Apparatus, South Natick, MA) connected to a bridge amplifier, which was in turn connected to a data acquisition system (Data Studio Pasco). According to Chaud *et al.*, myometrial strips were left to stabilize for 30 min until regular phasic contractions were achieved (Chaud *et al.* 1997). Afterward, 20 min spontaneous baseline contractile function was determined before the accumulative addition of a selective β2 –adrenoceptor agonist every 7-min intervals (salbutamol, 0.05ng–500 ng/mL) (Parida *et al.* 2013). The resultant contractile activity measurements included the amplitude and the frequency of contractions as well as the activity integrals (area under the time–force curve).

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) and $P < 0.05$ was considered statistically significant.

For contractile activity analysis, the amplitude, frequency, and integral activity comparisons among all concentrations of salbutamol and between control and obese rats were performed by two-way ANOVA with repeated measures followed by the Newman–Keuls test.

The frequencies of animals showing equal or unequal embryo distribution were analyzed by Fisher´s exact test. The significance of the remaining results was determined using Student's *t*-test.

Results

Maternal pre-gestational obesity causes excessive weight gain during pregnancy

After 60 days of diet protocol, body weight was higher in cafeteria diet-fed rats than those of rats fed standard diet (controls) (Table 2; $P < 0.01$), showing that obesity was induced after 2 months of cafeteria diet administration in rats. Moreover, body weight gain throughout pregnancy was significantly higher in obese rats than controls (Table 2; $P < 0.01$) despite all animals were fed standard chow during this period.

Table 2 Effects of cafeteria diet on body weight gain. Obesity was induced after 60 days of cafeteria diet administration in rats. Obese rats showed higher body weight gain than controls through pregnancy despite being fed control diet during this period; it became evident by comparing the dam body weights on gd 18.5. Values are expressed as the mean ± S.E.M.

	Body weight (g)	
	Control	Obese
Pre-diet	61.17 ± 1.08	60.67 ± 1.69
After 60 days of diet	282.2 ± 7.46	340 ± 16.04**
On gd 18.5	367.7 ± 17.07	496.7 ± 8.82**

n = 7/group. ** $P < 0.01$, *** $P < 0.001$ respect to the control group.

Maternal obesity causes aberrant intrauterine fetal positioning and macrosomia

The appearances of gestation day (gd) 18.5 implantation sites (IS) after fetal membranes removement are shown in Fig. 1; where fetuses, placentas and umbilical cords still attached to the uteri can be observed. Two intrauterine IS distribution phenotypes were found: equal and unequal IS distribution between uterine horns. Equal distribution occurs normally and is when each uterine horn holds approximately the same number of IS (Fig. 1 left), and the unequal distribution is considered when the number of IS differs between uterine horns (Fig. 1 right). The highest difference in the number of IS between both uterine horns detected in controls on gd 18.5 was 3. So, 3 was considered as cutting line for classifying as equal (≤3) or unequal (>3) distribution for all the foregoing results.

The results obtained using this criterion are summarized in Table 3: while all control dams showed equal intrauterine IS distribution, 85.74% of obese dams showed unequal IS distribution. Despite this, the total number of IS in each uterus was similar in control and obese rats. Ovulation rates were checked in all the gestational stages analyzed in this work and no difference between both ovaries were found in any case. Moreover, on gd 18.5, the post-implantation pregnancy loss rates were similar between control and obese rats (Table 3); showing that the unequal intrauterine IS distribution detected in obese rats is not consequence neither to unequal ovulation rates between its ovaries nor fetal re absorptions.

When the offspring was analyzed, it was found that fetuses from obese dams were heavier than those from controls and that it was independent of the uterine horn where they were implanted ($P < 0.05$; Fig. 2 and Table 4), demonstrating that maternal obesity leads to fetal macrosomia even when placental weights are not altered.

To identify when the unequal intrauterine IS distribution occurs in obese animals, IS distribution was analyzed in earlier gestational stages (gd 5.5 and 4.5).

Obesity alters the intrauterine embryo positioning prior to implantation

The intrauterine IS distribution just after implantation time was evaluated. On gd 5.5, all control and 20% of obese animals showed equal IS distribution, while 80 % of obese rats sowed unequal IS distribution (Fig. 3 and Table 5). The total number of IS was similar in control and obese animals. Ovulation rates were also similar in both ovaries in obese and control rats, indicating that the aberrant IS distribution is not consequence of unequal ovulation rates (not shown).

In addition, embryo spacing was analyzed on gd 5.5. As it can be easily seen in Fig. 3, the intrauterine IS spacing differed between control and obese animals. In control rats, spacing was as known, characterized by IS evenly spaced manner along the uterine horn (Fig. 3 right). However, in obese rats, this pattern was altered: an uneven embryo distribution was seen, with some IS remarkably close to each other and others very distant (Fig. 3 center and left).

