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ORIGINAL ARTICLE

Increase in endogenous estradiol in the progeny of obese rats is associated with precocious puberty and altered follicular development in adulthood

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Abstract Maternal obesity during pregnancy has been related with several pathological states in offspring. However, the impact of maternal obesity on reproductive system on the progeny is beginning to be elucidated. In this work, we characterize the effect of maternal obesity on puberty onset and follicular development in adult offspring in rats. We also propose that alterations in ovarian physiology observed in offspring of obese mothers are due to increased levels of estradiol during early development. Offspring of control dams and offspring of dams exposed to a high-fat diet (HF) were studied at postnatal days (PND) 1, 7, 14, 30, 60, and 120. Body weight and onset of puberty were measured. Counting of ovarian follicles was performed at PND 60 and 120. Serum estradiol, estriol, androstenedione, FSH, LH, and insulin levels were measured by ELISA. Hepatic CYP3A2 expression was determined by Western blot. HF rats had a higher weight than controls at all ages and they also had a precocious puberty. Estradiol levels were increased while CYP3A2 expression was reduced from PND 1 until PND 60 in HF rats compared to controls. Estriol was decreased at PND60 in HF rats. Ovaries from HF rats had a decrease in antral follicles at PND60 and PND120 and an increase in follicular cysts at PND60 and PND120. In this work, we demonstrated that maternal obesity in rats alters follicular development and induces follicular cysts generation in the adult offspring. We observed that maternal obesity produces an endocrine disruption through increasing endogenous estradiol in early life. A programmed failure in hepatic metabolism of estradiol is probably the cause of its increase.

Keywords Maternal obesity · Estradiol · Ovary · CYP3A2

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Introduction

Currently, obesity is one of the greatest concerns in worldwide public health systems. Obesity is closely related with a myriad of pathological states such as diabetes, cardiovascular diseases, and reproductive impairments [1–3]. In many countries such as the U.S, Chile, and Mexico, obesity is more prevalent in females than in males, exceeding 30 % of the female population, while in some regions over 50 % of women are overweight [4]. Maternal obesity is linked to several pregnancy complications like gestational diabetes mellitus, preeclampsia, and increased rate of spontaneous abortion [5]. More recently, it was demonstrated that obesity during

pregnancy may result in a higher predisposition of the progeny to develop endocrine and metabolic pathologies during adulthood, including obesity [6], hepatic steatosis [7–9], diabetes mellitus [10], and cardiovascular diseases [5, 11]. Some evidence has linked gestational obesity to reproductive alterations in mice progeny [12–14] but the exact nature of the alterations and the underlying cellular mechanisms are not known.

Maternal obesity in gestating mothers is associated with an early onset of puberty in female progeny. In humans, a high body mass index (BMI) of mothers was co-related to an early onset of menarche in daughters, but a higher BMI of offspring was not related to early puberty [15]. This evidence supports some effect of the uterine microenvironment on long-lasting reproductive function. Another study also showed a relation between a higher BMI and early age of menarche in humans [16]. In the rat model, obesity can be induced by administering a high-fat diet (HFD). HFD administration during gestation and/or lactation in rats results in early vaginal opening in the offspring [13, 14, 17]. In addition to early puberty, altered levels of steroidal hormones and estrous cycle abnormalities are observed when such rats reach adulthood [13]. Similarly, the administration of HFD in mice leads to altered ovarian follicular development, particularly a decrease in the number of antral follicles, and a decreased follicular reserve in the adult offspring [12].

Interestingly, some reproductive alterations observed in the female offspring of mothers who had obesity during pregnancy are similar to alterations produced by early exposure to estrogenic compounds. For example, early vaginal opening was observed in animals exposed to estradiol (E_2) or endocrine disrupting chemicals, during early postnatal development [18, 19]. In addition, adult rats exposed to estrogenic compounds in early life usually have a reduced number of healthy antral follicles in the ovary [18, 20, 21]. Finally, both exposure to either high-fat diet or ethinylestradiol during pregnancy are associated with an increased risk of developing breast cancer in rodents [22]. Taken together, and in light of the possible estrogenic effects of maternal obesity, we postulated that maternal obesity during pregnancy produces an increase in endogenous E_2 during early postnatal development in rats, which may be associated with altered follicular development in adulthood. In addition, we wanted to evaluate the mechanism of the increase in serum E_2 levels. For this purpose, we evaluated E_2 secretion from the ovary and expression of CYP3A2, a key hepatic enzyme metabolizing E_2 either to estriol (E_3) or catecholestrogens in rats [23, 24]. We selected CYP3A2 since other researchers demonstrated that maternal obesity in rodents produces a decrease in CYP3A2 in offspring [25], and also because CYP3A2 is homologous to human CYP3A4, the most abundant hepatic cytochrome in humans [26].

Materials and methods

Animals

Forty-two Sprague–Dawley female rats, each weighing 180–200 g, were used from the animal facility of the Faculty of Science, Universidad de Valparaíso. Rats were divided in two groups according to their diet; one group received the standard control food ($n = 15$, Control diet containing 13 % Kcal fat, Champion S.A., Nutrición Animal, Santiago, Chile) during the entire study, and the other group received a high-fat diet ($n = 27$, HFD 60 % Kcal fat, Research Diet, USA) for 1 month, following which these rats were mated with fertile males. The females continued to receive either control or HF diet during pregnancy and nursing. Offspring from both groups received control food from their weaning stage. Eighty six female offspring rats were subjected to euthanasia by decapitation at PND1 (control $N = 10$ and HF $N = 6$), PND7 (control $N = 6$ and HF $N = 5$), PND14 (control $N = 7$ and HF $N = 10$), PND30 (control $N = 4$ and HF $N = 8$), PND60 (control $N = 10$ and HF $N = 6$), and PND120 (control $N = 8$ and HF $N = 6$). The exact number of samples for each experiment is indicated in the legend of all tables and figures. Adult rats (PND60 and PND120) were subjected to euthanasia between 15:00 and 18:00 at the estrus stage. All rats remained in a temperature controlled room, in a 12 h light and 12 h dark cycle (light on at 08:00 am), and with access to food and water ad libitum. The Bioethics Committee of the Faculty of Science of Universidad de Valparaíso, and the Institutional Animal Experimentation Bioethics Board and the Science Council (FONDECYT) of Chile approved all experimental procedures. All efforts were made to minimize the number of animals used, and all procedures were undertaken in a humane manner.

