Maternal malnutrition during lactation alters the folliculogenesis and gonadotropins and estrogen isoforms ovarian receptors in the offspring at puberty

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Abstract

In this study, we aimed to evaluate whether maternal malnutrition during lactation alters the folliculogenesis and the expression of the gonadotropins and estrogen isoforms ovarian receptors in the offspring at puberty. At parturition, dams were randomly assigned to the following groups: control (C) group, with free access to a standard laboratory diet containing 23% protein and protein-energy-restricted (PER) group, with free access to an isoenergy and protein-restricted diet containing 8% protein. After weaning, the female pups had free access to standard laboratory diet. The maternal malnutrition caused a significant increase in the number of preantral ($C=13{\cdot}72\pm2{\cdot}87$; $PER=26{\cdot}36\pm3{\cdot}03$, $P<0{\cdot}01$) and small antral follicles ($C=9{\cdot}32\pm1{\cdot}35$; $PER=17{\cdot}64\pm2{\cdot}33$, $P<0{\cdot}01$) and decrease in the number of primordial ($C=11{\cdot}72\pm1{\cdot}37$; $PER=3{\cdot}92\pm0{\cdot}60$, $P<0{\cdot}01$) and Graafian follicles ($C=1{\cdot}84\pm0{\cdot}21$; $PER=0{\cdot}96\pm0{\cdot}11$, $P<0{\cdot}01$), and corpus luteum ($C=2{\cdot}00\pm0{\cdot}28$; $PER=0{\cdot}80\pm0{\cdot}31$, $P<0{\cdot}01$). The estradiol serum concentration was significantly higher ($C=67{\cdot}86\pm4{\cdot}39$; $PER=83{\cdot}29\pm2{\cdot}68$, $P<0{\cdot}05$) while testosterone serum concentration did not show statistical difference ($C=0{\cdot}09\pm0{\cdot}02$; $PER=0{\cdot}11\pm0{\cdot}01$, $P>0{\cdot}05$) in the PER group. In relation to the receptors expression, maternal malnutrition led to a significant increase in the amount of *Fshr* ($C=0{\cdot}89\pm0{\cdot}04$; $PER=1{\cdot}07\pm0{\cdot}03$, $P<0{\cdot}05$) and *Lhcgr* ($C=0{\cdot}87\pm0{\cdot}15$; $PER=1{\cdot}33\pm0{\cdot}08$, $P<0{\cdot}05$) transcripts and a significant decrease in the amount of *Ar* ($C=0{\cdot}59\pm0{\cdot}006$; $PER=0{\cdot}13\pm0{\cdot}080$, $P<0{\cdot}05$), *ERα* (*Esr1*) ($C=3{\cdot}33\pm0{\cdot}71$; $PER=0{\cdot}74\pm0{\cdot}50$, $P<0{\cdot}05$), *ERβ1* (*Esr2*) ($C=1{\cdot}33\pm0{\cdot}06$; $PER=0{\cdot}49\pm0{\cdot}36$, $P<0{\cdot}05$), and *ERβ2* (*Esr2*) ($C=3{\cdot}28\pm0{\cdot}60$; $PER=0{\cdot}62\pm0{\cdot}34$, $P<0{\cdot}05$) transcripts. In conclusion, perinatal maternal malnutrition can directly affect folliculogenesis at puberty probably as a consequence of changes in the ovarian expression of gonadotropins, androgen and estrogens isoforms receptors. Long-term sexual alterations could be expected in this experimental model, since a reduction in the primordial follicle number is observed, which can result in a decrease in the reproductive lifetime and an earlier termination of breeding capacity.

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Introduction

In mammals, the ability of the ovary to produce dominant follicles that ovulate their oocytes at mid-cycle is the basis of female fertility. In the adult ovary, folliculogenesis starts when follicles leave the pool of resting follicles to enter the growth phase. Thereafter, the early growing follicle undergoes a developmental process including a dramatic course of cellular proliferation and differentiation through primordial, primary, and secondary stages before acquiring an antral cavity. Most follicles fail to reach the preovulatory stage every cycle, dying in the process termed atresia (Gougeon 1996).

The gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), act by binding to and activating their specific receptors, LH receptor (LHR) and FSH receptor (FSHR; McFarland *et al.* 1989, Sprengel *et al.* 1990, Richards 1994). LHR is mainly expressed in ovarian theca cells, but it is also present in granulosa cells of preovulatory follicles and corpora lutea, whereas FSHR is expressed exclusively in granulosa cells (Themmen & Huhtaniemi 2000).

