Metabolic programming of ovarian angiogenesis and folliculogenesis by maternal malnutrition during lactation

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Objective: To evaluate whether maternal malnutrition during lactation programs ovarian folliculogenesis and the expression of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) and its receptors KDR, Flt-1, and FGFR.
Design: Experimental study.
Setting: University-based research laboratory.
Animal(s): Adult female rats from a urogenital research laboratory.
Intervention(s): Six rat dams randomly assigned to the following groups: control group (C), with free access to a standard laboratory diet containing 23% protein; and a protein-energy-restricted group (PER), with free access to an isoenergy and protein-restricted diet containing 8% protein. After weaning, the female pups had free access to the standard laboratory diet until 90 days of age, when they were sacrificed at the proestrum stage.
Main Outcome Measure(s): Quantification of ovarian follicles, vessels, and expression of growth factors and their receptors.
Result(s): Maternal malnutrition during lactation caused a significant reduction in the number of primordial (C = 6.60 ± 0.24, PER = 5.20 ± 0.20), primary (C = 5.80 ± 0.66, PER = 4.00 ± 0.31), and Graafian follicles/section (C = 2.18 ± 0.29, PER = 1.08 ± 0.37), in KDR (C = 0.22 ± 0.04, PER = 0.09 ± 0.01), Flt-1 (C = 0.28 ± 0.05, PER = 0.12 ± 0.02), and FGFR mRNA expression (C = 0.34 ± 0.05, PER = 0.13 ± 0.05) and in the vessel density of follicles (C = 17.26 ± 2.30, PER = 9.96 ± 0.97).
Conclusion(s): Maternal malnutrition during lactation programs the follicular development by a reduction of VEGF and FGF mRNA receptors expression, probably from a direct action on the follicular development or a reduction in vasculature resulting in a decreased delivery of folliculotrophic substances in PER animals. (Fertil Steril® 2009;■:■–■. ©2009 by American Society for Reproductive Medicine.)

Key Words: Folliculogenesis, vascular endothelial growth factor, basic fibroblast growth factor, lactation, malnutrition, rat, ovary, angiogenesis

The organs of the female reproductive system undergo cyclic changes that are associated with intense growth of new blood vessels (1), which is very important for follicular development, corpus luteum formation, and uterine endometrial proliferation during the menstrual cycle (2, 3). Among the many endothelial regulators, vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) have been characterized as potent promoters of angiogenesis (3–5).

The human VEGF gene is organized into eight exons, and differential alternative splicing results in the synthesis of multiple VEGF isoforms of 121, 145, 165, 189, and 206 amino acids (VEGF121, VEGF145, VEGF165, VEGF189, and VEGF206, respectively) (6); the corresponding murine forms are shorter by one amino acid (7). VEGF120 and VEGF164 are expressed in the mammalian ovary (8, 9) and are associated with follicular angiogenesis during follicular development (10, 11). VEGF immunoreactivity in granulosa cells is weak or absent from the primordial to multilayered stages of primary follicles but increases gradually when follicles differentiate into secondary and then into antral follicles (12, 13). VEGF expression in granulosa cells is associated with thecal vasculature growth during ovarian follicular development (14–16).

The FGF makes up a large family of 23 related polypeptides (17, 18). Basic FGF, also called FGF-2, is a 146–amino acid polypeptide that is restricted to the oocytes of primordial and primary follicles of many species, including rats (19), and also restricted to the granulosa cells of preantral and antral follicles (20, 21). Basic FGF is important in regulating a wide range of ovarian functions including granulosa cell mitosis (22–25), differentiation (26), steroidogenesis (27), apoptosis (28), and initialization of follicular development (29).

Both FGFs and VEGFs act through their specific cell surface tyrosine kinase receptors. The best-characterized VEGF receptors are VEGFR1 or Flt-1 (Fms-like tyrosine kinase-1) and VEGFR2 or Flk-1 (fetal liver kinase)/kinase-insert domain receptor (KDR) (6). Both receptors are located in the endothelial cells of the theca of late secondary follicles and increase in the theca of tertiary follicles and decrease in atretic follicles (30). FGFs interact with a family of four distinct receptors, designated FGFR-1 to -4 (31). FGFR1 is highly expressed in small microvessels at all stages and in large microvessels, especially in the late luteal stage of follicular development (32).

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The VEGF synthesis is stimulated by FSH and LH in the granulosa cells (33–35). The expression of the VEGF164 gene and its receptor Flk-1 is stimulated by E_2 and FSH in bovine granulosa cells, while VEGF expression is inhibited by P in a dose-dependent way, suggesting a hormone-dependent expression pattern of VEGF isoforms during follicular development (36, 37). In humans, VEGF and its receptors are also under gonadotropin control (38). FSH also stimulates the expression of FGF-2 receptors in granulosa cells (19).