Control of weight and estrous cycle

All mother rats were weighed at least three times a week and the estrous cycle was followed by performing daily vaginal smears [27]. Only rats with regular cycles were selected for the study. After 1 month on either HFD or control diet, the rats were placed with fertile males in the afternoon of the proestrus stage. The next day, successful mating was confirmed by the presence of sperm in the vaginal smear. If no mating occurred, rats were again placed with a fertile male in the subsequent proestrus stage for only one more time. If rats were not become pregnant

after second attempt, they were classified as "unsuccessful pregnancies" and were excluded from the study (Table 1). In offspring female rats, the external genitalia were observed every day from weaning to determine the day of vaginal opening. The estrous cyclicity was determined by performing vaginal smears at the onset of vaginal opening.

Histology and morphometric analyses

All right ovaries of PND60 and PND120 rats were immersed in Bouin's fixative, embedded in paraffin, cut into 6 μm sections and stained with hematoxylin and eosin. Morphometric analysis of follicles was performed as previously reported [18]. All follicular structures were followed through all slices and were counted when the nucleus of the oocyte was visible or had reached the maximum diameter, in cases where the oocyte was absent. Primordial follicles were counted every three slices to avoid overcounting. Follicles were classified according to Cruz et al. [18]. Follicular structures were classified as cystic follicles if they satisfied the following conditions: lack of oocyte, absence of atresia criteria, and a large antral cavity.

Hormones and transaminases determination

We collected rat blood samples during euthanasia at: PND1, PND7, PND14, PND30, and PND60. We centrifuged the blood samples at 850 g for 10 min to obtain serum, which was then stored at −80 °C until further analyses. E_2, E_3, insulin, FSH, and LH levels were determined by enzyme immunoassay according to the manufacturer's protocol for E_2 and E_3 (Alpco Diagnostic, USA), FSH and LH (Cusabio Biotech Co, USA), and for insulin (Merck Millipore, USA). Intra- and inter-assay variations were less than 5 % for E_2, less than 3 % for E_3, less than 6 % for insulin and less of 15 % for FSH and LH. The minimum detectable for each hormone were E_2 10 pg/mL, E_3 75 pg/mL, FSH 0.25 mIU/mL, LH 0.3 mIU/mL, and insulin 0.2 ng/mL, respectively. Serum glutamic oxaloacetic transaminase (GOT) and alanine transaminase (GPT) were measured using VITROS Chemistry Products AST Slides and ALT slides (Ortho-Clinical Diagnostics, Inc, USA).

Estradiol secretion from the ovaries

E_2 secretion from the ovaries was assessed in an in vitro assay following the protocol from Barria et al. [28]. Briefly, the left ovaries of PND30 rats were incubated in 2 mL Krebs bicarbonate-albumin, pH 7.4 (in mM: 118.6 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 2.5 $CaCl_2$, 25 glucose, 0.6 ascorbic acid, 0.15 EDTA, and 0.1 g/L albumin). The right ovaries were incubated in the same solution plus the hormone hCG to stimulate E_2 secretion (2.5 UI/2 mL hCG, Sigma). The incubation protocol was performed in the presence of a mix of carbogen gas (95 % O_2/5 % CO_2) for 3 h. Buffer solution was then collected and stored at −20 °C until enzyme immunoassay was performed.

Liver total fat content

Total lipids were extracted from liver homogenates using a modified Bligh and Dyer extraction procedure [29]. Liver samples were homogenized in distilled water and the lipid components were extracted with a 1:2 chloroform:ethanol solution, followed by centrifugation (2000*g* for 10 min at

Table 1 Mothers and offspring weight

	Control		HF	*p* value
Mother body weight (g) before pregnancy	Pre-diet	232.0 ± 12.8	233.0 ± 13.0	0.0047**
	1 month diet	260.8 ± 13.7	293.0 ± 16.9	
Successful pregnancy (% and number)	80 % (12/15)		41 % (11/27)	–
Offspring (number)	11.7 ± 0.4		9.0 ± 1.4	0.07
Offspring body weight (g)				
PND1	5.58 ± 0.13		6.88 ± 0.17	0.045*
PND7	11.56 ± 0.40		15.08 ± 0.40	0.0428*
PND14	21.88 ± 0.49		30.42 ± 0.57	0.0002**
PND30	71.66 ± 1.51		97.75 ± 1.33	<0.0001****
PND60	182.30 ± 3.54		206.80 ± 4.18	<0.0001****
PND120	246.91 ± 5.04		276.30 ± 7.06	<0.0001****

PND postnatal day. For PND1–60, control $N = 10$ and HF $N = 6$ and for PND120, control $N = 8$ and HF $= 6$. Data represent mean ± SEM. Significance was obtained by a Student *t* test. $p < 0.05 =$ *, $p < 0.01 =$ **, and $p < 0.001 =$ *** for control versus HF in each condition

room temperature). After extraction of the chloroformic phase, the solvent was allowed to evaporate to determinate the total fat content. Values were expressed as g of fat/100 g of liver.

Western blot analyses

Western Blot was used to detect hepatic CYP3A2 protein using liver samples from rats of different ages. The samples were separated by SDS-PAGE on 10 % polyacrylamide gels under reducing conditions. GAPDH was detected as an internal loading control. Proteins were transferred to nitrocellulose membrane, blocked with 5 % milk for 1 h and probed with either rabbit polyclonal anti-CYP3a2 antibody (AB1276 Merck Millipore, Germany) (1:250, overnight incubation) or rabbit polyclonal anti-GAPDH antibody (G9545, Sigma-Aldrich Co, LLC) (1:40,000, 1 h incubation). The antibody complexes were detected using a Goat Anti-Rabbit IgG Fc (HRP) ab97200, Abcam (1:10,000). For detection, we used EZ-ECL Kit Enhanced Chemiluminescence Detection Kit (Biological Industries, Israel). Chemiluminescence was captured using C-digit Blot Scanner (LI-COR Bioscience, USA). Results were analyzed by measuring the pixel intensities of bands using the semi-quantification tool of the Image Pro Plus 6.0 program (Media Cybernetics, Inc., USA). All Western blots were performed thrice for each liver sample.