These results indicate that obesity not only alters the IS distribution between uterine horns but also alters the spacing in each uterine horn, and both phenomena preceded the implantation process.

Embryo transport from the oviduct to the uterus is delayed by maternal obesity

To analyze embryo distribution just before embryo implantation, uteri from dams on gd 4.5 were flushed and embryos were collected. When control dams were analyzed, it was found that both uterine horns

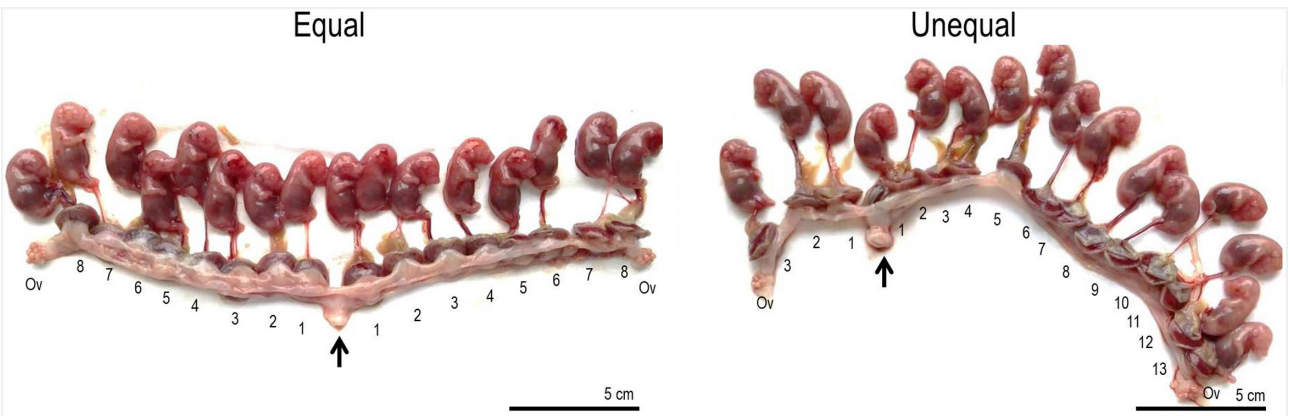

Figure 1 Effects of obesity on the intrauterine fetal positioning. Control and obese dams on gd 18.5 were sacrificed, implantation sites (IS) were exposed by fetal membranes removal and photographed. Representative images of fetuses, placentas and umbilical cords still attached to the uteri are shown here. Arrows indicate the uterine cervix and the numbers of IS present in each uterine horn are listed. Unequal IS distribution was considered when the difference in the number of IS between both uterine horns was greater than 3. Ov: Ovary. Bars: 5 cm; $n = 7$/group.

Table 3 The intrauterine fetal positioning is altered by obesity. Control and obese rats on gd 18.5 were sacrificed and implantation sites (IS) were exposed and analyzed. Unequal IS distribution was considered when the difference in the number of IS between both uterine horns was greater than 3. Post-implantation losses were calculated as the difference between the number of IS and the number of live fetuses ×100. The values are expressed as the mean ± S.E.M. or as percentages.

	Intrauterine IS distribution phenotypes			
	Equal		Unequal	
	Control	Obese	Control	Obese
% animals	100 (7/7)	14.29 (1/7)**	0	85.71 (6/7)**
Total number of IS	16.67 ± 1.48	15.00	–	16.67 ± 0.56
Post-implantation pregnancy loss rate	7.53	–	–	5.52

** $P < 0.01$ compared to the respective control group.

contained approximately the same number of viable embryos, and thus, were all classified as showing equal intrauterine embryo phenotype. Among obese dams, 68.75% showed unequal embryo distribution. When the number of corpora lutea in each ovary was analyzed, no differences were found, showing that the unequal embryo distribution was not due to unequal ovulation rates between ovaries (not shown).

When the total number of embryos per uteri (calculated by adding the embryos found in both horns) was analyzed, it was found that it was lower in obese dams than in controls (Table 6; $P < 0.05$). Since this result was not seen in 18.5 nor 5.5 gd (previously described in this manuscript), we analyzed if some embryos were still in the oviducts. For that purpose, oviductal flushings were performed. No embryos were detected in the oviducts from control dams; however, embryos were detected in the oviducts from obese rats (Fig. 4). In those animals, one oviduct held more embryos than the contralateral ($P < 0.01$; Fig. 4). The oviduct showing the highest number of embryos was ipsilateral to the uterine horn that contained the lowest number of embryos, suggesting that obesity delays embryo transport from the oviduct to the uterus, specially in one flank.