Adverse metabolic conditions, such as malnutrition, obesity, anorexia nervosa, or intense exercise, are known to be associated with reduced or abolished reproductive function (39, 40). The concept of metabolic programming is a permanent change related to a particular function as a result of some event that occurs during the perinatal period (41). An early food restriction can change the original program of organs, especially those in developmental phases, which can result in long-term changes in metabolism (42, 43). Malnutrition in early life is associated with alterations in adulthood, such as type 2 diabetes, hypertension, and cardiovascular disease (41, 44–48), follicular growth and ovulation rate (49–51), uterus morphology (52), and fertility (53).

To better understand the follicular development in malnourished animals, it would be valuable to investigate the mRNA expression of both VEGF and FGF, their receptors, and their relationship with E_2 serum levels. Furthermore, the effect of malnutrition during lactation and the possible metabolic programming on these genes has not yet been studied. We hypothesized that malnutrition during lactation would cause an ovarian metabolic programming, leading to a permanent reduction in the mRNA expression of both angiogenic factors and their receptors that are important to a normal follicular development.

MATERIALS AND METHODS

Animals

The handling of animals was approved by the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro, which based their analysis on the *Guide for the Care and Use of Laboratory Animals* (54). The study design was approved by the local Ethics Committee for the care and use of laboratory animals.

We used Wistar rats that were kept in a room with controlled temperature (25 ± 1C) and an artificial dark-light cycle (lights on from 7:00 to 19:00 hours). Virgin female rats aged 3 months were caged with one male rat 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.

Experimental Design

After delivery, six pregnant Wistar rats were separated into two groups: the control group (C), with free access to a standard laboratory diet containing (in grams per 100 g) 23 protein, 66 carbohydrate, 11 fat, 17,038.7 total energy (kJ/kg); and a protein-energy-restricted group (PER), 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 (55). The protein-restricted diet was prepared at our laboratory by using the control diet (Nuvilab-Nuvital Ltd., Paraná, Brazil), with the 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. Vitamins and mineral mixtures were formulated to meet the American Institute of Nutrition AIN-93G recommendation for rodent diets (56). Within 24 hours of birth, excess pups were removed so that only six pups were kept per dam because it has been shown that this procedure maximizes lactation performance (57). Malnutrition of the studied rats 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 90 days of age. Then only the animals at the proestrum stage were sacrificed with a lethal dose of pentobarbital.

To evaluate the nutritional state, food consumption of the offspring was monitored each day from weaning onward, while body weight and linear growth (nose-tail) were monitored each 5 days from birth until the experiment end. The blood was collected by cardiac puncture, and the serum was kept at −20C for subsequent hormonal assessment. Ovaries were excised, dissected, and weighted. One ovary was kept at −80C for subsequent measurements of basic fibroblast growth factor (bFGF), VEGF, FGFR, Flt-1, and KDR transcripts by reverse transcriptase–polymerase chain reaction (RT-PCR). The other ovary was paraffin embedded, sectioned at 5-μm thickness, and processed by routine histological analyses. Some samples were stained with hematoxylin-eosin to confirm tissue integrity.

Morphologic Classification of Follicles

Paraffin sections of 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-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 ×400 for primordial and primary follicles and of ×100 for preantral, antral, and Graafian follicles as well as corpus luteum. Photographs of ovarian follicles were analyzed using Image Pro Plus for Windows (version 1.3.2; Media Cybernetics, Bethesda, MD) (51). 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, and antral follicles were distinguished by the presence of an antrum within the granulosa cell layers enclosing the oocyte (58). 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 postantral follicles, were counted in the same way as the follicles in the antral class. In addition, the vascular density of follicles was assessed. All vessels in the sections of the whole follicular theca interna were counted under ×400 magnification.

RNA Extractions

Total RNA from ovary tissue was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA) 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 determination of the ratio 260 nm/280 nm and by electrophoresis on a 1% agarose gel. The samples were stored at −80C until use.

Semiquantitative RT-PCR

All RNA samples were rid of contaminating DNA by using DNAse-free reagents (Invitrogen) according to the manufacturer's protocol. Then 1 μg of RNA sample was used in a 20-μL cDNA reaction using oligo-dT and the superscript III 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). To quantify VEGF, bFGF, FGFR, Flt-1, and KDR transcripts, we determined the optimal number of amplification cycles for each gene (Fig. 1).