Statistical analysis

All data are expressed as mean ± SEM. Student *t* test was used to compare hormone serum levels, number of follicles, mRNA levels, and CYP3a2 levels between HFD and control groups. Two-way ANOVA test was used to compare basal and stimulated E_2 secretion from the ovaries of control and HFD groups. Statistical analyses were carried out with GraphPad Prism v6.0 (GraphPad Software, San Diego, CA, USA).

Results

Maternal obesity during gestation and nursing results in early vaginal opening and increased weight in the offspring

Figure 1a and Table 1 show the weight of HF rats compared to controls. The weight was higher in HF rats than in controls from PND1 until PND120. In addition, as shown in Fig. 1b, the vaginas of HF rats opened earlier than control rats (Controls: 41.0 ± 0.6, $n = 10$; HF: 38.2 ± 0.7, $n = 6$). Moreover, Table 1 shows that obese rats have a lower capacity to become pregnant, and also have a tendency to produce a lower number of pups.

Maternal obesity during gestation and nursing produces an increase in serum E_2 independently of ovarian E_2 secretion in the offspring

Figure 2a–e shows the serum levels of E_2 at PND1, PND7, PND14, PND30, and PND60. HF rats had higher E_2 levels compared to controls from PND1 until PND60. Figure 2f shows that even when the HFD exposure induced a permanent increased estradiol levels in offspring, the pattern of serum levels trough life was unaltered. To assess the origin of this E_2 increase, we selected PND30 rats and performed a secretion experiment. Figure 3 shows the basal and stimulated secretion of E_2 from the ovary. No differences were observed in basal secretion of E_2 between HF rats and controls. Although hCG produced an increase in E_2 secretion both in controls and in HF rats (Fig. 3a), no differences in stimulated E_2 secretion were detected between HF rats and controls (Fig. 3b).

Maternal obesity during gestation alter hepatic function and decreases expression of CYP3A2 and E3 levels in the offspring

Table 2 shows some characteristics of hepatic function at PND14, PND30, and PND60. Hepatic weight increased from PND14 until PND60 in HF rats compared to controls. Together with this, total fat content, GOT and GOT/GPT ratio were increased at PND30 and PND60. In histological analysis of liver, we observed signs of hepatic steatosis in some HF rats but no sign of steatosis in control rats (Not shown). In addition, Fig. 4a–e) shows the semi-quantification of CYP3A2 protein levels by Western blotting. The protein CYP3A2 decreased from PND1 until PND60 in HF rats compared to control rats. To assess if the E_2 increase was due to lower biotransformation of the hormone by CYP3A2, we measured E_3 (a metabolic product of E_2) at PND14, PND30, and PND60. E_3 serum levels at PND60 are shown in Fig. 5a. Serum levels of E_3 decreased at PND60 and the ratio E_3/E_2 also decreased in the HFD group (Fig. 5b). Unfortunately, levels of E_3 at PND14 and PND30 were below the sensitivity of this assay.

Maternal obesity during gestation alters ovarian follicular development and hormonal profile in the adult offspring

Morphometric analysis of ovarian follicles is shown in Fig. 6 for PND60 rats and in Fig. 7 for PND120 rats. At PND60, we found no differences in primordial and primary follicles between HF and control rats (Fig. 6a, b). In addition, we

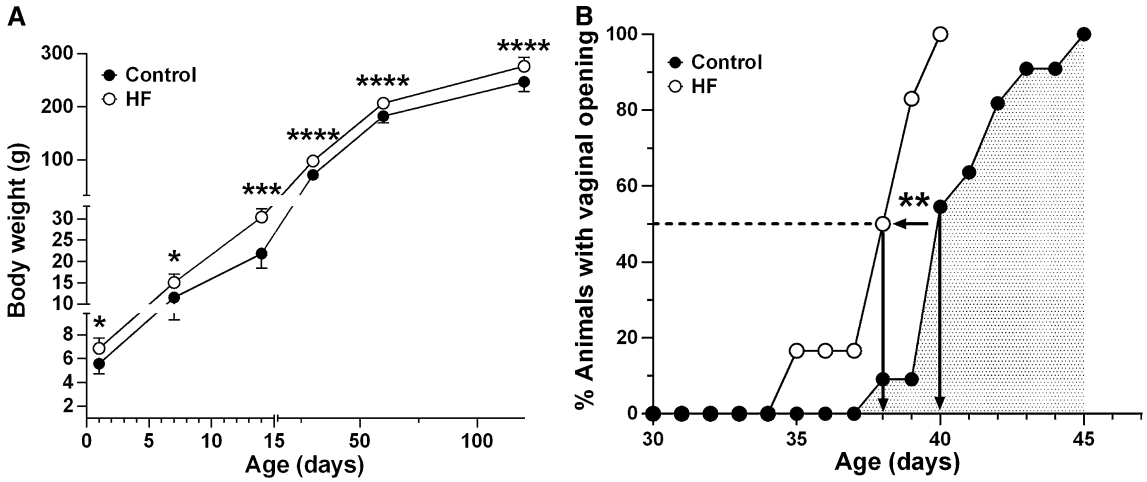

Fig. 1 Weight curve and onset of puberty in offspring of obese dams. **a** This panel shows the average body weight of the offspring from dams fed with a high-fat diet (*open circles*) or control chow diet (*closed circles*) during the development. **b** Onset of puberty assessed by the observation of vaginal opening, HF (*open circles*) and control chow (*closed circles*). Data are shown as mean ± SD. $p < 0.05$ = *, $p < 0.01$ = **, and $p < 0.001$ = *** for control versus HF in each age (PND1 up to PND60: control, $N = 10$; HF, $N = 6$, and PND120: control, $N = 6$; HF, $N = 6$)