Fully differentiated preovulatory follicles are responsive to FSH and characterized by an increased capacity to synthesize large amounts of estradiol via aromatization of thecal-derived androgens, formation of a large fluid-filled antrum, and the acquisition of LHR (Richards *et al.* 2002, Couse *et al.* 2005). The LH surge promotes major changes in ovarian preovulatory follicles, including terminal differentiation of follicular cells and oocyte maturation. These events are required for the ovulation of a fertilizable egg (Hizaki *et al.* 1999), but it is clear that many other signaling events are critical for the final follicle growth and eventual ovulation. These include specific nuclear hormone receptors as progesterone receptors (Lydon *et al.* 1995, Robker *et al.*

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2000, Conneely *et al.* 2001), leptin (Ryan *et al.* 2003, Ricci *et al.* 2006), several local growth factors (Vitt *et al.* 2000, Shimada *et al.* 2006), prostaglandins (Wong & Richards 1991, Sirois *et al.* 1992, Davis *et al.* 1999, Joyce *et al.* 2001), insulin (Tamura *et al.* 2007), and others.

Besides gonadotropins, sex steroids play important roles in the growth and differentiation of reproductive tissues and in the maintenance of fertility. The biological actions of estrogens are mediated by binding to one of the two specific estrogen receptors (ERs), ERα or ERβ, which belong to the nuclear receptor superfamily (Clark *et al.* 1992, Korach *et al.* 1995).

ERα and ERβ are products of distinct genes on different chromosomes (Chu & Fuller 1997, Chun *et al.* 1998). Several ERα and ERβ splicing variants have been described. Unlike ERα, several splice variants of ERβ are expressed in tissues and the isoforms *ERβ1* and *ERβ2* mRNAs coexist in most rat organs including the ovary and uterus (Petersen *et al.* 1998, Price & Handa 2000). The ERβ1 mRNA sequence represents all eight exons identified so far in the *ERβ* gene. *ERβ2* contains an additional 54 nt between exons 5 and 6. Thus, this transcript encodes a protein with 18 additional amino acids in the ligand-binding domain (Maruyama *et al.* 1998, Petersen *et al.* 1998). ERβ2 has a lower ligand-binding affinity when compared with ERβ1 (Petersen *et al.* 1998). ERβ2 acts as a homodimer and it does not have the ability to induce transcription and even inhibits transcriptional activity of *ERα* and *ERβ1* (Maruyama *et al.* 1998).

Although ERα and ERβ share similar mechanisms of action, several differences in the transcriptional abilities of each receptor as well as distinct phenotypes between gene-null animals have been identified, suggesting that these receptors may regulate distinct cellular pathways. When ERs are coexpressed, ERβ exhibits an inhibitory action on ERα-mediated gene expression (Paech *et al.* 1997, Liu *et al.* 2002, Lindberg *et al.* 2003).

Estrogens acting via its receptors have direct proliferative and differentiative influences on follicle development, depending on the stage of folliculogenesis (Drummond & Findlay 1999). The ovary contains both ER subtypes, with a predominance of ERβ over ERα in granulosa cells (Byers *et al.* 1997, Drummond *et al.* 1999).

The follicular production of estrogen is dependent on the production of testosterone in theca cells and aromatase activity in the granulosa cells of the ovary in response to LH. The presence of androgen receptors (AR) suggests that this hormone can have specific actions in the ovary (Kimura *et al.* 2007). Besides serving as estrogen precursors, in rats, androgens appear to promote follicular growth (Horie *et al.* 1992, Wang *et al.* 2001) and corpus luteum formation (Hu *et al.* 2004). Therefore, multiple interactions between the cell types forming the follicle involve the interplay of several hormonal, paracrine, and autocrine factors that are essential for ovarian steroid hormone production and follicular maturation.

The prenatal and early postnatal nutritional status plays a critical role in postnatal growth and development. Early malnutrition may change the original programming of organs, especially those in developmental phases, which can result in metabolism long-term changes (Lucas 1994, 1998, Barker 2000). Some authors have shown that lactation could be a critical period in determining the future endocrine status of the progeny (Moura *et al.* 1997, Passos *et al.* 2000). Recently, we have shown that maternal protein and energy malnutrition during lactation leads to growth retardation and delay in the onset of puberty in female pups (Faria *et al.* 2004). Also, protein and energy restriction during lactation leads to an atrophy of the uterine endometrial glands of the offspring at puberty (Brasil *et al.* 2005).

The maternal nutritional state during lactation is equivalent and possibly even more important than that during gestation, as evidenced by a study by Léonhardt *et al.* (2003), which showed that the offspring whose dams were malnourished during lactation had more drastic consequences on gonadal development when compared with the offspring whose dams were malnourished only during pregnancy, or during pregnancy and lactation. Guzman *et al.* (2006) showed similar results. Based on those papers, we decided to analyze the effects of malnutrition only during the lactation time.

The goal of this study was to evaluate whether maternal malnutrition during lactation alters folliculogenesis, the ovarian expression of gonadotropins receptors, AR, and the different isoforms of ERs in the offspring at puberty.

Materials and Methods

Animals

Wistar rats were kept in a room with controlled temperature (25±1°C) and an artificial light: darkness cycle (lights on from 0700 to 1900 h). Virgin female rats of 3 months of age were caged with male rats at a proportion of 2:1. After mating, determined by the presence of a vaginal plug, each female was placed in an individual cage with free access to water and food until delivery. The handling of the animals was approved by the Animal Care and Use Committee of the Biology Institute of State University of Rio de Janeiro, which based their analysis on the Guide for the Care and Use of Laboratory Animals (Bayne 1996), and the study design was approved by the local Ethical Committee for the care and use of laboratory animals.