The applied PCR primers used are the following: bFGF (sense: 5′-gaaccggtacctggctatga-3′; antisense: 5′-ccgttttggatccgagttta-3′), VEGF (sense: 5′-gcccatgaagtggtgaagtt-3′; antisense: 5′-actccagggcttcatcatt-3′), FGFR (sense: 5′-ctctgtggtgccttctgaaca-3′; antisense: 5′-ttcacctcgatgtcttcag-3′), KDR (sense: 5′-ccaagctcagcacacaaaaa-3′; antisense: 5′-ccaaccactctgggaactgt-3′), and Flt-1(sense: 5′-tttatcagcgtgaagcatcg-3′; antisense: 5′-ccgaatagcgagcagatttc-3′). Thermocycling conditions were the same for all genes (2 minutes

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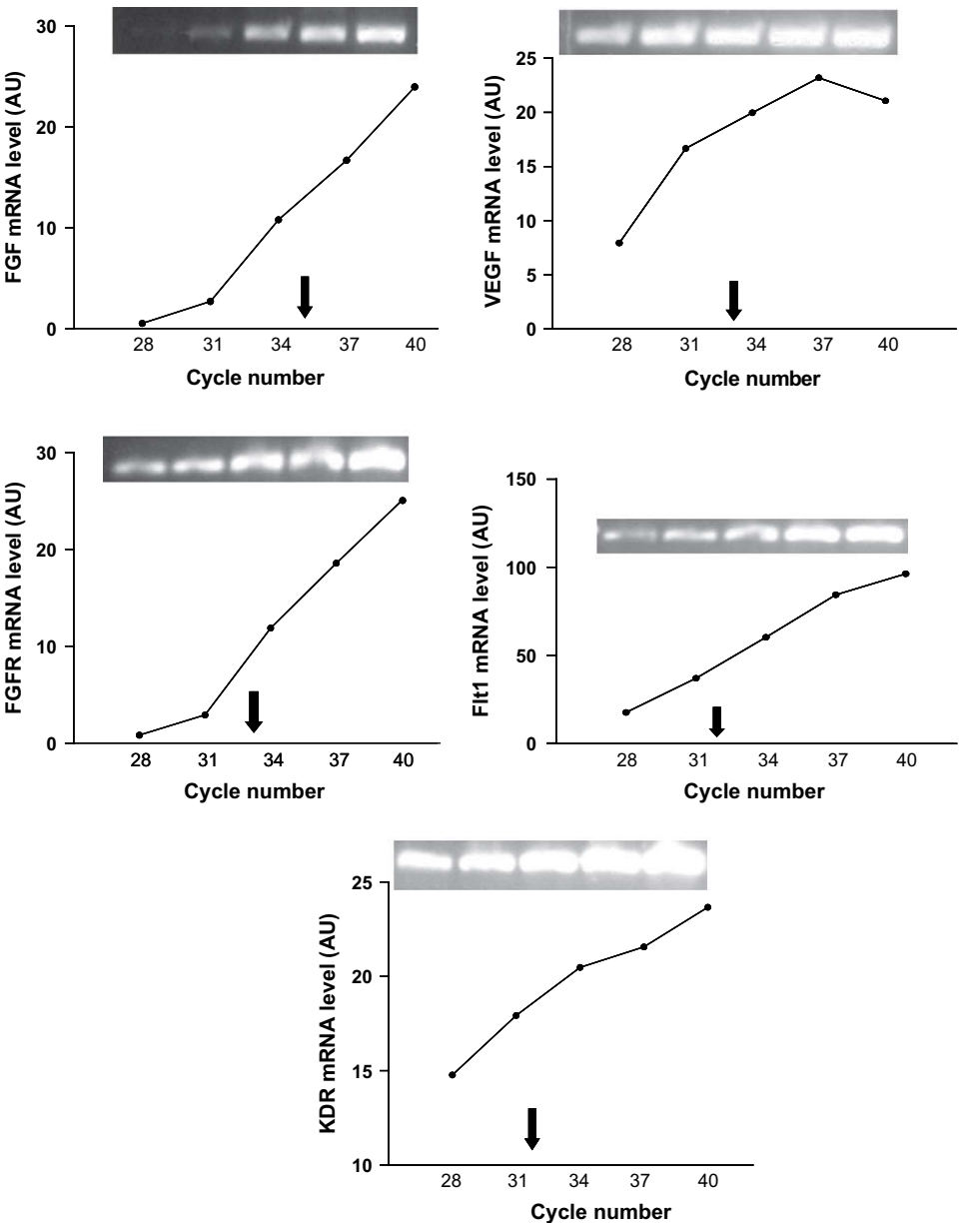
FIGURE 1

Optimization of RT-PCR conditions for semiquantitative determination of target mRNAs. For amplification in the exponential phase of PCR, different numbers of cycles were tested for each message. Quantitative analysis of the cycle dependency for the generated PCR signals revealed a strong linear relationship among cycles 28–40 in the case of FGF (correlation coefficient = 0.9774), FGFR (correlation coefficient = 0.9763), VEGF (correlation coefficient = 0.7511), Flt-1 (correlation coefficient = 0.9902), and KDR (correlation coefficient = 0.9720). The arrow indicates that the cycle number was chosen for each gene.