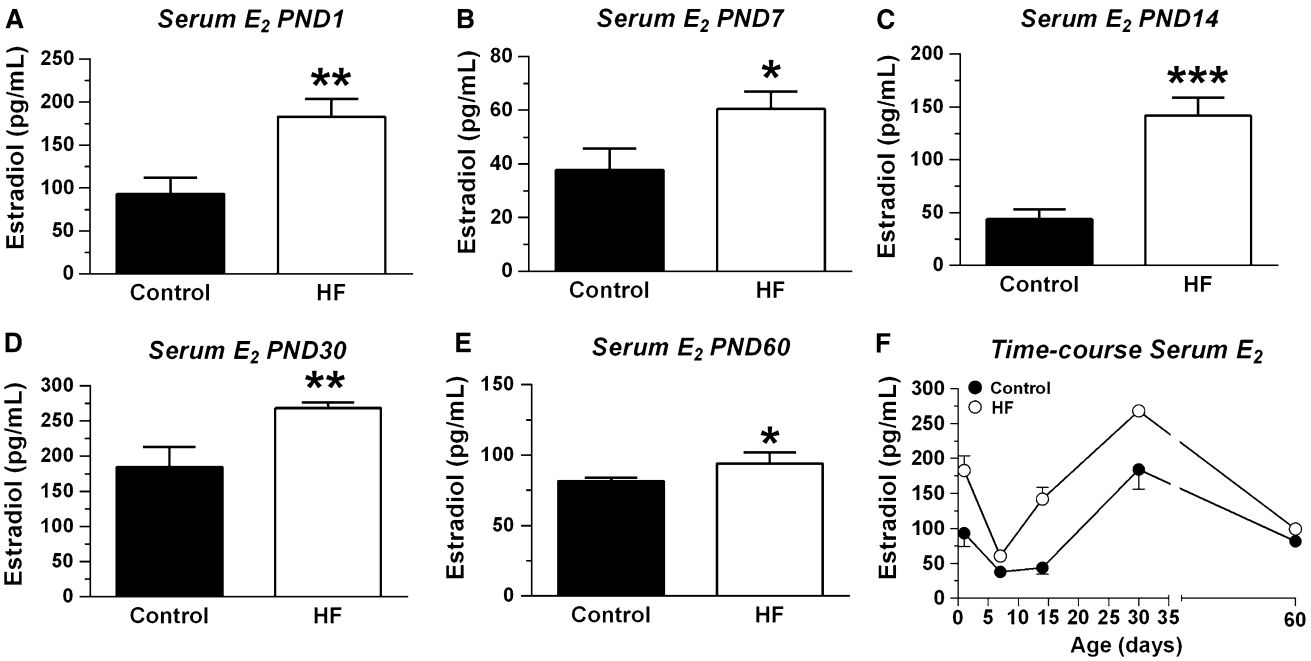

Fig. 2 Serum estradiol over the lifespan of the offspring. **a** E_2 at PND1, control, *black bar* ($N = 5$) and HF, *white bar* ($N = 4$), B) E2 at PND7, control $N = 6$ and HF $N = 5$, **c** E_2 at PND14, control $N = 6$ and HF $N = 10$, **d** E_2 at PND30, control $N = 4$ and HF $N = 8$. **e** E_2 at PND60, control $N = 7$ and HF $N = 4$, and **f** Changes in time course serum of E_2 trough the life span of the rats, control (*closed circles*) and HF (*open circles*). Serum samples were measured in triplicated. Data are shown as mean ± SEM. Significance was obtained by a Student *t* test. $p < 0.05$ = *, $p < 0.01$ = **, and $p < 0.001$ = *** for control versus HF in each age

found a decrease in healthy secondary, healthy antral and atretic antral follicles in HF rats compared to controls (Fig. 6c, e and f). Despite this, the *corpora lutea* did not change in HF rats compared to controls (Fig. 6h). In contrast, we found cystic follicles in PND60 HF rats (Fig. 6i), which were missing in controls. For PND120 rats, we did not find changes in primordial, primary and secondary follicles (Fig. 7a–d, respectively) between HF and control rats. However, an increase in atretic antral follicles and a decrease in healthy antral follicles were observed in HF rats compared to controls (Fig. 7e, f). In addition, the *corpora lutea* did not change and cystic follicles increased in HF rats compared to control rats at PND (Fig. 7h, i). On the other hand, we found an altered hormone profile in prepubertal (PND 30) and adult

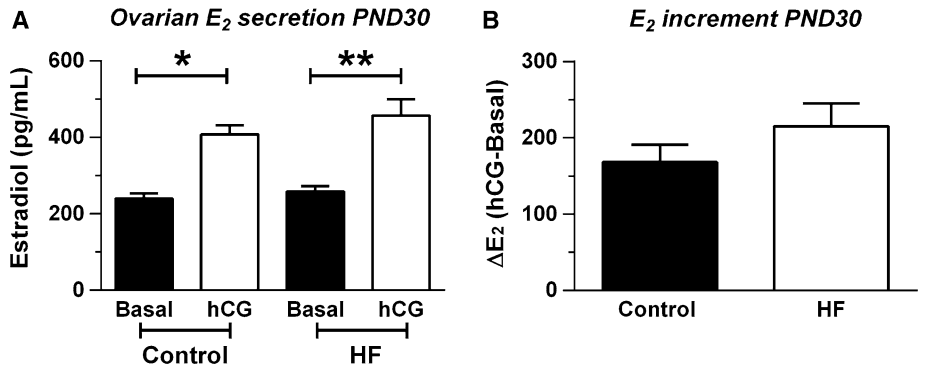

Fig. 3 Ovarian estradiol secretion. **a** E2 levels of incubation media collected from an in vitro secretion assay of ovaries from 30-day-old rats. *Black bars* basal secretion of E2 and white bars: E2 secretion under a stimulus of 2.5 UI of hCG. **b** Difference in the level of estradiol between control and hCG as the comparison of delta changes of E2 previous and after hCG stimulation control: ovaries from offspring of dams fed with chow standard food $N = 4$, and HF: ovaries from offspring of dams fed with a high-fat diet $N = 8$. Significance was obtained by a Two-way ANOVA test. $p < 0.05 = *$, $p < 0.01 = **$ for control versus HF

Table 2 Liver profile of offspring of obese mothers

	Control	HF	*p* value
Offspring liver weight (g)			
PND14	0.79 ± 0.08	0.94 ± 0.06	0.0016**
PND30	3.72 ± 0.23	4.28 ± 0.14	0.0008***
PND60	6.12 ± 0.42	7.35 ± 0.58	0.0002***
Offspring serum GOT (U/L)			
PND30	146.8 ± 11.89	223.0 ± 35.91	0.0394*
PND60	94.9 ± 7.02	146.4 ± 32.85	0.0502
Offspring serum GPT (U/L)			
PND30	63.2 ± 7.42	61.6 ± 5.53	0.4335
PND60	47.0 ± 1.53	49.4 ± 4.52	0.2888
Offspring serum GOT/GPT ratio			
PND30	2.4 ± 0.14	3.6 ± 0.43	0.0129*
PND60	2.2 ± 0.08	2.8 ± 0.39	0.0286*
Offspring total liver fat (%)			
PND30	4.9 ± 0.47	6.8 ± 0.78	0.0371*

