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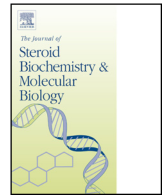

Maternal protein restriction during gestation and lactation programs offspring ovarian steroidogenesis and folliculogenesis in the prepubertal gilts

Shiyan Sui, Bin He, Yimin Jia, Runsheng Li, Demin Cai, Xi Li, Haogang Song, Longfei Jia, Ruqian Zhao *

Key Laboratory of Animal Physiology and Biochemistry, Ministry of Agriculture, Nanjing Agricultural University, Nanjing 210095, China

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ABSTRACT

Maternal malnutrition may disrupt ovarian functions in adult offspring. Steroidogenesis and folliculogenesis in the offspring ovary appear to be the major targets of nutritional programming. Nevertheless, the mechanism by which maternal low-protein diet affects the offspring steroidogenesis and folliculogenesis, and the possible pathway linking these two processes remain unclear. In this study, Landrace × Yorkshire crossbred sows were fed either standard (SP) or low-protein (LP, 50% of the SP) diets throughout gestation and lactation. Female offspring were fed the same diet after weaning until 6 months of age. LP offspring had higher serum 17β-estradiol level ($P < 0.01$), which was accompanied by lower mRNA ($P < 0.05$) but higher protein ($P < 0.05$) expression of cytochrome P450 aromatase (CYP19A1) in the ovary. CYP19A1 protein up-regulation was associated with lower ovarian expression of drosha ($P < 0.05$) and miRNAs targeting CYP19A1 ($P < 0.05$). LP offspring had less graafian follicles with more apoptotic granulosa cells ($P < 0.05$), as well as higher caspase 3 activity ($P < 0.05$) and FasL expression ($P < 0.05$) in the ovary. *FasL* gene up-regulation was associated with higher ER_α protein expression ($P < 0.05$) and binding to *FasL* gene promoter. These results suggest that a maternal LP diet in pregnancy and lactation elevated serum 17β-estradiol level by activating CYP19A1 through miRNA-mediated mechanism, and induced granulosa apoptosis in graafian follicles through ER-activated Fas/FasL-caspase 3 pathway.

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1. Introduction

Perinatal malnutrition programs the reproductive function in adult life [1]. It has been reported that maternal undernutrition during pregnancy and/or lactation altered serum concentrations of sex steroid hormones [2,3] and the number of ovarian follicles [4,5] in adult rat offspring. Therefore, steroidogenesis and folliculogenesis in the offspring ovary appear to be the major targets of maternal nutritional programming.

Steroidogenesis, the production of sex steroid hormones, is regulated by factors including steroidogenic acute regulatory protein (StAR), cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A1), 3β-hydroxysteroid dehydrogenase (3β-HSD), cytochrome P450 17a-hydroxylase (CYP17A1), 17β-hydroxysteroid dehydrogenase (17β-HSD), and cytochrome P450 aromatase (CYP19A1) [6]. CYP19A1 is the rate-limiting enzyme which aromatizes androgens to 17β-estradiol (E2) in granulosa cells [7]. Maternal undernutrition significantly affected the mRNA expression of CYP19A1 [8] in the testis at weaning and 3β-HSD [4] in the ovary of adult rat offspring, associated with altered estrogen concentration.

Folliculogenesis is the process of follicle development from primordial follicles to graafian follicles with the fate of either ovulate or die by atresia [9]. The delicate balance of follicular cell proliferation and apoptosis determines the destiny of the follicles [10]. Uncontrolled follicle apoptosis would reduce the number of available follicles [11]. Fas/FasL system plays an essential role in

Abbreviations: 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; BMP4, bone morphogenic protein 4; BW, body weight; ChIP, chromatin immunoprecipitation; CYP11A1, cytochrome P450 cholesterol side-chain cleavage enzyme; CYP17A1, cytochrome P450 17a-hydroxylase; CYP19A1, cytochrome P450 aromatase; E2, 17β-estradiol; ERα, estrogen receptor α; ERβ, estrogen receptor β; FasL, Fas ligand; LP, low-protein; miRNAs, microRNAs; OW, ovary weight; P4, progesterone; PCNA, proliferating cell nuclear antigen; SP, standard protein; StAR, steroidogenic acute regulatory protein; T, testosterone; TUNEL, transferase-mediated deoxyuridine triphosphate-biotin nick end labeling; UTR, untranslated regions.

* Corresponding author. Tel.: +86 2584395047.
E-mail address: zhao.ruqian@gmail.com (R. Zhao).

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the apoptosis of granulosa cells, through activating caspases, the key effecters in the apoptosis process [12]. It has been reported that maternal nutrient restriction during pregnancy increased the expression of caspase 3 in the liver of fetal baboon [13].

Steroidogenesis and folliculogenesis in the developing ovary are closely related processes in the ovary development. Granulosa cells in the fully differentiated preovulatory follicles have an increased capacity to synthesize large amounts of estradiol through the aromatization of thecal-derived androgens [14]. Conversely, progesterone (P4) and estrogen were reported to inhibit early follicle development in rodents [15,16]. Faria et al. found that perinatal malnutrition directly affected folliculogenesis at puberty probably as a consequence of altered ovarian expression of estrogen receptors [17]. Nevertheless, the mechanism by which maternal low-protein diet affects the offspring steroidogenesis and folliculogenesis, and the possible pathway linking these two processes remain unclear.