Experimental design

Six pregnant Wistar rats were separated after delivery into two groups: control (C) group, with free access to a standard laboratory diet containing 23% protein and protein-energy-restricted (PER) group, with free access to an isoenergy and protein-restricted diet containing 8% protein. The PER group, in spite of having free access to diet, consumed about 60% of that consumed by the control group (Passos *et al.* 2000). The low-protein diet was prepared in our laboratory

and its composition is shown in Table 1. Vitamins and mineral mixtures were formulated to meet the American Institute of Nutrition AIN-93G recommendation for rodent diets (Reeves *et al.* 1993). Within 24 h of birth, excess pups were removed so that only six female pups were kept per dam, as it has been shown that this procedure maximizes lactation performance (Fishbeck & Rasmussen 1987). Malnutrition of the studied rats was started at birth, which was defined as day 0 of lactation (d0) and was ended at weaning (d21). After weaning, female pups of the same treatment group were housed in groups of three animals per cage and given unlimited access to food and water until puberty (day 40). Then, only the animals on the diestrum stage were killed with a lethal dose of pentobarbital. To evaluate the nutritional state, food consumption of the offspring was monitored every day from weaning onward, while body weight and linear growth (nose–tail) were monitored every 5 days from birth until the end of the experiment. The blood was collected by cardiac puncture and the serum kept at −20 °C for subsequent determination of hormonal parameters. Ovaries were excised, dissected, weighted, and then divided into two parts: one was kept at −80 °C for subsequent measurements of androgen receptor, ERs α and β, FSHR, and LHR transcripts by RT-PCR. The other part of the ovary was paraffin embedded, sectioned at 5 μm thickness, and processed by routine histological analyses. Some samples were stained with hematoxylin and eosin to check the integrity of the tissue.

Morphologic classification of follicles

Paraffin sections (5 μm) from the left ovary of five animals from each group were taken at intervals of 50 μm and mounted on slides. The total number of sections analyzed was 15–20 per ovary. Routine hematoxylin and eosin staining was performed for histological examination under a light microscope. Sections from each ovary were digitized using a video camera coupled to a light microscope with a final magnification of X400 for primordial and primary follicles, and X100 for preantral, antral, and Graafian follicles and corpus luteum. Photographs of ovarian follicles were analyzed using Image Pro Plus for Windows (version 1.3.2; Media Cybernetics, Silver Spring, MD, USA). Follicle types in ovarian cross-sections were defined as follows. Primary follicles consisted of an oocyte surrounded by a single layer of cuboidal granulosa cells. Preantral follicles comprised an oocyte surrounded by two or more layers of granulosa cells with no antrum. Antral follicles were distinguished by the presence of an antrum within the granulosa cell layers enclosing the oocyte (Cheng *et al.* 2002). To avoid double counting, in the growing class only those follicles that showed the nucleus of the oocyte were counted, and in the antral class the follicles were compared with previous sections. The corpora lutea, which are in fact post-antral follicles, were counted in the same way as the follicles in the antral class.

Table 1 Composition of control and protein-energy-restricted diets

	Control[a]	Protein restricted[b]
Ingredients (g/kg)		
Total protein[c]	230·0	80·0
Cornstarch	676·0	826·0
Soybean oil	50·0	50·0
Vitamin mix[d]	4·0	4·0
Mineral mix[d]	40·0	40·0
Macronutrient composition (%)		
Protein	23·0	8·0
Carbohydrate	66·0	81·0
Fat	11·0	11·0
Total energy (kJ/kg)	17 038·7	17 038·7

[a]Standard diet for rats (Nuvilab-Nuvital Ltd, Paraná, Brazil).
[b]The protein-restricted diet was prepared in our laboratory by using the control diet, with replacement of part of its protein content with cornstarch. The amount of the latter was calculated to replace the same energy content of the control diet.
[c]The principal protein resources are soybean wheat, steak, fish, and amino acids.
[d]Vitamin and mineral mixtures were formulated to meet the American Institute of Nutrition AIN-93G recommendation for rodent diets (Reeves *et al.* 1993).

RNA extractions

Total RNA from ovary tissue was extracted using TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. Briefly, ovaries were homogenized in 1 ml of TRIZOL reagent per 50–100 mg of tissue. Then, RNA was extracted by a phenol/chloroform solution and precipitated by isopropyl alcohol. After washing with 75% ethanol, the RNA was dried and dissolved with diethyl pyrocarbonate-treated water. The quality of RNA samples was verified by the determination of the ratio 260/280 nm and by electrophoresis on a 1% agarose gel. The samples were stored at −80 °C until utilization.