PND postnatal day. For PND60, control $N = 10$ and HF $N = 6$ and for PND30, control $N = 6$ and HF $= 7$. Data represent mean ± SEM. Significance was obtained by a Student *t* test. $p < 0.05 = *$, $p < 0.01 = **$, and $p < 0.001 = ***$ for control versus HF in each condition

(PND 60) rats. Table 3 shows that FSH increased at PND30, whereas no changes were observed at PND60. LH did not change neither at PND30 nor at PND60. Insulin levels were elevated at PND60 in offspring of obese rats, compared to controls.

Discussion

Maternal obesity is strongly associated with the development of several pathologies including cardiovascular and metabolic diseases in the offspring. Some studies have also associated maternal obesity to reproductive abnormalities in female offspring [12–14]. In the present work, we mimic these reproductive alterations, and study the ovarian effects of maternal obesity, particularly on follicular development, in detail. In addition, we show that these reproductive alterations could result from increased levels of endogenous E_2 in the serum during early postnatal development. Finally, we propose that a defect in hepatic E_2 metabolism is the mechanism responsible for endogenous E_2 increase in the offspring.

In humans, a higher body mass index is related to an advanced menarche in daughters [15, 16]. This was reproduced by several authors in rodents using early vaginal opening as an index of precocious puberty [13, 14, 17]. Similarly, we also found early vaginal opening in HF rats compared to controls (Fig. 1b). The underlying mechanism resulting in early puberty in the offspring of obese mothers is poorly understood. Connor et al. [13] suggest that the energy demands of reproductive maturation are covered in HF rats earlier than in controls, due to fat accumulation, and this leads to early puberty. Similarly, the HF rats in our study that reached reproductive maturity more quickly than controls had higher body weight from PND1 until adulthood (Fig. 1a). Nevertheless, we propose an alternative explanation for our observation. Precocious puberty is usually associated with an exposure to estrogenic compounds during early postnatal life in rats because an early activation of GnRH neurons [18, 30–33]. We found an increase in FSH levels at PND30 that could support an early activation of GnRH neurons that probably leads to early development of follicles and hence to advance the first ovulation. Thus, we thought that maternal obesity would increase endogenous E_2 during early postnatal development. Further evidence to support our hypothesis is the strong relation between maternal obesity

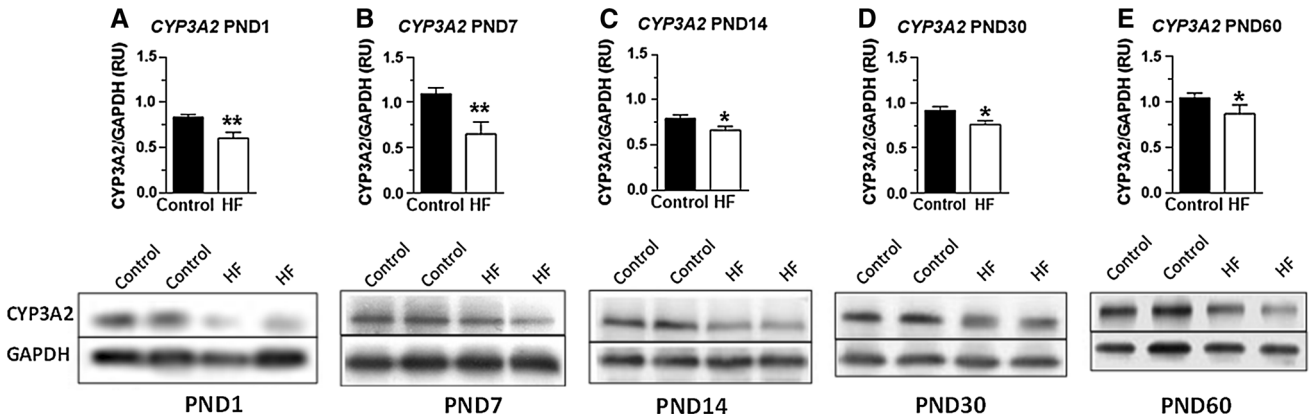

Fig. 4 Hepatic CYP3A2 expression. Each graph shows the mean of the quantification of pixels for CYP3A2 bands divided by the quantification of pixels for GAPDH bands. Below each graph a representative picture of two samples for control and HF is shown. PND1 $N = 9$ for control and 4 for HF, **b** PND7 $N = 6$ for control and 4 for HF, **c** PND14 $N = 7$ for control and 8 for HF, **d** PND: 30 $N = 4$ for control and 6 for HF, **e** PND60 $N = 10$ for control and 5 for HF. Data are shown as mean ± SEM. Significance was obtained by a Student t test. $p < 0.05 = *$, $p < 0.01 = **$, and $p < 0.001 = ***$ for control versus HF in each age

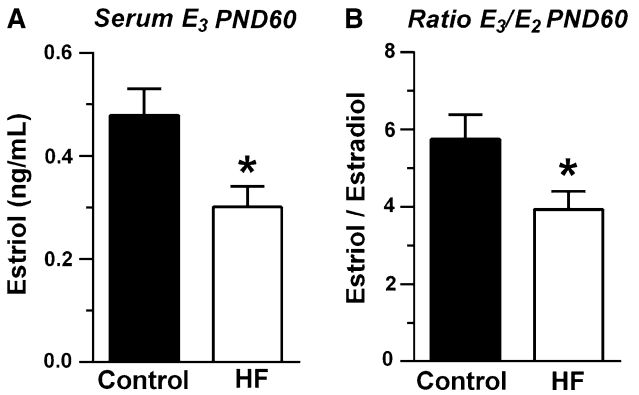

Fig. 5 Serum estriol of PND60 offspring measured by EIA. **a** E_3 at PND60, control, *black bar* ($N = 10$) and HF, *white bar* ($N = 6$). All serum samples were measured in duplicate. **b** The ratio E_3/E_2 was obtained by the division of each serum sample. Data are shown as mean ± SEM. Significance was obtained by a Student t test. $p < 0.05 =$ for control versus HF in each age

and the development of breast cancer in offspring [17, 22], since breast cancer is traditionally related to increased E_2 levels or estrogen exposure [34, 35]. In this study, we found that endogenous E_2 was elevated not only during early postnatal development, but remained high until adulthood. Therefore, this increase in endogenous estradiol during early life, could trigger a plethora of pathological conditions similar to those already observed in other models of exposure to estrogenic compounds [36].