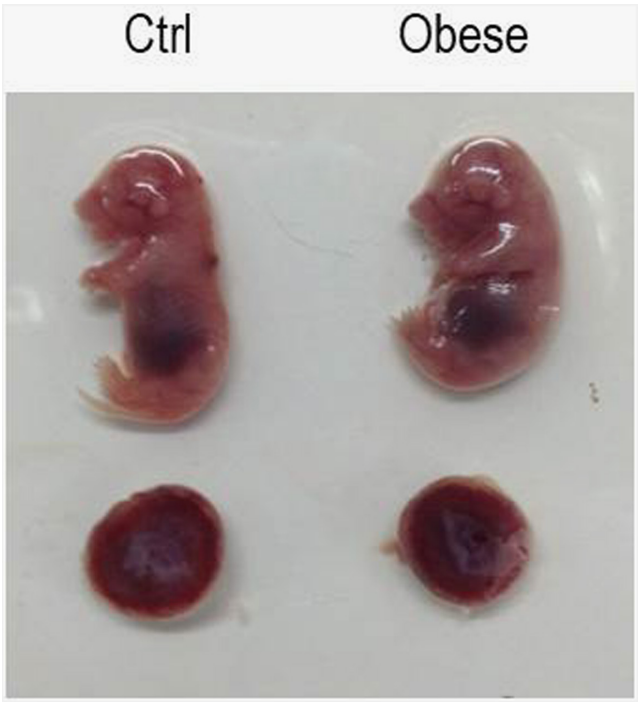

Figure 2 Effects of maternal obesity on fetal and placental development. Representative images of pups (upper panel) and placentas (bottom panel) from control and obese dams on gd 18.5 are shown here; $n = 7$/group.

Embryos from obese dams are in early developmental stage prior to implantation

When viable embryos flushed on gd 4.5 were classified according to their developmental stage, a higher number of morulae and a lower number of initial and expanded blastocysts were found in obese dams compared to controls (Fig. 5; $P < 0.001$), showing that embryos from obese dams were in early developmental stage during the pre-implantation period.

Uterine B2AR upregulation is caused by obesity at pre-Implantation

Embryo positioning and implantation is known to be controlled by several factors that include adrenergic receptor (AR) signaling (Chen *et al.* 2011); so, the expression profile of different adrenergic receptors in uteri on gd 4.5 was screened. No expression of mRNA codifying for α1B AR, β1 AR and β3 AR isoforms was detected in uteri neither from control nor from obese

Table 4 Fetal body weigh, but not placental, is altered by maternal obesity. Fetuses and placentas from control and obese dams on gd 18.5 were dissected and weight. Placental and fetal body weight were compared between control and obese dams; as well as between uterine horns for each dam. The uterine horn with the highest number of IS was called H1, while the other uterine horn was named H2.

	Control	**Obese**
Fetal weight (g)	1.33 ± 0.06	1.47 ± 0.03*
H1	1.28 ± 0.08	1.50 ± 0.03
H2	1.37 ± 0.08	1.39 ± 0.06
Placental weight (g)	0.65 ± 0.02	0.62 ± 0.02
H1	0.65 ± 0.02	0.64 ± 0.02
H2	0.65 ± 0.02	0.63 ± 0.02

*$P < 0.05$ respect to controls; $n = 7$ dams/group.

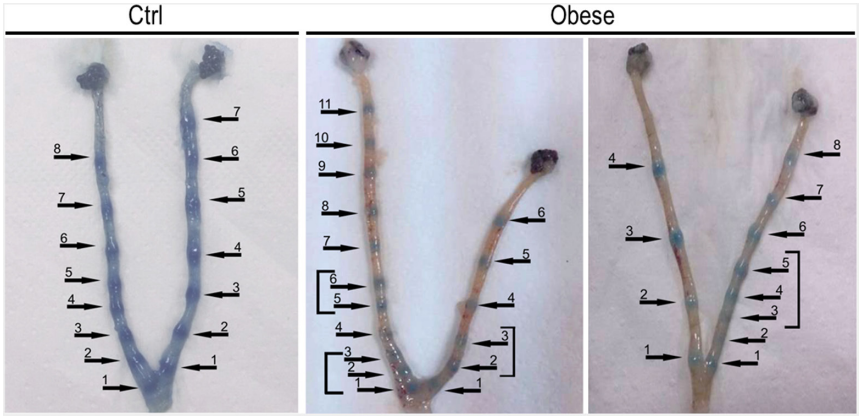

Figure 3 Effects of obesity on intrauterine embryo positioning. Control and obese rats on gd 5.5 (n = 5/group) were injected with Evans Blue dye, sacrificed and uteri were exposed and photographed. Representative images of the uteri are shown here, where each implantation site (IS) is seen as blue bands by the blue dye method. Arrows indicate IS and IS present in each uterine horn are listed. Brackets indicate IS that are closer to each other than the average of all IS in this animal, showing the irregularity in the spacing of the IS in the obese group.

dams. However, α1A, α2C, α2A, α2B and β2 AR isoforms were detected in uteri from both obese and control animals. Among the later, the β2 AR isoform showed a dominant expression (Fig. 6A), showing an intense myometrial expression and relatively weak expression at luminal and glandular epithelium (Fig. 6D) both in control and obese rats. Both mRNA (Fig. 6B) and protein (Fig. 6C) β2 AR levels were higher in uteri from obese than control animals. β2 AR levels were similar in both uterine horns from each animal, both in control and obese dams (data not shown).