Semi-quantitative RT-PCR

All RNA samples were rid of contaminating DNA by using DNA-free reagents (Invitrogen) according to the manufacturer's protocol. Then, 2 μg of RNA sample were used in a 20 μl cDNA reaction using Oligo dT and the Superscript cDNA synthesis system (Invitrogen) according to the manufacturer's protocol. PCRs were prepared using the equivalent of 2 μl cDNA per 50 μl reaction (triplicate) for each respective primer set using PCR reagents and Platinum Taq polymerase (Invitrogen). In order to quantify glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), *Ar*, *ERα*, *ERβ1*, *ERβ2*, *Fshr*, and *Lhcgr* transcripts, we determined the optimal number of amplification cycles for each gene. The applied PCR primers and the cycle profiles used are described in Table 2. All amplified cDNA fragments were run on a 1·5% agarose gel stained with ethidium bromide, visualized under u.v. transillumination, and analyzed with the

Table 2 Oligonucleotide sequences used for the amplification of RT-PCR and cycling conditions for the different sets of pairs

Gene	Sequence (5′–3′)	Cycle profile	Number of cycles
Gapdh	5′-ACC AC AGTCC ATGCC ATC AC-3′ 5′-TCCACCACCCTGTTGCTGTA-3′	94 °C/3 min, 94 °C/30 s, 58 °C/2 min, 72 °C/2min,	30 cycles
Ar	5′-GGAGAACTCTTC AGAGC AAG-3′ 5′-AGCTGAGTCATCCTGATCTG-3′	94 °C/2 min, 94 °C/1 min, 60 °C/1 min, 72 °C/1 min,	32 cycles
Erα (*Esr1*)	5′-GGCTACGTCAAGTCGATTCC-3′ 5′-ATCTTGTCCAGGACTCGGTG-3′	94 °C/1 min, 60 °C/50 s, 72 °C/2 min,	30 cycles
Erpβ (*Esr2*)	5′-GAGCTCAGCCTGTTCGACC-3′ 5′-GGCCTTCACACAGAGATACTCC-3′	94 °C/1 min, 60 °C/50 s, 72 °C/2 min,	30 cycles
Fshr	5′-CTCATCAAGCGACACCAAGA-3′ 5′-GGAAAGGATTGGCACAAGAA-3′	94 °C/2 min, 94 °C/1 min, 60 °C/50 s, 72 °C/2 min,	36 cycles
Lhcqr	5′-ATGGCCATCCTCATCTTCAC-3′ 5′-TGGATTGGCACAAGAATTGA-3′	94 °C/2 min, 94 °C/1 min, 60 °C/50 s, 72 °C/2 min	33 cycles

Scion Image software (http://www.meyerinst.com/html/scion/scion_image_windows.htm).

Steroid determinations

The estradiol and testosterone serum concentrations were determined using a specific RIA for each hormone (MP Biomedicals, Solon, OH, USA). The intra- and inter-assay variation coefficients were 4·6 and 7·5% for testosterone and 6·4 and 5·9% for estradiol respectively. Sensitivity of the RIA was 0·04 ng/ml for testosterone and 0·8 pg/ml for estradiol (Teixeira *et al*. 2007).

Statistical analysis

All results are means ± S.E.M. Statistical analysis was performed by Student's *t*-test. Values of $P<0·05$ were considered significant.

Results

Figure 1 shows food consumption, body weight, and linear growth of the groups throughout the experiment. Food consumption of the offspring was evaluated from weaning until killing. The PER group had lower food intake than the C group ($P<0·001$) from weaning until the end of experiment (Fig. 1A). Overall, at each time point of measurement from day 4 after birth until day 40, body weight in the PER group was significantly lower when compared with controls ($P<0·001$; Fig. 1B). Similarly, linear growth showed the same pattern, that is the PER group had significantly lower linear growth ($P<0·001$) when compared with controls from day 6 after birth until day 40 (Fig. 1C).

Figure 2 shows the number of ovarian follicles. The offspring whose dams were submitted to PER diets during lactation presented, at puberty, alteration in the number of ovarian follicles. The number of preantral ($C=13·72\pm2·87$; PER $=26·36\pm3·03$, $P<0·01$) and small antral follicles ($C=9·32\pm1·35$; PER $=17·64\pm2·33$, $P<0·01$) was significantly increased by maternal malnutrition, while the number of primordial follicles ($C=11·72\pm1·37$; PER $=3·92\pm0·60$, $P<0·01$), Graafian follicles ($C=1·84\pm0·21$; PER $=0·96\pm0·11$, $P<0·01$), and corpora lutea ($C=2·00\pm0·28$; PER $=0·80\pm0·31$, $P<0·01$) was significantly reduced. The number of primary follicles was increased, but this difference was not significant ($C=2·80\pm0·52$; PER $=4·84\pm0·90$, $P>0·05$).