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of initial denaturation at 94C, 1 minutes of additional denaturation at 94C, 1 minute of annealing at 55C, 1 minute of extension at 72C). The number of cycles for each gene is shown in Figure 1. All amplified cDNA fragments were run on a 1.5% agarose gel stained with ethidium bromide visualized under UV transillumination and analyzed with the Image J software. In addition, to provide an appropriate internal control, coamplification of a 450-bp fragment of the GAPDH mRNA was carried out in each sample using the primer pair (sense: 5′-accacagtccatgccatcac-3′; antisense: 5′-tccaccacctgttgctgta-3′) at the thermocycling conditions of 3 minutes of denaturation at 94C, 30 seconds of additional denaturation at 94C, 2 minutes of annealing at 58C, and 2 minutes of extension at 72C.

Steroid Determinations

The E_2 and T serum concentrations were determined by a specific radioimmunoassay for each hormone (ICN Pharmaceuticals, Inc., Costa Mesa,

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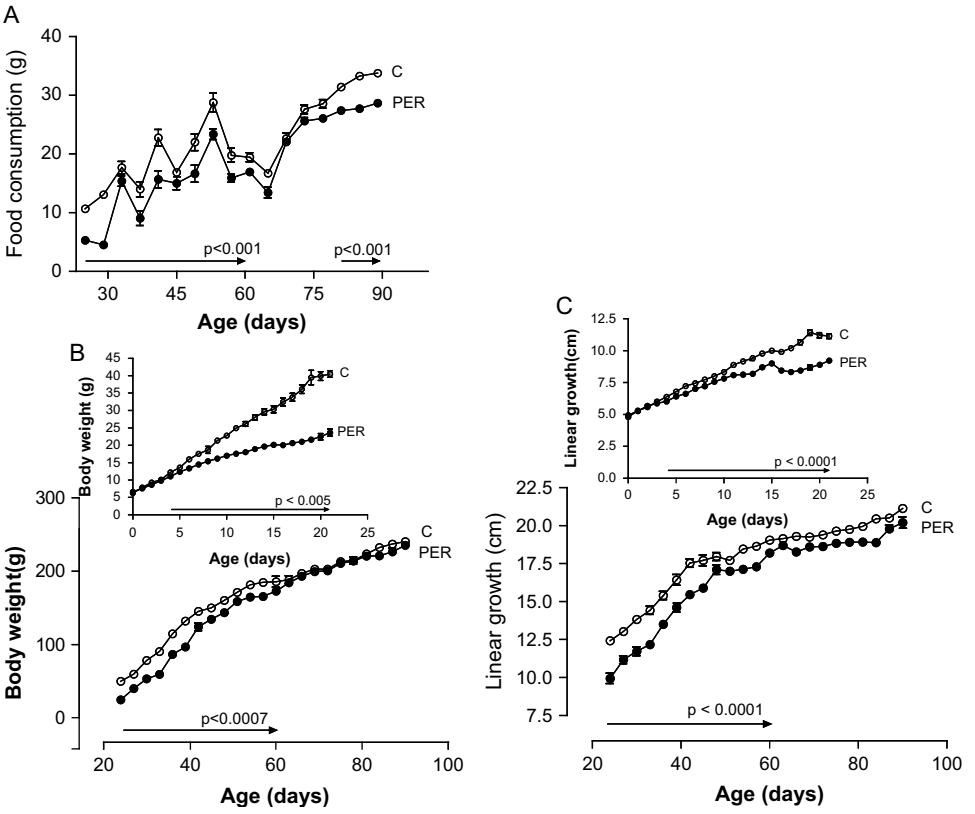
FIGURE 2

Food consumption (panel A), body weight (panel B), and linear growth (panel C) in the control (C) and protein-energy restricted (PER) groups. Values are given as mean ± SEM of 14 animals per group.



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CA). The intra- and interassay variation coefficients were 4.6% and 7.5% for T and 6.4% and 5.9% for E_2. The sensitivity of the radioimmunoassay was 0.04 ng/mL for T and 0.8 pg/mL for E_2 (59).

Statistical Analysis

All results are mean ± SEM. Statistical analysis was performed by Student's *t*-test. $P<.05$ was considered statistically significant.