Obesity increases the spontaneous uterine contractile activity and alters its response to a β2 AR agonist during pre-implantation

Adrenergic receptors are known to control intrauterine embryo spacing through modifying uterine responsiveness and contractile activity (Sakamoto *et al.* 2005, Akyol *et al.* 2009, Chen *et al.* 2011), so the uterine contractile activity was studied *ex vivo*.

Examinations were only performed on myometrial strips which showed regular spontaneous contractile activity as those illustrated in Fig. 7A. No differences in the contractile activity were detected when both uterine horns from each animal were analyzed in any case (data not shown). So, the media between both uterine horns for each animal is included for the foregoing results.

Table 5 Aberrant intrauterine implantation site distribution during the early post-implantation period as a consequence of maternal obesity. The number of implantation sites (IS) in each uterine horn was analyzed for control and obese dams on gd 5.5. Unequal distribution was considered when the difference in the number IS between uterine horns was greater than 3. The values are expressed as the mean ± S.E.M. or as percentages.

	Intrauterine IS distribution phenotypes			
	Equal		Unequal	
	Control	Obese	Control	Obese
% animals	100 (5/5)	20 (1/5)*	0	80 (4/5)*
IS, total n	16.25±1.60	15	–	14.50±1.04

* $P < 0.05$ compared to the respective control group.

The analysis revealed that the amplitude (Fig. 7B) and the activity integral of uterine contractions (Fig. 7D) were increased by maternal obesity. However, the frequency of the spontaneous contractions was not modified by obesity (Fig. 7C).

The fact of finding an increase in the amplitude of spontaneous uterine contractions concomitantly with higher levels of β2AR, that is known to promote uterine relaxation, lead us to analyze whether obesity alters β2AR activation in the uterus. For that purpose, we next analyzed the response of the myometrium to salbutamol, a selective agonist of β2 AR, in control and obese rats on gd 4.5 (Fig. 8A). In the control group, salbutamol significantly decreased the amplitude of contractions compared to the spontaneous contractile activity at 5, 50 and 500 ng/mL ($P < 0.05$, $P < 0.001$ and $P < 0.001$, respectively), while in obese rats it occurred at 0.5, 5, 50 and 500 ng/mL ($P < 0.01$–0.001). Besides, this reduction in the amplitude of contractions was greater in the obese group than in controls for 5 and 50 ng/mL of salbutamol (Fig. 8B; $P < 0.05$).

In the control group, only a 500 ng/ml concentration of salbutamol significantly decreased the frequency of contractions compared to the spontaneous contractile (Fig. 8C, $P < 0.01$); while in the obese group significant differences were evidenced for 5, 50 and 500 ng/mL ($P < 0.01$–0.001). The reduction in the frequency of

Table 6 The intrauterine embryo positioning is altered by maternal obesity prior to implantation. Control and obese rats on gd 4.5 were sacrificed, their uteri were flushed and embryos were collected and viable embryos were quantified. Unequal distribution was considered when the difference in the number of embryos between uterine horns was greater than 3. The values are expressed as the mean ± S.E.M. or as percentages.

	Intrauterine embryo distribution phenotypes			
	Equal		Unequal	
	Control	Obese	Control	Obese
% animals	100 (5/5)	20 (1/5)*	0	80 (4/5)*
Embryos, total n	15.67 ± 2.74	13.00 ± 4.00	–	12.27 ± 2.72$^{\#}$

* $P < 0.05$ compared to the respective control group. $^{\#}$ $P < 0.05$ compared to controls showing equal phenotype.