Ovarian sections of offspring are shown in Fig. 3. The primordial follicle consists of an oocyte surrounded by a single layer of relatively undifferentiated granulosa cells (Fig. 3A). Primary follicles consist of an oocyte surrounded by a single layer of cuboidal granulosa cells (Fig. 3B). The preantral follicles present a central oocyte surrounded by several layers of granulosa cells and bounded by thecal cells, which form a fibrous theca externa and an inner theca interna with no antrum. In antral follicles, fluid appeared between the granulosa cells, and the drops coalesced to form follicular fluid within the follicular antrum (Fig. 3C). In Graafian follicles, the follicular antrum is clearly developed, leaving the oocyte surrounded by a distinct and denser layer of granulosa cells, the cumulus oophorus. The corpus luteum is formed by luteal cells and abundant capillaries (Fig. 3D).

The serum estradiol concentration was significantly increased ($C=67·86\pm4·39$; PER $=83·29\pm2·68$, $P<0·05$) in PER group while testosterone did not show statistical difference ($C=0·09\pm0·02$; PER $=0·11\pm0·01$, $P>0·05$; Fig. 4).

We performed semi-quantitative RT-PCR in order to determine whether the expression of *Ar*, *ERα*, *ERβ1*, *ERβ2*, *Fshr*, and *Lhcqr* genes in ovary are affected by protein-energy diet during lactation. Protein-energy maternal restricted diet led to a significant increase in the FSHR ($C=0·89\pm0·04$; PER $=1·07\pm0·03$, $P<0·05$), and the LHR ($C=0·87\pm0·15$; PER $=1·33\pm0·08$, $P<0·05$; Fig. 5). However, there was a decrease in the relative quantity of androgen receptor ($C=0·59\pm0·006$; PER $=0·13\pm0·080$, $P<0·05$), ESR1 ($C=3·33\pm0·71$; PER $=0·74\pm0·50$, $P<0·05$), ERβ1 ($C=1·33\pm0·06$; PER $=0·49\pm0·36$, $P<0·05$), and ERβ2 ($C=3·28\pm0·60$; PER $=0·62\pm0·34$, $P<0·05$) in the PER group (Fig. 6).

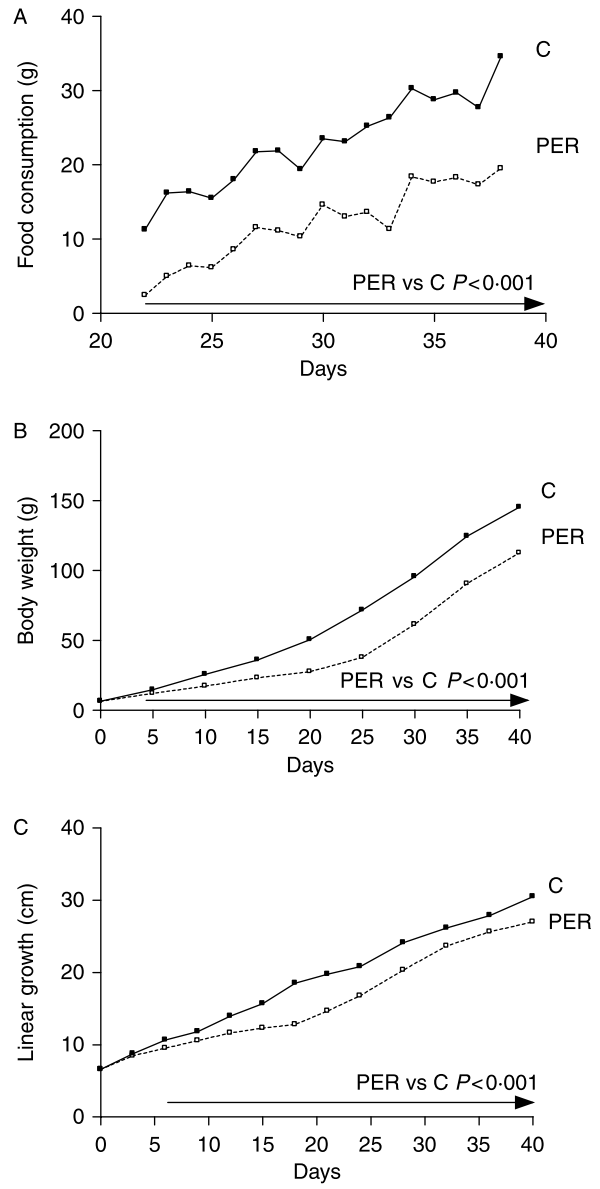

Figure 1 (A) Food consumption, (B) body weight, and (C) linear growth (nose–tail) in control (C) group and protein-energy-restricted (PER) group. Values are given as mean ± S.D. of 14 animals.