RESULTS

Figure 2 shows the food consumption, body weight, and linear growth of the C and PER. Compared with the C, the PER had a significant decrease in food consumption ($P<.001$) from weaning to day 60, with an additional reduction after day 80 to the end of the experiment (Fig. 2A). There was a significant decrease in the PER body weight from day 4 to day 60 ($P<.005$; Fig. 2B). The PER group had also a significant decrease in linear growth from day 4 to day 60 ($P<.0001$; Fig. 2C).

The offspring whose dams were submitted to protein-energy-restricted diets during lactation presented a reduction in the number of all ovarian follicles per section: primordial (C = 6.60 ± 0.24, PER = 5.20 ± 0.20; $P<.01$), primary (C = 5.80 ± 0.66, PER = 4.00 ± 0.31; $P<.04$), preantral (C = 3.00 ± 0.44, PER = 2.08 ± 0.48), antral (C = 4.74 ± 0.72, PER = 3.40 ± 0.77), Graafian (C = 2.18 ± 0.29, PER = 1.08 ± 0.37; $P<.05$), and corpus luteum (C = 3.98 ± 0.65, PER = 3.36 ± 0.27).

The offspring whose dams were submitted to protein-energy-restricted diets during lactation presented a reduction in the vessel density of follicles (C = 17.26 ± 2.30, PER = 9.96 ± 0.97; $P<.01$).

Ovarian sections of offspring are shown in Figures 3 and 4. 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. 3A). 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. 3B). 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. 3C).

Figure 4 represents the vessels, considering arterial, capillary, or venous counted inside the theca layer of preantral, antral, and Graafian follicles.

The T (ng/mL) serum concentration was not detected in either group. E_2 (pg/mL) serum concentration did not show statistical difference between the C and PER (C = 125.4 ± 20.4, PER = 116.6 ± 16.2).

A protein-energy maternal restricted diet did not change the mRNA bFGF (C = 0.34 ± 0.03, PER = 0.36 ± 0.04) and VEGF

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FIGURE 3

Photomicrographs showing ovaries from female rats in the control (C) and protein-energy restricted (PER) groups. (A) Primordial follicles [1]; primary follicles [2]. (B) Preantral follicles [3]; antral follicles [4]. (C) Graafian follicles [5]; corpus luteum [6]. Magnification, A = ×400; B = ×100; C = ×40.