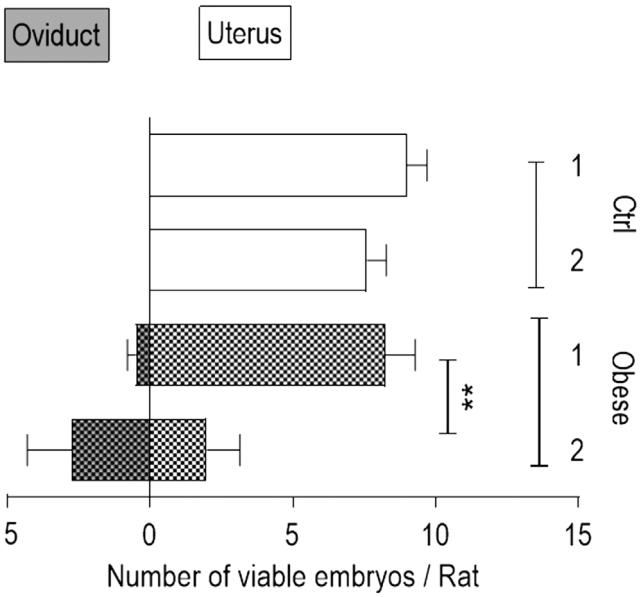

Figure 4 Effect of obesity on embryo localization during the pre-implantation stage. Control and obese rats on gd 4.5 were sacrificed, their uteri and oviducts were flushed and embryos were collected and viable embryos were counted according to their position. The uterine horn with the highest number of embryos (and its ipsilateral oviduct) were called 1, while the contralateral were named 2. Data are expressed as the mean ± S.E.M.; n = 5/group; ** $P < 0.01$.

contractions was significantly different between the obese and control group for a 50 ng/mL salbutamol concentration (Fig. 8C; $P < 0.05$).

When the activity integrals were calculated, it was found that, in the control group, salbutamol induced a significant decrease in activity among 0.5–500 ng/mL compared to the spontaneous contractile activity ($P < 0.01$–0.001). In the obese group, the decrease was detected for all the concentrations evaluated (0.05–500 ng/mL) ($P < 0.05$–0.001). The activity integral was lower in the obese group compared to controls in response to 0.5 and 5 ng/mL of salbutamol (Fig. 8D; $P < 0.001$ and $P < 0.01$, respectively).

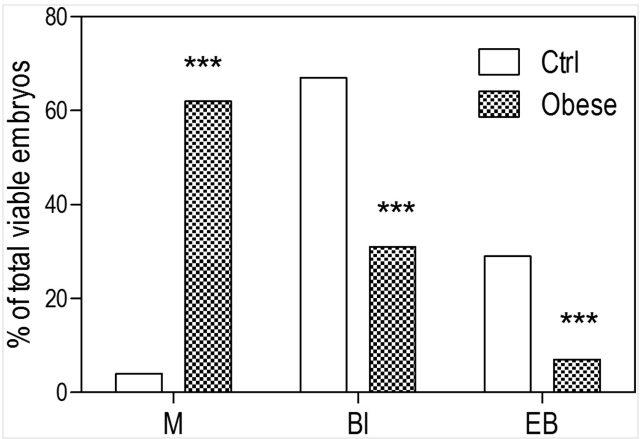

Figure 5 Effects of maternal obesity on *in vivo* embryo differentiation during the pre-implantation stage. Control and obese rats on gd 4.5 were sacrificed, their uteri and oviducts were flushed, viable embryos were collected and classified according to their quality and developmental stage: M, morulas; BI, initial blastocysts; and EB, expanded blastocysts. Data are expressed as percentages respect to the total number of viable embryos collected; n = 5/group; *** $P <$ 0.001 respect to control group.

Discussion

There is strong evidence from epidemiological and animal studies showing that obesity induces endometrial alterations that leads to subfertility (Bellver *et al.* 2013, Provost *et al.* 2016). Moreover, maternal obesity brings about long-term consequences in offspring, promoting the development of adiposity and metabolic diseases, and it seems to be due to the alteration in the intrauterine environment that it produces (Alfaradhi & Ozanne 2011). This study provided strong evidence that obesity prior to gestation is enough condition for altering the uterine milieu, disrupting embryo implantation and the foregoing offspring development.

In this study, we used cafeteria diet-induced obesity as an animal model, in which rats become obese when offered a varied and palatable diet that mimics the so-called Western diet of humans. Cafeteria diet-induced obesity mainly results from hyperphagia since it increases average meal size as well as the meal frequency (Lutz & Woods 2012). In experimental studies in rats, several parameters, such as body weight, have commonly been used to demonstrate increased adiposity and investigate the mechanisms underlying obesity (Leopoldo *et al.* 2016); and that was the criteria used in this study. As previously described by others and us, here we found that cafeteria diet administration for 60 days increases body weight in rats, inducing obesity (Akyol *et al.* 2009,2012, Bazzano *et al.* 2017,2018). Studies using different animal models have generally concluded that maternal overfeeding and obesity before and/or during pregnancy sensitizes the offspring to the development of postnatal adiposity and excess weight gain (Shankar *et al.* 2008, Borengasser *et al.* 2013, Murabayashi *et al.* 2013). In those studies, dams were fed an obesogenic diet prior and during gestation; so, discrimination between the effects of maternal obesity from those of maternal diet during gestation was difficult. Our work highlights the importance of maternal body weight at conception time since maternal weight during pregnancy was higher in obese dams than controls although all dams were fed standard chow during this period.

