Rumen-protected glucose stimulates the secretion of reproductive hormones and the mTOR/AKT signaling pathway in the ovaries of early postpartum

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Abstract

This study was conducted to determine the response of the reproductive hormones and the mTOR/AKT pathway in the ovaries of post-partum dairy cows with the dietary rumen-protected glucose (RPG) supplementation. Twelve Holstein cows were randomly assigned to two groups (n = 6 / group): control group (CT) and RPG group. Cows were fed with the diets from 1 week prepartum to 2 weeks postpartum, and euthanized on d 22 postpartum. Blood samples were collected from cows in each group on d 1, 7 and 14 after calving. The plasma hormones were determined using a bovine specific enzyme linked immimosorbent assay method. The gene expressions of the reproductive hormone receptors and mTOR/AKT pathway in the ovarian tissues were detected using real-time polymerase chain reaction, Western blot, and immunohistochemistry, respectively. The results showed that the RPG addition significantly increased the plasma LH, E2, and P4 concentrations on d14 after calving. The mRNA and protein expressions of the estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), 17β-hydroxysteroid dehydrogenase (17β-HSD), FSHR, LHR and cytochrome p450 (CYP17A1) were upregulated, while the mRNA and protein expression of steroidogenic acute regulatory protein (StAR) was downregulated in cows of the RPG group compared with the CT group. Immunohistochemical analysis identified higher expressions of the FSHR and LHR in the ovaries of RPG-fed cows versus CT cows. Furthermore, the ratios of the protein expressions of p-AKT to total AKT and p-mTOR to total mTOR ratio were significantly increased in the ovaries of RPG-fed cows compared with the CT group cows. The current results indicated that dietary RPG supplementation regulated the secretion of gonadal hormones, and stimulated the expressions of hormone receptors and the mTOR/AKT pathway in the ovaries of early postpartum dairy cows, and might beneficial for ovarian activity recovery in the post-calving dairy cows.

Introduction

Ovarian resumption of cows after calving is critical to ensure the next reproductive cycle [1]. However, the early post-partum cows are in negative energy balance (NEB) due to the dual stress of reduced feed intake and increased milk production [2], and NEB was associated with delayed ovulation [3]. Glucose is a critical nutrient in the postpartum dairy cow. It can induce the milk synthesis and cell growth in mammary epithelial cells [4]. In addition, the uptake of glucose by ovarian tissues during the oestrous cycle of ruminants can modulate the function of ovary [5]. However, little glucose is absorbed from the diet by ruminants, as dietary carbohydrate provides glucogenic precursors in the form of propionate absorbed from the rumen. But glucose can be absorbed directly from the gastrointestinal tract if they by-pass rumen digestion [6], which can be more sufficient to meet the nutrient demands for milk production [7]. Because direct absorption of glucose from the small intestine can be more energy-efficient [8]. For instance, 1,000 g glucose infusion through the jugular vein can rapidly change the hormonal and metabolic profiles and improve the reproduction in postpartum cows [9]. A more direct way of enhancing the net glucose absorption for ruminants is to feed the rumen-protected glucose (RPG) coated with fat. Our previous study also confirmed that dietary RPG supplementation could replenish energy, improved

milk production, and stimulate the insulin-like growth factor system in the endometrium of early postpartum dairy cows [10,11].

In ovarian function, the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mechanistic target of rapamycin complex1 (mTOR) pathway plays a key role, including steroidogenesis, granulosa proliferation, corpus luteum survival, and oocyte maturation [12]. Growth and proliferation of granulosa cells are crucial to ovarian follicular development. Steroid hormones are synthesized by steroidogenic enzymes such as cytochrome p450 aromatase (CYP19A1), cytochrome p450 (CYP17A1), and cytochrome P450scc (CYP11A1) [13]. The nutritional status of dairy cows affects fertility by providing nutrients required for gamete development, insulin-like growth factor 1 (IGF-1) production and luteinizing hormone (LH) in the blood [14]. Indeed, LH stimulates cytochrome p450 (CYP17A1) mRNA expression in bovine theca cells via activation of the PI3K/AKT pathway [15]. Follicle-stimulating hormone (FSH) can also activate the PI3K pathway, and a delicate interplay has been shown to exist between cAMP/protein kinase A and PI3K signaling in the regulation of steroidogenesis by FSH in rat granulosa cells. Meanwhile, it is known that Akt causes mTOR activation through various mechanisms [16], synergistically stimulating follicles growth [17]. Our previous study has also indicated that the mTOR/AKT pathway was involved in regulating the proliferation of endometrial cells in early postpartum cows and might be beneficial for uterine recovery [11].