We postulated two mechanisms that may lead to increased serum levels of E_2 in HF rats: (1) An increase in E_2 secretion from the ovary and (2) a decrease in hepatic metabolism of E_2. To evaluate the secretion of E_2, we selected PND30 rats and performed an in vitro experiment in which ovaries were incubated both in basal and stimulated conditions, to measure the E_2 secreted from them into the incubation media. We found no differences in the basal or the hCG-stimulated secretion of E_2 from the ovaries of control and HF rats, which rule out an ovarian origin of serum estradiol increase. We then explored the second possibility and measured the expression of CYP3A2 protein in the liver using Western blot. HF rats had reduced CYP3A2 levels, from PND1 until PND60, compared to controls. Similarly, other authors evaluated the expression and activity of CYP3A in the offspring of mice fed on a high-fat diet during pregnancy, and found a decrease in the expression of CYP3A at different ages [25]. Rat CYP3A2 is homologous to human CYP3A4 [23], and is predicted to also catalyze the hydroxylation of E_2 to catecholestrogens and E_3, the latter being the major metabolite [24]. To confirm a decrease in metabolism of E_2 in HF rats, we measured serum E_3 at PND14, PND30, and PND60. We found that E_3, and hence the E_3/E_2 ratio, were decreased in PND60 HF rats compared to controls, which confirmed decreased metabolism of E_2 in adult rats. Unfortunately, the E_3 measurements performed at PND14 and PND30 were below the sensitivity of detection.

Lower metabolism through CYP enzymes has been associated to non-alcoholic fatty liver disease (NAFLD) [37–39]. This condition could explain the lower expression of CYP enzymes in the offspring of obese rats, since in the present study and in other studies, we can observe that maternal high-fat diet exposure causes hepatic steatosis and dysfunctions of the liver [7, 40]. In our study, the liver weight was increased at PND14, 30 and 60. We assessed hepatic histology of HF and control livers and found that only some HF rats had evident intracellular lipid accumulation in the liver (not shown). However, the quantitative study of total fat showed an increase in total fat in livers of

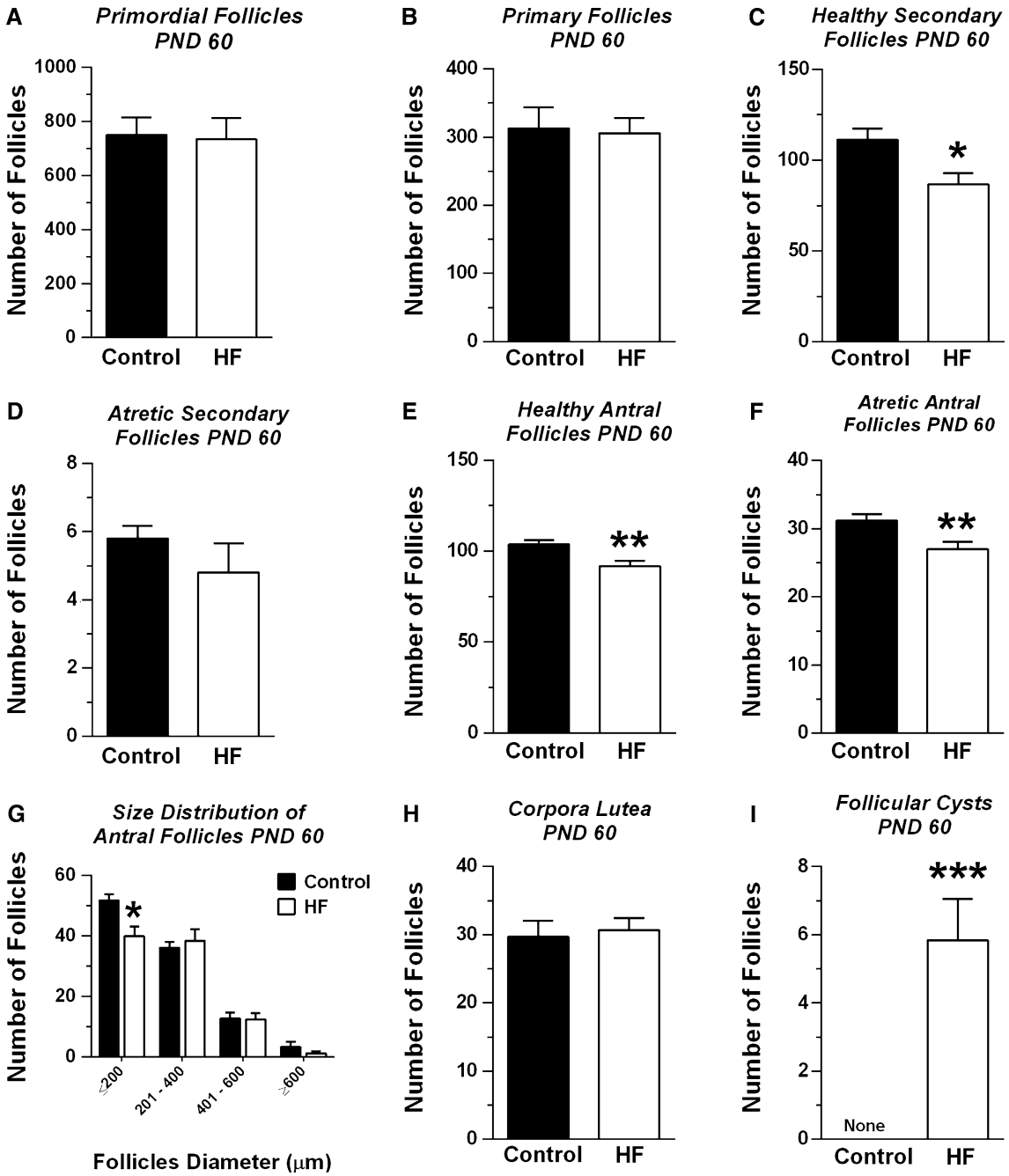

Fig. 6 Follicular development at PND60. Counting of follicles at different stages is shown. *Black bars* control rats; *white bars* HF rats. **a** Primordial follicles, **b** primary follicles, **c** healthy secondary follicles, **d** atretic secondary follicles, **e** healthy antral follicles, **f** atretic antral follicles, **g** size distribution of healthy antral follicles, **h** Corpora Lutea, **i** follicular cyst. Data are shown as mean ± SEM. Significance was obtained by a Student *t* test. $p < 0.05$ = for control versus HF in each age. $N = 6$ for control rats and $N = 6$ for HF rats