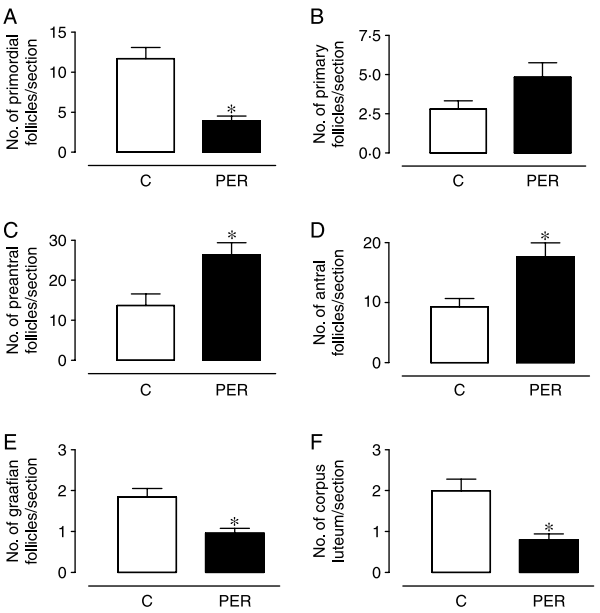

Figure 2 The number of (A) primordial follicles, (B) primary follicles, (C) preantral follicles, (D) small antral follicles, (E) Graafian follicles, and (F) corpus luteum in control (C) and protein-energy-restricted (PER) groups, at 40 days. The total number of sections analyzed was 15–20 per ovary. Values are given as mean ± S.E.M. of five animals. *$P<0·05$ compared with C.

The pattern of expression of the different ER isoforms was different between the C and PER groups. Considering ERβ as the sum of ERβ1 and ERβ2, the ERβ/ERα ratio was 1·3 in the C group and increased to 1·5 in the PER group. The ERβ2/ERβ1 ratio was 2·5 in the C group and decreased to 1·2 in the PER group.

Discussion

The present data show that maternal malnutrition during lactation leads to alterations in the ovarian expression of FSH, androgen and estrogens isoforms receptors. The follicular development is altered showing great number of primary, preantral, and small antral follicles and a reduced number of Graafian follicles, corpus luteum, and primordial follicles.

Some authors reported that intrauterine growth-retarded rats have a significantly lower number of follicles in the ovaries at vaginal opening whereas postnatal food-restricted rats have a normal number of follicles, but impaired follicular maturation (Engelbregt *et al.* 2000). Leonhardt *et al.* (2003) showed that maternal food restriction during lactation increased the number of antral follicles of small size whereas the number of antral follicles of large size (Graafian follicles) was reduced. In spite of using a different malnutrition experimental model, our data show that maternal malnutrition during lactation disturbed the follicular development, with an increment in the number of growing follicles and a reduction in the Graafian follicles number, suggesting an impaired follicular maturation since not all growing follicles reach the ovulatory stage.

FSH is, undoubtedly, the primary stimulus for the differentiation of follicles from the preantral to preovulatory stage. As the ovarian follicles grow and differentiate, increasing amounts of estrogen are produced (Fortune 1994), which, in turn, upregulate the synthesis and release of the pituitary gonadotropins, thereby promoting the upregulation of FSHR and consequent ovarian follicular growth that culminates in ovulation (Lapolt *et al.* 1992, Tano *et al.* 1999). Female mice null for FSH signaling due to the loss

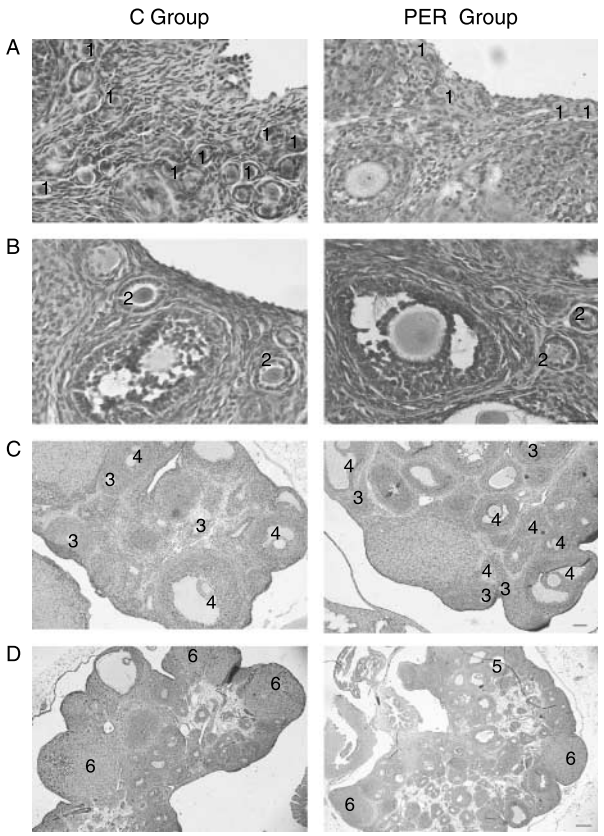

Figure 3 Photomicrographs showing ovaries from female rat pups at puberty in control (C) and protein-energy-restricted (PER) groups. (A) Primordial follicles (1); (B) primary follicles (2); (C) preantral follicles (3), antral follicles (4); (D) Graafian follicles (5), corpus luteum (6). Magnification: (A) ×400; (B) ×400; (C) ×100; (D) ×40 (bar 100 μm).