By using a pair-feeding model, White *et al.* (White *et al.* 2009) concluded that maternal obesity and not the consumption of dietary fat *per se* is the primary mechanism driving offspring obesity. On the other hand, Zambrano *et al.* (Zambrano *et al.* 2010) described that dietary intervention in rats prior to pregnancy partially

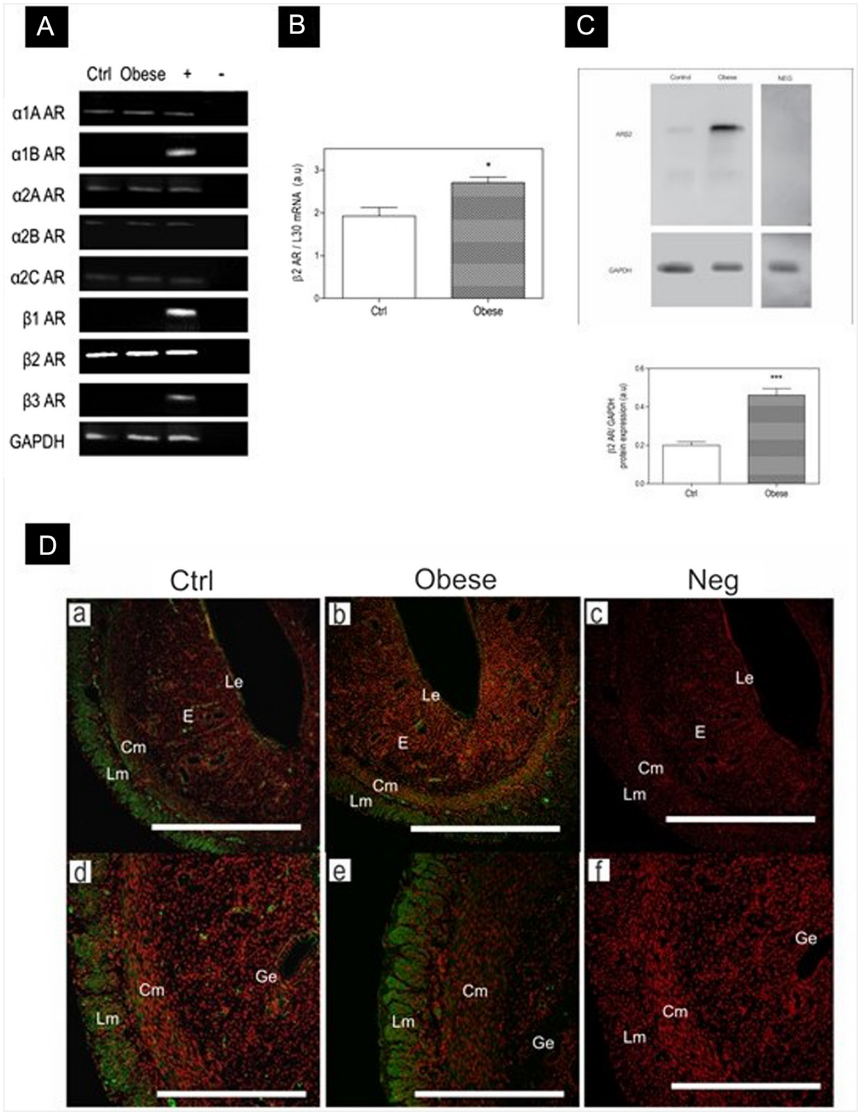

Figure 6 Effect of obesity on the uterine β2 AR expression during pre-implantation. Uteri from control and obese dams on gd 4.5 were used: to perform a screening of all adrenergic receptors (AR) mRNA isoforms (A), to compare the β2 AR expression at gene (B) and at protein levels (C); as well as to analyze the β2 AR localization (D). In (A) −, negative control; +, positive control. In (B) and (C) data are expressed as the mean ± S.E.M.; $n = 5$/group; ***$P < 0.001$ respect to control group. In (D) Alexa 488-labeled β2-AR antibody is seen in green and propidium iodide-labeled nuclei are in red. Negative controls (Neg) were performed by omitting the primary antibody. E, stroma; Le, luminal epithelium; Ge, glandular epithelium; Lm, longitudinal muscular layer; Cm, circular muscular layer.

reverses adverse metabolic outcomes of maternal obesity associated with high-fat diet feeding in the male offspring. Our results agree with these works since offspring of obese dams were large for gestational age although maternal diets were like those of controls during gestation, suggesting that maternal weight prior to pregnancy is the primary mechanism driving to offspring macrosomia.

It has also been shown that babies who are large for gestational age have a higher risk for developing serious health complications such as diabetes, obesity, and metabolic syndrome later in life (Johnsson *et al.* 2015). So, our results suggest that maternal obesity prior to pregnancy is enough condition to susceptible the offspring for developing metabolic complications in adulthood by inducing fetal macrosomia.