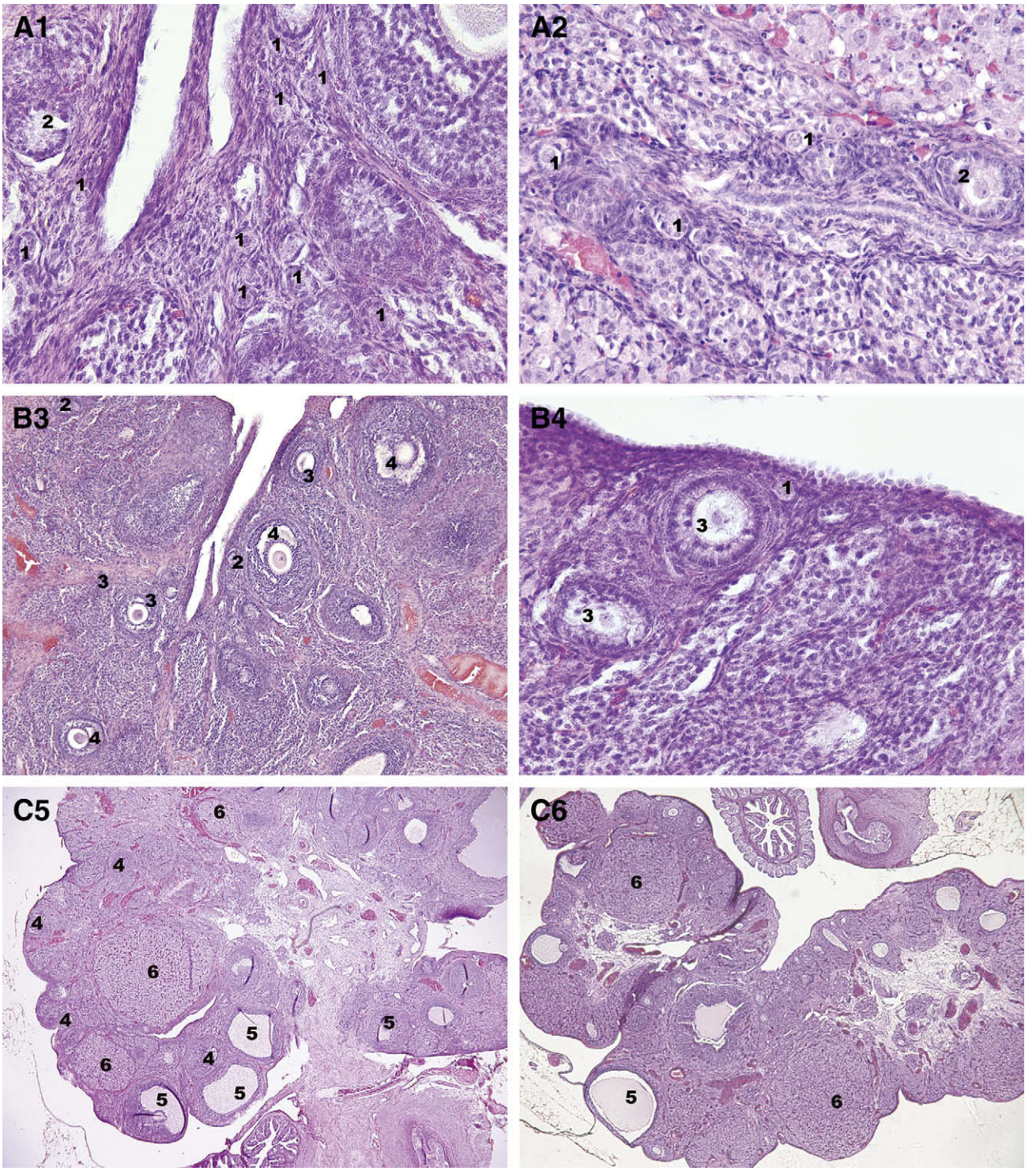

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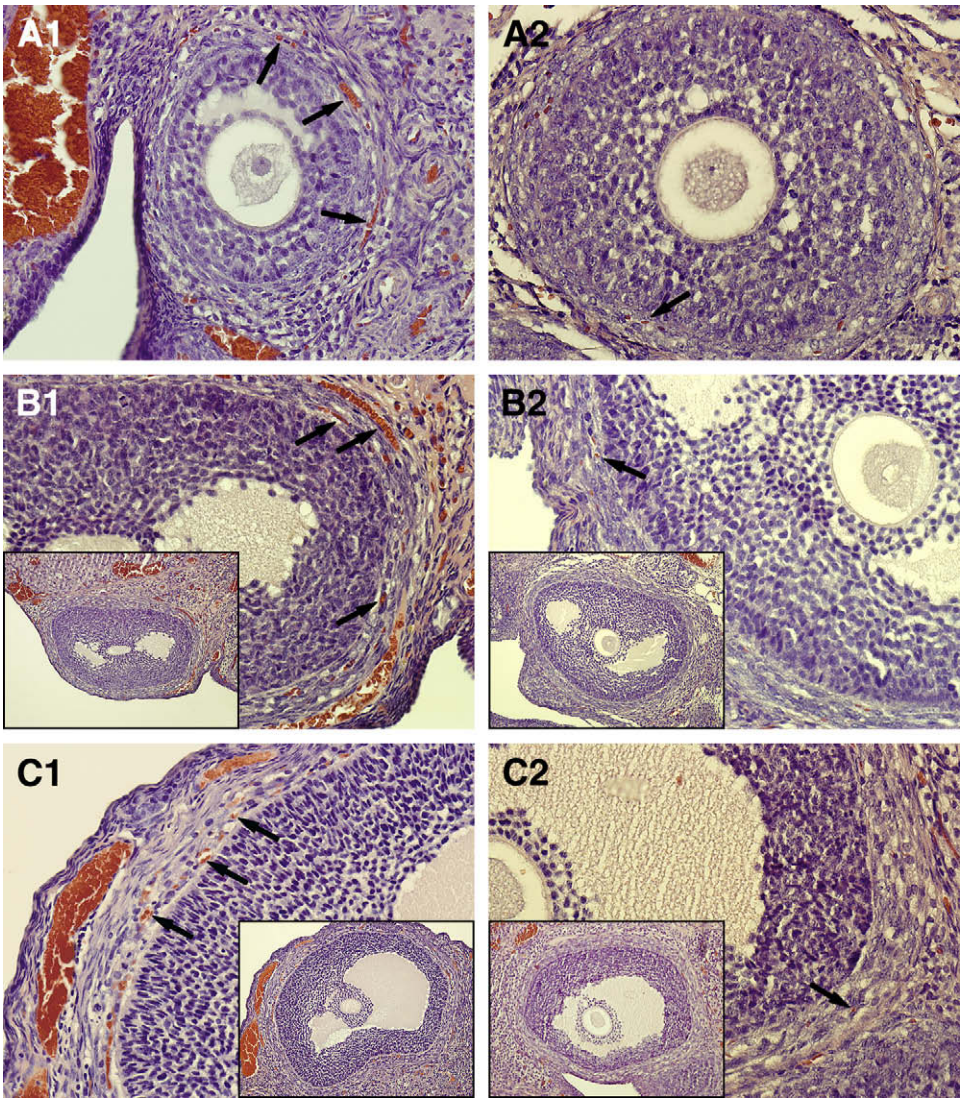
FIGURE 4

Photomicrographs showing ovaries from female rats in the control (C) and protein-energy restricted (PER) groups. (A) Preantral follicles. (B) Antral follicles. (C) Graafian follicles. The arrow indicates vessels counted in the theca layer of follicles. The image magnification is ×400. The inset magnification is ×200.