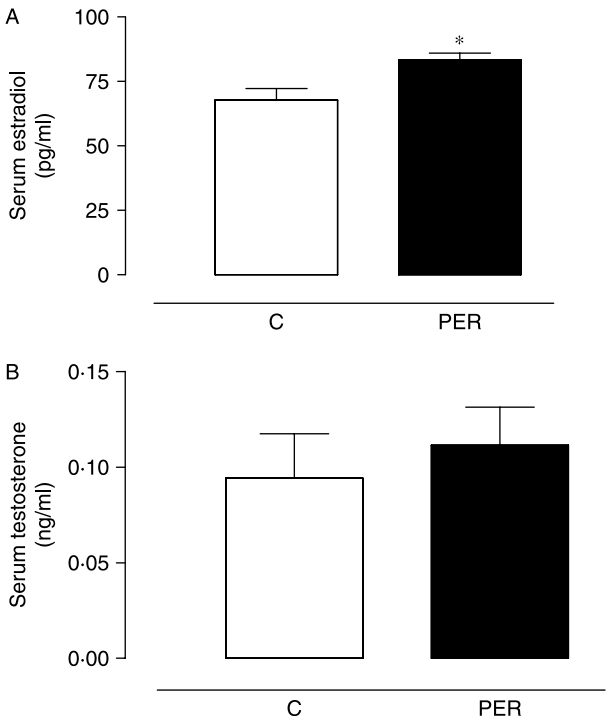

Figure 4 (A) Serum estradiol concentration and (B) serum testosterone concentrations in control (C) and protein-energy-restricted (PER) groups, at 40 days. Values are given as means ± S.E.M. of seven animals per group. *$P<0{\cdot}05$ compared with C.

of hormone (Kumar *et al.* 1997, Burns *et al.* 2001) or receptor (Dierich *et al.* 1998, Danilovich *et al.* 2001) exhibit follicles that fail to differentiate beyond the preantral stage and produce minimal estradiol and hence are infertile. However, FSH alone does not provide for complete follicle and granulosa cell differentiation. Instead, the synergistic actions of estradiol are required to maximize FSH induction of antrum formation (Hirshfield 1991, Emmen *et al.* 2005) and LH responsiveness (Knecht *et al.* 1984, 1985*a*,*b*). Also, estrogen seems to directly increase the number and size of ovarian follicles (Goldenberg *et al.* 1972, Nakayama *et al.* 1981, Nakano *et al.* 1982, Gore-Langton & Daniel 1990, Nayudu & Osborn 1992, Hulshof *et al.* 1995), regulate corpus luteum formation, and control luteal maintenance (Krege *et al.* 1998, Britt *et al.* 2000, Dupont *et al.* 2000, Rosenfeld *et al.* 2000). So, the increment observed in the number of primary, preantral, and small antral follicles in the offspring at puberty following maternal malnutrition during lactation could be consequent to the increase in the ovary FSHR expression and estradiol serum concentration presented by these animals.

In this paper, we confirm previous studies which show that there is a predominance of ERβ over ERα expression in the ovary (Byers *et al.* 1997, Drummond *et al.* 1999). We also show that the ERβ/ERα ratio is even higher after maternal malnutrition during lactation, probably due to the increase observed in the number of growing follicles in this group, since previous results show that ERβ increases in synergy with the proliferation of granulosa cells (Drummond *et al.* 1999).

A number of variant forms of ERβ have been identified with different patterns of expression (Chu & Fuller 1997). According to our data, Drummond *et al.* (1999) showed that ovary of immature rats expresses more ERβ2 than ERβ1 isoform, and also that the ERβ2/ERβ1 ratio increases with age. Maternal malnutrition during lactation caused a reduction in the ERβ2/ERβ1 ratio. This could be explained by the fact that these animals present growth retardation and delay in the onset of puberty (Faria *et al.* 2004), suggesting that they are more immature than the control group.

We have no previous knowledge about how maternal malnutrition influences the expression of ER isoforms or how the ER isoforms expression alteration showed here could affect the ovarian function. However, we can hypothesize that the follicular growth alteration in the malnourished group can be related to the change of balance among ERα, ERβ1, and ERβ2 expressions since the ERβ isoforms can differentially modulate estrogen action (Peng *et al.* 2003).