Despite this known association between maternal obesity during pregnancy and fetal overgrowth, which strongly predicts the appearance of childhood obesity and metabolic syndrome during adulthood (Boney *et al.* 2005, Reilly *et al.* 2005, Sewell *et al.* 2006); the mechanisms by which the excess of maternal adipose mass affects fetal weight gain are not well understood. The fact of finding that fetal macrosomia is induced by maternal obesity without altering placental weight led us to suggest that the placental nutrient transport may be altered by obesity, as previously reviewed (Howell & Powell 2017). It has been shown that changes in placental function not only play a critical role in the development of pregnancy complications but may also be involved in linking maternal obesity to long-term health risks in the infant. Maternal adipokines and metabolic hormones have a direct impact on placental function by modulating placental nutrient transport.

In this work, the number of pups was not altered by maternal pre-conceptional obesity. However, a

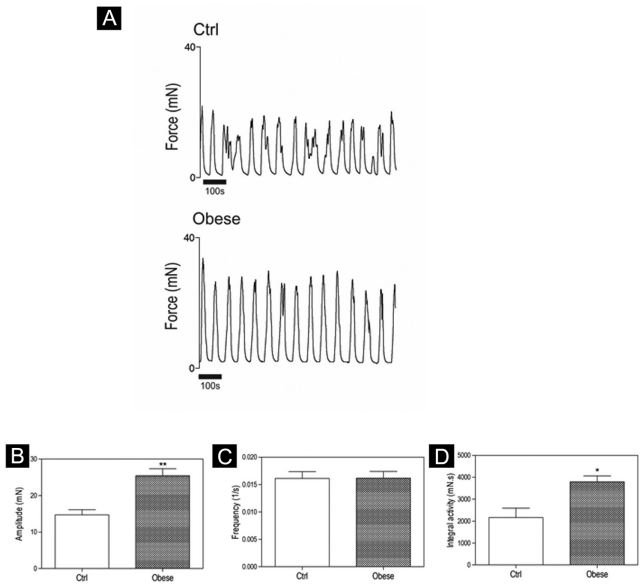

Figure 7 Obesity alters the spontaneous uterine activity during pre-implantation in rats. (A) Representative recordings of spontaneous contractile activity in uteri from control (top trace) and obese (bottom trace) dams on gd 4.5. (B) Amplitude, (C) frequency and (D) integral activity of 20 min of uterine spontaneous contractions in control and obese rats. Data are expressed as the mean ± S.E.M.; n = 5/group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the control group.

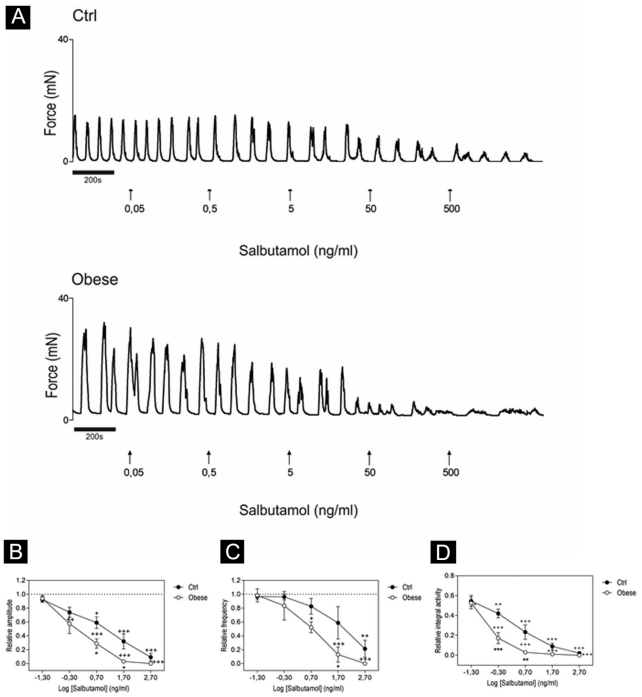

Figure 8 Obesity alters the uterine contractile response to salbutamol during pre-implantation in rats. (A) Representative recordings of uterine contractions in control (top trace) and obese (bottom trace) rats on gd 4.5 in response to the accumulative addition of salbutamol, a selective agonist of β2 adrenoceptor (0.05 ng/ml to –5000 ng/mL). (B) Amplitude, (C) frequency and (D) integral activity of uterine contractions in response to each concentration of salbutamol in control (open circles) and obese (filled circles) rats. Each dose was analyzed during 7 min and normalized to a spontaneous activity. Data are expressed as mean ± S.E.M.. n = 5/group; $^{\Phi}P < 0.05$, $^{\#}P < 0.01$ and $^{\delta}P < 0.001$ respect to the spontaneous activity. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ respect to the control group for each dose of salbutamol.