MicroRNAs (miRNAs), a class of small (19–25 bp) non-coding RNAs, play important roles in post-transcriptional regulation of gene expression through mRNA degradation or translation repression [18]. Several miRNAs have been reported to regulate estradiol production directly by targeting the aromatase in granulosa cells [19] or indirectly by targeting the main transcriptional factors regulating the aromatase expression [20,21]. Meanwhile, some miRNAs were found to be critical for the ovary development. For instance, miRNA-143 inhibited the formation of primordial follicles through suppressing the proliferation of pregranulosa cells [22], and miRNA-145 was able to maintain primordial follicle quiescence by targeting transforming growth factor beta receptor 2 (TGFβR2) [23]. Moreover, the expression miRNAs in offspring liver [24], muscle [25] and brain [26], were reported to change in response to maternal nutritional intervention, suggesting possible roles of miRNAs in the nutritional programming of offspring tissue functions. However, knowledge is lacking regarding whether miRNAs are involved in the regulation of folliculogenesis and steroidogenesis in offspring ovary in response to the maternal protein restriction during gestation and lactation.

Therefore, the present study was designed to investigate the effects of maternal low-protein diet during gestation and lactation on the folliculogenesis and steroidogenesis in the ovary of offspring prepubertal gilts. Moreover, the underlying mechanisms and the possible pathways linking these two processes were investigated. The results will help to understand the impact of maternal malnutrition on ovarian dysfunction in mammalian offspring.

2. Materials and methods

2.1. Animals

The Animal Ethics Committee at Nanjing Agricultural University reviewed the protocol and approved this study specifically, with the project number 2012ZX08009-103B. The slaughter and sampling procedures complied with the "Guidelines on Ethical Treatment of Experimental Animals" (2006) No. 398 set by the Ministry of Science and Technology, China.

Landrace × Yorkshire crossbred sows in the second parity were artificially inseminated, at the observation of estrus, with a mixture of Duroc semen samples obtained from two littermate boars. One week after artificial insemination, sows were randomly divided into standard- (SP) and low-protein (LP) diet groups ($n = 8$ per group). SP sows were fed diets containing 15% and 18% of crude protein during gestation and lactation, respectively. LP sows were fed an isoenergy and protein-restricted diet containing 7.5% and 9% of crude protein, respectively, during gestation and lactation. The diet composition is shown in Table 1. All sows were kept in the same house with

Table 1
Ingredients and calculated composition of the experimental diets.

	Pregnancy		Lactation	
	SP	LP	SP	LP
Ingredient, g/kg				
Corn	370	615	332.50	547
Wheat	300	100	100	100
Bran	80	100	50	50
Soybean meal	170	0	253	30
Maize starch	0	100	150	150
Lignocellulose	30	40	0	15
$CaHPO_4$	20	25	20	30
Soybean oil	10	0	34.50	38
Fish meal	0	0	40	20
Premix[a]	20	20	20	20
Calculated composition				
Digestible energy, MJ/kg	13.10	13.10	14.39	14.39
Crude protein, %	15	7.50	18	9
Crude fiber, %	4.50	4.30	2.30	2.50
Calcium, %	0.84	0.85	0.90	0.91
Phosphorous, %	0.65	0.61	0.70	0.70

[a] The premix contains (per kilogram): retinol: 1100 kIU; cholecalciferol: 350 kIU; vitamin K3: 0.4 g; vitamin B-1: 0.4 g; vitamin B-2: 1640 mg; vitamin B-6: 0.65 g; vitamin B-12: 4.4 mg; lysine: 72 g; niacin: 4.5 g; pantothenic acid: 2.5 g; d-pantothenic acid: 2 g; folic acid: 5.2 g; biotin: 30 mg; d-biotin: 16 mg; choline chloride: 30 g; vitamin C: 20 g; manganese: 0.8 g; zinc: 7 g; ferrous: 7 g; copper: 2 g; selenium: 20 mg; sodium chloride: 3 g; β-xylanase: 8000 kIU; antioxidant; 0.19 g; acidifier; 2.5 g. LP, low protein; SP, standard protein.

a constant temperature maintained at 25 °C and a 12-h light/dark cycle. Sows were fed three times daily (05:00, 10:00 and 17:00 h) during gestation and two times daily (05:00 and 17:00 h) during lactation, with the ration of 1.2 kg/d, 1.8 kg/d and 2.4 kg/d during early, middle and late stage of pregnancy, respectively. The quantity of feeding increased to 4.2 kg/d during lactation. All the piglets were weaned at 21 days of age and were fed the same diet according to the feeding standards of the farm until the marketing age.

2.2. Tissue collection

Prepubertal gilts ($n = 8$) were sacrificed at 6 months of age. Blood was collected immediately and serum was separated by centrifugation at 3000 × g for 10 min at 4 °C and stored at −20 °C. The right ovaries of prepubertal gilts were collected within 20 min, snap-frozen in liquid nitrogen and stored at −80 °C for subsequent RNA extraction. The left ovaries were immediately fixed in 4% paraformaldehyde (0.1% M, pH 7.2) for histological processing.

2.3. Hormone assays

The serum concentrations of P4 (B08PZB), testosterone (T, B10PZB) and 17β-estradiol (E2, B05PZB) were measured using respective commercial ^{125}I-RIA kits (Beijing North Institute of Biological Technology, China), according to the manufacturer's manuals. The limit of detection was 0.05 ng/mL for P4, 0.02 ng/mL for T, and 5 pg/mL for E2. The intra-assay coefficient was 10% for all the assays. The cross reactivity of T RIA was 0.011% with dihydrotestosterone, 0.021% with E2, 0.032% with P4, and less than 0.01% with androstenedione and estriol. The cross reactivity of E2 RIA was 0.016% with estriol, 0.01% with T and less than 0.01% with P4. All samples were measured in duplicate in one assay.

2.4. Morphological classification of follicles

The left ovaries were embedded in paraffin and sections (6 μm) were stained with hematoxylin and eosin following the routine method [3,17]. The images were digitized using a computer coupled to a light microscope with a final magnification of 400× for

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