PND30 HF rats compared to controls, which is consistent with hepatic steatosis, since they had more than 5 % of total fat. This condition was also associated with increase GOT/GPT ratio, which is a feature of hepatic steatosis associated to insulin resistance and diabetes mellitus II [41]. In fact, our rats had increased levels of insulin in serum compared to controls. NAFLD is related to increased levels of pro-inflammatory cytokines, such as interleukin-1 beta, interleukin-6, and tumor necrosis factor-alpha, which have demonstrated to reduce the expression of CYP3A and other cytochromes in the liver [42]. In addition, Tajima et al. found a decrease in the nuclear receptor CAR, which is related with up-regulation of CYP3A expression. They propose that a decrease in p-ERK reduces CAR expression,

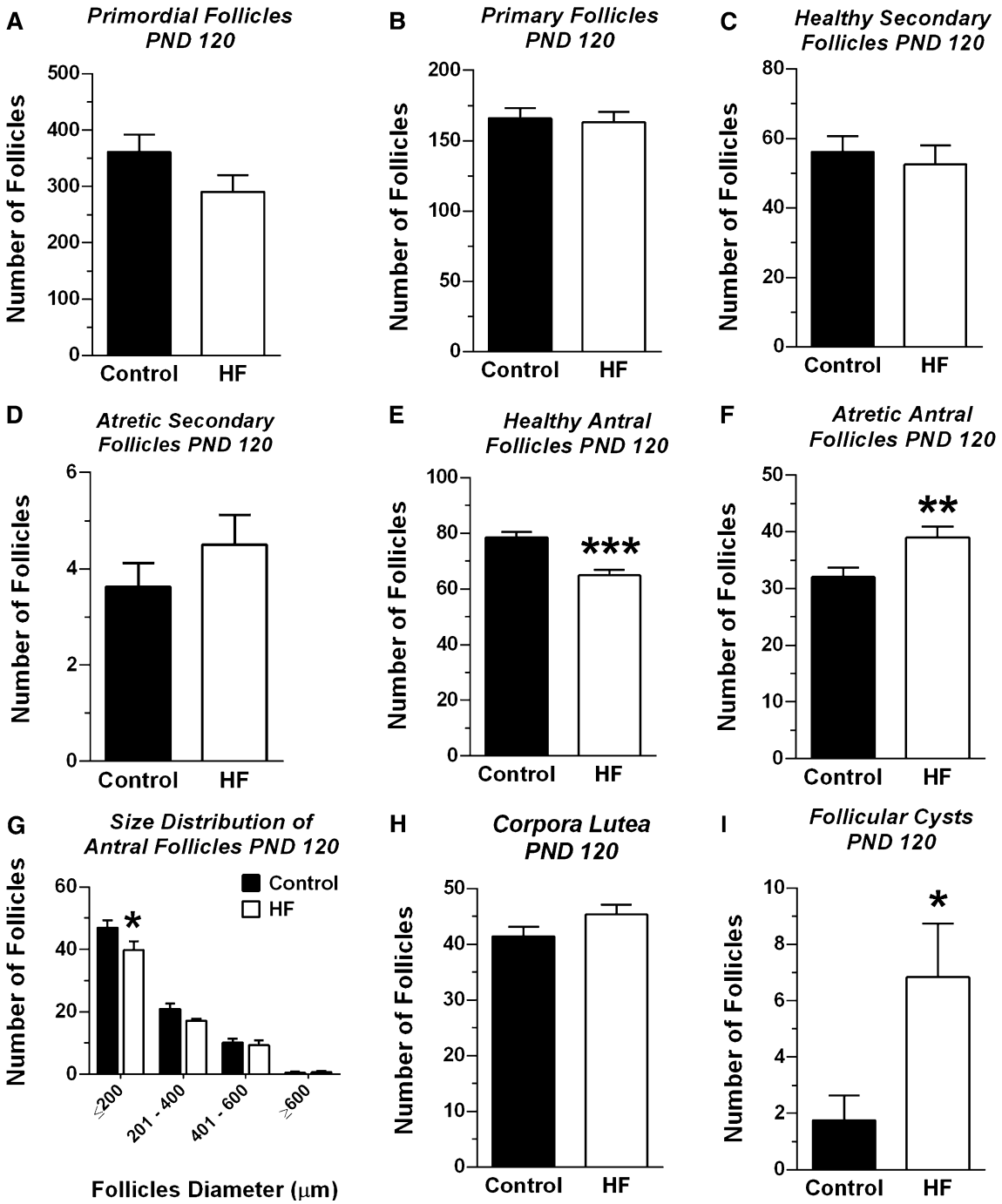

Fig. 7 Follicular development at PND120. Counting of follicles at different stages is shown. *Black bars* control rats; *white bars* HF rats. **a** Primordial follicles, **b** primary follicles, **c** healthy secondary follicles, **d** atretic Secondary follicles, **e** healthy antral follicles, **f** atretic antral follicles, **g** size distribution of antral follicles, **h** Corpora Lutea, **i** follicular cyst. Data are shown as mean ± SEM. Significance was obtained by a Student *t* test. $p < 0.05$ = for control versus HF in each age. $N = 8$ for control rats and $N = 6$ for HF rats

which leads to reduced expression of CYP3A in offspring of mice exposed to a high-fat diet [25]. Taken together, hepatic failure may be the primary event that leads to increased serum E_2 levels, and hence to reproductive abnormalities in the adult offspring of obese mothers. In fact, if the increase in estradiol synthesis were the primary cause of increased serum estradiol, we would expect an increase in the expression of cytochromes, since estrogenic compounds induce their expression in both rats and humans [43, 44].