surprising finding was found when dissecting the uteri to extract the pups; it was the way in which IS were distributed in most of the obese dams. Most of the obese dams showed unequal intrauterine IS/embryo distribution, that is, different quantity of fetuses/embryos in both uterine horns, on gd 18.5 but also on gd 5.5. This phenotype could be explained if the ovulation rate differed between ovaries in those rats; nevertheless, that possibility was ruled out since the number of corpora lutea in both ovaries was checked, and it was similar in both ovaries in all dams. Therefore, the two plausible explanations we found for explaining that inequality was that maternal obesity: (1) diminishes the quality of embryos in the uterine horn that contains fewer IS/embryos and/or (2) reduces the number of embryos that reach that uterine horns in the pre-implantation period. The first option was ruled out since the number of non-viable gd 4.5 embryos was similar in both uterine horns in those animals (not shown). To test the second hypothesis, the number of gd 4.5 embryos in each uterine horn was analyzed, and it was different in both uterine horns in the obese group. So, obesity diminishes the number of embryos that reach one uterine horn on gd 4.5 morning, indicating that maternal obesity alters the intrauterine embryo positioning prior to implantation.

Serendipity, when the total number of embryos (by summing them up on both horns) was analyzed, we found that it was lower in uteri from obese dams than controls. In view of these data, we evaluate whether some embryos were still in the oviducts on gd 4.5 in obese dams. Oviductal flushings showed that, indeed, in obese dams, some embryos were still in the oviduct at the predicted time of initial embryo attachment (gd 4.5 at 12:00 h), while no embryos were detected in oviduct from control dams; showing that embryo transport from the oviduct to the uterus is delayed by maternal obesity. Furthermore, the developmental stage of those gd 4.5 embryos was also altered by maternal obesity. Despite these alterations, some of the oviductal embryos seem to be implanted afterwards, since the total number of IS was similar in control and obese dams on gd 5.5 and 18.5, suggesting that obesity is modifying the implantation window. New experiments are being designed to evaluate the expression of markers of uterine receptivity to confirm this suggestion.

In view of all the above, it can be concluded that obesity delays embryo transport from oviduct to uterus in a flank-dependent manner. In one flank, some embryos are kept in the oviduct, so, a lower number of embryos reach that uterine horn at the predicted time of initial embryo attachment. Furthermore, oviductal embryos

seem to be implanting 'later' in the contralateral flank. It seems to be a 'compensatory' mechanism between both flanks; the low number of embryos in one uterine horn, may be modifying the implantation window, allowing some embryos that were still in the contralateral oviduct to descend and implant. The fact of finding that gd 4.5 embryos from obese dams were in earlier developmental stage than those of controls supports this suggestion.

All these results cannot explain the reason for the lower number of embryos in one flank; however, what we do show here is that is not due to lower ovulatory rate nor to worse embryo quality in that flank. A possible explanation for the mentioned embryo inequality could imply a differential uterine contractile activity between both uterine horns that controls embryo positioning for implantation. This plausible explanation was ruled out since no differences in the spontaneous contractile activity were detected between both uterine horns from each animal in any case. So, to explain the mentioned embryo inequality new experiments are being designed.

Nevertheless, an increase in the spontaneous uterine contractile activity was detected in obese dams compared to controls on gd 4.5. This is not a minor fact, given that higher uterine contractile activity is associated to lower pregnancy rate after *in-vitro* fertilization (Fanchin *et al.* 1998), it has been shown that obesity negatively impacts outcomes of assisted reproduction due to lower pregnancy rates (Bellver *et al.* 2010), and we have previously shown that the fertility rates are lower in our animal model compared to controls (Bazzano *et al.* 2017). Moreover, uteri from obese dams showed a higher sensitivity to a relaxation stimulus (salbutamol, selective agonist of β2 AR) than controls. This higher sensitivity can be explained due to the upregulation in the β2 AR gene and protein uterine expression we found in these animals. The fact that uteri from obese dams show an increase in the contractile activity although showing higher β2AR levels suggest that the latter could be a compensatory mechanism in response to the higher spontaneous uterine contractibility.

Despite the difficulty in understanding these novel mechanisms, we want to highlight the usefulness of our animal model for these kinds of studies. This model not only allows detecting the modification of the implantation window but also shows the alteration in the intrauterine embryo positioning, which is only possible to be observed in species with multiple IS. If these disruptions would take place in species with only one IS, as it is in humans, implantation would have not occurred, and its reasons could not be figured out.

In conclusion, our animal model allowed us to show here that obesity delays embryo transport from the oviduct to the uterus, specially in one animal flank, alters the intrauterine embryo positioning and modifies the implantation window. Furthermore, the uterine environment is modified by maternal obesity, programming those embryos that get over to all the mentioned alterations and inducing fetal macrosomia. This seems to be the mechanism that guarantees the perpetuity of obesity, explaining its increasing incidence worldwide.

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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Author contribution statement

EE contributed to the conception and design of the research, MVB and GBS performed research and the acquisition of data. The analysis and interpretation of data were at MVB, GBS, MVDA and EME's expenses. EME drafted the article, all authors revised it critically and approved the version to be published.

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