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(C = 0.81 ± 0.04, PER = 0.79 ± 0.11) expression in ovaries. However, there was a significant decrease in the mRNA FGFR (C = 0.34 ± 0.05, PER = 0.13 ± 0.05; $P<.03$), Flt-1 (C = 0.28 ± 0.05, PER = 0.12 ± 0.02; $P<.05$), and KDR (C = 0.22 ± 0.04, PER = 0.09 ± 0.01; $P<.04$) expression in ovaries (Fig. 5).

DISCUSSION

Epidemiological data and studies in animals have focused on the concept of metabolic programming, which specifies that the quantity and quality of nutrition in the perinatal period generate consequences in adulthood (45, 51, 53, 55, 60). Restricting food during the perinatal period is associated with a reduction in growth rate. The present results of low body weight and linear growth are in agreement with the literature (49–53, 61–63). It seems that changes in body weight and linear growth are associated with food intake, since the reduction in food intake was accompanied by a reduction in body weight and linear growth up to 60 days of age. After this period, there was a normalization of consumption, body weight, and growth. However, at about 80 days, there was an additional reduction in food consumption, which was not accompanied by changes in body weight or linear growth. The evaluation of these parameters for a longer period is needed to confirm whether this last change in food consumption would be accompanied by changes in body weight and linear growth. It was recently demonstrated that male rats, whose mothers were submitted to the same experimental protocol, showed a reduction in body weight up to 180 days of age, despite the fact that food consumption had normalized around day 50 (60).

Follicular development with adequate maturation of primordial follicles to the mature stage of the Graafian follicle is essential for

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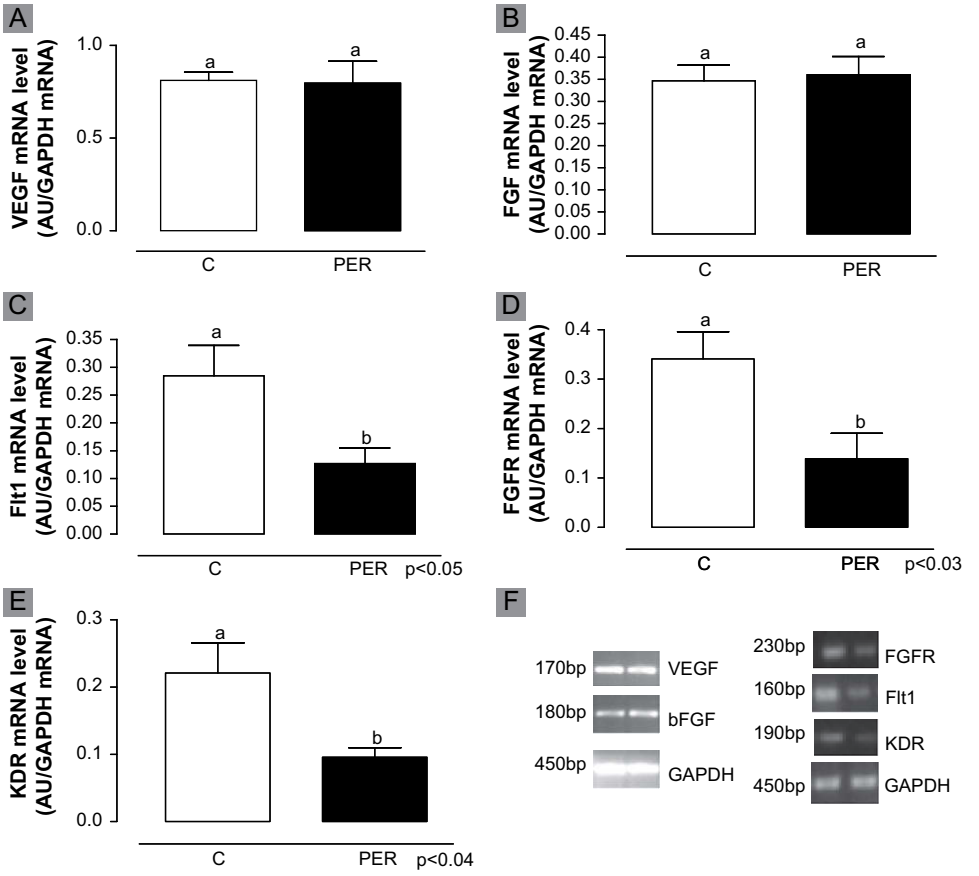
FIGURE 5