Exposure to exogenous estrogenic compounds during early postnatal life has deleterious effects on the functions

Table 3 Serum hormones of offspring of obese mothers

	Control	HF	*p* value
Offspring serum insulin (ng/mL)			
PND30	0.067 ± 0.0136	0.084 ± 0.0083	0.1512
PND60	0.67 ± 0.101	1.15 ± 0.154	0.0025*
PND60 HOMA-IR	7.53 ± 1.081	9.68 ± 0.739	0.0759
Offspring serum FSH (mIU/L)			
PND30	13.67 ± 2.495	24.57 ± 4.577	0.0315*
PND60	38.32 ± 4.479	50.04 ± 11.59	0.1426
Offspring serum LH (mIU/L)			
PND30	0.67 ± 0.263	1.16 ± 0.483	0.3902
PND60	2.49 ± 0.472	1.66 ± 0.261	0.0814
Offspring LH/FSH ratio			
PND30	0.046 ± 0.0105	0.057 ± 0.0182	0.341
PND60	0.055 ± 0.0150	0.023 ± 0.0051	0.0782
Serum androstenedione (ng/mL)			
PND60	0.35 ± 0.023	0.45 ± 0.463	0.024*

PND postnatal day. For PND60, control $N = 10$ and HF $N = 6$ and for PND30, control $N = 6$ and HF $= 7$. Data represent mean ± SEM. Significance was obtained by a Student *t* test. $p < 0.05 = *$ for control versus HF in each condition

of the brain, ovary, and other reproductive tissues [18, 21, 45, 46] [47, 48]. We hypothesized that the increase in endogenous estradiol through early life in rats who are descendants of obese mothers, is associated with altered ovarian follicular development in adulthood, similar to what occurs in rats exposed to environmental estrogens. While preparing this manuscript, Cheong et al. [12] reported for the first time that the offspring of obese mothers have altered follicular development with a decrease in both primordial follicles and healthy antral follicles, in mice. However, in this study follicles were only counted every 50 µm, and other follicular structures such as follicular cysts, were not counted. In our study, we analyzed ovarian follicle morphometry in each histological slice, and counted all ovarian follicles, *corpora lutea* and follicular cysts through the whole ovary in rats. The decrease in antral follicles observed by Cheong et al. was confirmed in our rat model, but we did not find a decrease in primordial follicles. The decrease in the number of antral follicles observed in HF rats, is similar to the results obtained during exposure to endocrine disruptors or synthetic estrogens [18, 21, 45, 49, 50]. However, the magnitude of the decrease is less dramatic in HF rats, probably since dosing results in higher plasmatic levels of estrogenic compounds compared to the levels triggered by maternal obesity. As shown in Figs. 6g and 7g, among the antral follicles, the smaller ones are decreased in HF rats compared to controls. This is consistent with the fact that secondary follicles are decreased in HF rats at PND60. The development of these two types of follicles is primarily under the control of follicle-stimulating hormone (FSH). We measured serum FSH and LH levels and found no changes in adult rats. This could mean that follicles are less responsive to FSH effect. On the other hand, the higher levels of FSH that are responsible for recruit follicles during estral cycle are produced proestrus and early estrus in the rat. Since we done our studies at late estrus, we cannot assume that low follicular development is independently of an eventual hypothalamic failure. We think that these issues should be studied in the future. On the other hand, an FSH increase in prepubertal period could imply an early maturation of hypothalamic GnRH control of folliculogenesis and ovulation. Since the hypothalamic control of ovarian function matures after puberty and rats may not be fully mature at PND60, we followed a group of rats until PND120 to evaluate if the effect of maternal obesity in the offspring's follicular development is prolonged with age. We also found a decrease in healthy antral follicles and an increase in atretic antral follicles, showing that the alteration in follicular dynamics remains through reproductive life.

In addition to the decreased development of follicles, HF rats showed an increase in cystic follicles in the ovary, both at PND60 and PND120. Some authors postulate that polycystic ovary syndrome (PCOS) may be trigged by early exposure to estrogenic or androgenic compounds [51, 52]. In our model, we observed only a modest increase in follicular cysts compared to that observed following early exposure to endocrine disrupting chemicals. In fact, follicular cysts coexist with normal numbers of corpora lutea as usually occur in humans. In addition to early exposure to estradiol, higher insulin levels observed in our model could contribute to the development of follicular cyst. In fact, insulin favors androgens production from the ovary in a model of polycystic ovary in the rat [53]. In this context, we measured serum androstenedione in the offspring of obese rats and found an increase. Interestingly, the participation of insulin resistance and metabolic syndrome in PCOS has led to consider metformin as a possible drug in the management of the syndrome [54, 55] that could be evaluated in offspring of obese mothers.

Although maternal obesity is likely to only weakly induce reproductive abnormalities, due to early exposure to endogenous estradiol, its impact would be highly significant if another risk factor (i.e., stress) is added. On the other hand, offspring of obese mothers have a higher weight related to insulin resistance and also have several impairments such as increase in oxidative stress [56], predisposition to neuropsychiatric disorders [57], endothelial dysfunction [58], increase adrenergic tone and hypertension [59], hepatic steatosis [40], and higher breast cancer predisposition [22], among others. All these features

could also be directly produced by obesity and hence, estradiol increase in offspring of obese mothers adds complexity to the effects described above and could contribute to the multisystem damage produced by maternal obesity on the offspring.

Conclusion

Obesity is a public health concern whose impact is being determined, and thus it has a strong motive for intense research. In the present work, we found that serum E_2 levels increase from postnatal day (PND) 1 until adulthood (PND60), while hepatic CYP3A2 expression decreases through early postnatal development in the offspring of obese mothers. These changes were associated with early vaginal opening, altered follicular development, and the presence of ovarian cysts. The primary event appears to be a hepatic dysfunction related to NAFLD which decrease CYP3A2 expression, however, disorders related to obesity and the increase estradiol levels could combine to produce the reproductive diseases in offspring of obese mothers. It will be of interest in the near future to attempt to prevent this hepatic failure and its consequences, using pharmacological and non-pharmacological treatment of obesity during gestation. This will likely avert reproductive and metabolic alterations in offspring.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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