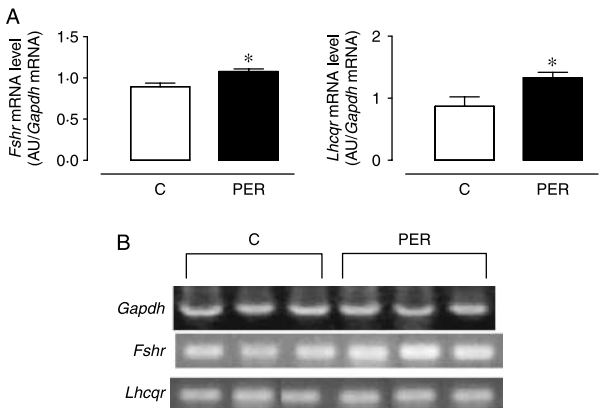

Figure 5 Expression of *Fshr* and *Lhcqr* genes in ovaries of control (C) and protein-energy-restricted (PER) groups. After RT-PCR reactions, the amplified fragments were run on a 1·5% agarose gel and visualized by u.v. transillumination. (A) Graphical representation of the data. (B) A representative ethidium bromide-stained gel depicting products for the expression of *Gapdh, Fshr,* and *Lhcqr* genes in ovaries. The ratios between the signal intensities (arbitrary units) of *Fshr* and *Lhcqr* are represented as means±S.E.M. of three animals. *$P<0·05$ compared with C.

Despite normal testosterone and high estradiol serum concentrations, the low ERα, ERβ, and AR expressions could have contributed to the alterations in the antrum and corpus luteum formation leading to a reduction in the number of Graafian follicles and corpus luteum. It has been shown that AR and ERβ double knockout mice do not undergo luteinization and corpus luteum formation (Fisher *et al.* 1998, Couse *et al.* 1999, Britt *et al.* 2000, Dupont *et al.* 2000). Also, since some signaling events that are critical for the final follicle growth and eventual ovulation are altered by perinatal malnutrition, like leptin (Teixeira *et al.* 2002), insulin (Moura *et al.* 2002) etc., we can suggest that a high LHR expression was not capable of stimulating the final maturation of Graafian follicles without the presence of those signaling factors.

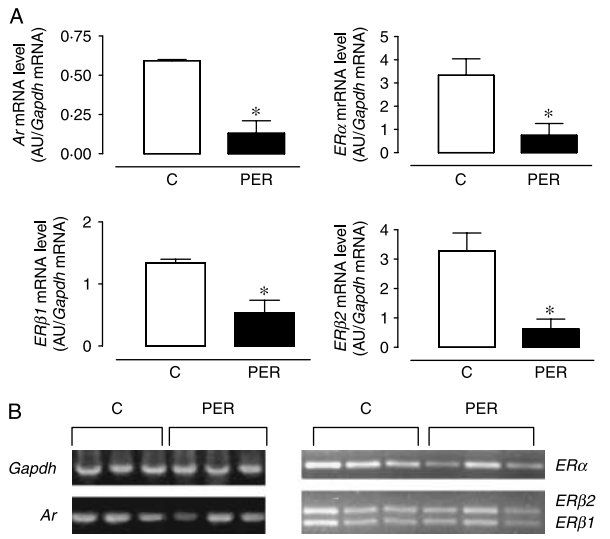

Figure 6 Expression of *Ar, ERα, ERβ1,* and *ERβ2* genes in ovaries of control (C) and protein-energy-restricted (PER) groups. After RT-PCR reactions, the amplified fragments were run on a 2% agarose gel and visualized by u.v. transillumination. (A) Graphically representation of the data. (B) A representative ethidium bromide-stained gel depicting products for the expression of *Gapdh, Ar, ERα, ERβ1,* and *ERβ2* genes in ovaries. The ratios between the signal intensities (arbitrary units) of *Ar, ERα, ERβ1,* and *ERβ2* are represented as means±S.E.M. of three animals. *$P<0·05$ compared with C.

Primordial follicles are the stock from which all growing follicles are derived. Some hypothesis could explain the reduced number in the primordial follicles observed in the offspring whose mothers were submitted to malnutrition during lactation. First of all, in rodents, the primordial follicles are formed by day 3 of age, and the first wave of follicles develop into antral follicles over the next 3 weeks (Rajah *et al.* 1992, Gelety & Magoffin 1997, McGee *et al.* 1997). We have published recently that, in this period, these animals show significant alterations in their body weight and thyroid function as well as in the milk composition (Passos *et al.* 2000, 2001, Ramos *et al.* 2000). Thus, it is possible that the decrease observed in the number of primordial follicles could result from a direct action of malnutrition in the ovary of the pups in the first days of life when primordial follicles are being formed. Secondly, the increase in the primary, preantral, and antral follicles observed in these animals could result in a gradual decrease in the original follicle pool, which is stimulated to enter the growth phase.

Recently, Guzman *et al.* (2006), using a similar model of malnutrition during lactation, have shown that 1-year-old rats presented an increase in the estrous cycle length and reduced fertility rate. Our data of reduced primordial follicle number could explain this fact, suggesting that the reduction of follicular reserve that serves as a ticking clock to the onset of senescence could be responsible for the decrease in the fertility rate.

In conclusion, perinatal maternal malnutrition can directly affect folliculogenesis at puberty probably as a consequence of changes in the ovarian expression of gonadotropins, androgen and estrogen isoform receptors. Long-term sexual alterations could be expected in this experimental model, since a reduction in the primordial follicle number is observed, which can result in a decrease in reproductive lifetime and an earlier termination of breeding capacity.

Declaration of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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