Expression of VEGF (A), bFGF (B), Flt-1 (C), FGFR (D), and KDR (E) genes in ovaries of the 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 UV transillumination. Panel F shows a representative ethidium bromide–stained gel depicting products for expression of VEGF, bFGF, FGFR, Flt-1, KDR, and GAPDH genes in ovaries. The ratios between the signal intensities (arbitrary units) of VEGF, bFGF, FGFR, Flt-1, and KDR are represented as means ± SEM of five animals per group. Different letters mean statistical significance.



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releasing the oocyte and maintenance of female fertility. In rodents, the primordial follicles are formed by day 3 of age, and the first wave of follicles develops into antral follicles over the next 3 weeks (64–66). In agreement with the literature (55, 67, 68), our study showed that during this period, the PER group presents significant alterations in body weight. In addition, thyroid function and milk composition are altered at this time (55, 67, 68). Thus, it is possible that the decrease observed in primordial follicles number could result from a direct action of malnutrition in the ovaries of the pups in the first days of life when primordial follicles are being formed. A reduction in the developing follicles number is also shown after maternal malnutrition during lactation, suggesting that a developmental effect exists.

Angiogenesis plays an important role in follicular development. The capillary network in dominant follicles is both more extensive and more permeable than that in other follicles (69), and such follicles are able to acquire an increased uptake of serum gonadotropins, a variety of hormones, and growth factors (70). The capillary network limited to the thecal cell layer during follicular development is stimulated by angiogenic factors (71). VEGF and FGF have been characterized as potent promoters of angiogenesis (3–5).

VEGF may also have direct mitogenic effects on granulosa cells in vitro and could directly stimulate follicle growth in the rat ovary (72). Evidence has been reported that bFGF is able to induce primordial follicles to initiate development (29). Therefore, we could hypothesize that any alteration of the angiogenic factors receptors expression could lead to a reduction in the follicular growth and/or maturation process.

Several investigators have demonstrated a primary role of VEGF in corpus luteum angiogenesis by neutralizing VEGF activity (73–75). Despite the production of other angiogenic factors, neutralization of VEGF activity prevented normal development and function of the corpus luteum. Neutralization of VEGF activity with neutralizing antibodies (76) or a soluble form of the VEGF receptor (30) disrupts follicle growth and granulosa cell proliferation in monkeys.

All evidence showing VEGF and FGF to be responsible for angiogenesis and follicular growth suggests that the reduction of VEGF and FGF mRNA receptors expression after maternal malnutrition during lactation could be responsible, at least in part, for the reduction in follicle growth. This effect may be the consequence of the direct action of both factors in the follicular development. A reduction in

the vascularity could also be responsible for the reduction observed in the follicular development, probably by decreasing the delivery of folliculotrophic substances. Despite the presence of VEGF and FGF from primordial to antral follicles (12, 13, 19–21), their receptors are expressed only in the theca cells of late secondary follicles (30). Therefore, the reduction in the VEGF and FGF receptors expression after maternal malnutrition could be related to the growing follicles and not to primordial follicles. The absence of theca cells in the primordial follicles reinforces the hypothesis of a direct action of malnutrition in the first days of life when the primordial follicles are being formed.

It has been shown that both the growth factors VEGF and FGF and their receptors are regulated by gonadotropins (33, 34) and E_2 (35, 77, 78). Also, it seems that the expression of Flk-1/KDR mRNA is significantly increased in the VEGF-injected ovaries when compared with those of the saline groups (16). The unchanged E_2 serum levels could explain the normal expression of both VEGF and bFGF, but it is possible that it is not related to the reduction of growth factors receptors expression. Thus, the normal expression of both VEGF and bFGF factors, besides the reduction of the VEGF and FGF receptors expression, raises the possibility that some regulatory step is missed in the ovary after maternal malnutrition during lactation.

Our results suggest that maternal malnutrition during the lactation program affects follicular development, which is probably related to a reduction of VEGF and FGF mRNA receptors expression. This effect could be the consequence of a direct action of both factors in follicular development or it could be due to a vasculature reduction, resulting in a decreased delivery of folliculotrophic substances in the PER animals.

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1 **Metabolic programming of ovarian angiogenesis and folliculogenesis by maternal malnutrition during lactation**

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Maternal malnutrition during lactation programs follicular development by a reduction in vasculature consequent to reduced mRNA expression of vascular endothelial growth factor and fibroblast growth factor mRNA receptors.

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