The objective of this study was to determine the effects of supplementing RPG from d -7 ± 2 to 14 postpartum on the secretion of the gonadal hormones (FSH, LH, E2, P4) along with the expressions of their receptors, and the mTOR/AKT pathway in the ovaries of the early post-partum dairy cows.

Results

Ovary Weight Index. The ovarian weight on 14 d post-partum was significantly higher ($P = 0.049$), and the ovary index was tended to be higher ($P = 0.094$) among cows in the rumen-protected glucose (RPG) group as compared to the control diet (CT) group (Fig. 1).

Post-Partum Plasma Hormones Concentrations. The concentrations of E2 and LH were significantly affected by the RPG addition. The E2 concentrations were significantly increased in the RPG group compared with the CT group at d 7 ($P = 0.004$) and 14 ($P = 0.006$) postpartum, and were comparable between two groups at d1 postpartum (Fig. 2A). The LH concentrations in the RPG group were higher than that in the CT group (Fig. 2D) on d1 ($P = 0.032$) and d 14 ($P = 0.01$) postpartum, and tended to be increased ($P = 0.078$) in the RPG group at d 7 postpartum compared with the CT group. The concentrations of P4 and FSH were significantly affected by the RPG addition ($P = 0.003$, $P = 0.000$, respectively), the collection time ($P = 0.002$, $P = 0.003$, respectively), and their interactions ($P = 0.001$, $P = 0.001$, respectively). Furthermore, P4 concentrations were comparable between two groups on d 1 and 7 postpartum and were significantly increased ($P = 0.01$) in the RPG group compared with the CT group on d 14 postpartum (Fig. 2B). Meanwhile, the FSH concentrations were significantly increased in the RPG

group on d 1 (P = 0.004) and 7 (P = 0.006) postpartum compared with the CT group, and were comparable between two groups at d14 postpartum (Fig. 2C).

Gene Expression Profile. As illustrated in Fig. 3, the FSHR, LHR, estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), 17β-hydroxysteroid dehydrogenase (17β-HSD) and CYP17A mRNA expression levels were significantly increased (P = 0.004, P = 0.004, P = 0.004, and P = 0.004, respectively) in the ovaries of the RPG group cows compared with the CT group cows. However, there were no differences in the mRNA expression levels of mPRα (P = 0.20), mPRβ (P = 0.201), CYP11A1 (P = 0.15) and CYP19A1 (P = 0.631). The mRNA expression levels of 3β-hydroxysteroid dehydrogenase (3β-HSD) and steroidogenic acute regulatory protein (StAR) were significantly decreased (P = 0.013 and P = 0.004, respectively) in the ovaries of the RPG group cows compared with the CT group cows.

Protein Expression Pattern. As shown in Fig. 4, the ratios of the protein expression levels of p-AKT to total AKT and p-mTOR to total mTOR were significantly increased (P = 0.004, P = 0.004, respectively) in the ovaries of RPG-fed cows compared with the CT group cows. However, the ratio of p-PI3K to total PI3K was comparable (P = 0.386) between two groups. Meanwhile, the protein expression levels of 17βHSD, FSHR, LHR, Erα, Erβ, and CYP17A1 were significantly increased (P = 0.043, P = 0.021, P = 0.021, P = 0.021, P = 0.021 and P = 0.021, respectively) in the ovaries of RPG-fed cows versus CT cows (Fig. 5A, 5C, 5D, 5E, 5F, 5G). However, the protein expression level of StAR was decreased (P = 0.021, Fig. 5B) in the ovaries of RPG-fed cows versus CT cows.

Ovarian Histology. In the CT group, visible primordial follicles (Black arrows), growing follicles (Yellow arrows), cuboidal granulosa cells, no follicular atresia and corpus luteum were observed (Fig. 6A). However, the volume and number of growing follicles were increased in the RPG group compared to the CT group, and there was no newly formed follicular atresia and corpus luteum in the RPG group (Fig. 6B). There was no difference in the percentage of primordial follicles between two groups (Fig. 6C, P = 0.468), but an increased trend in the proportion of follicles growing was observed (Fig. 6D, P = 0.09) in the RPG group.

Expression of FSHR and LHR in Ovarian Tissue Determined by Immunohistochemistry. The FSHR protein expression in ovarian tissues of dairy cows was significantly increased (P = 0.046) in RPG cows compared with the CT cows (Fig. 7A). In addition, the LHR protein expression was tended to increase (P = 0.098) in the RPG-fed cows compared with the CT cows (Fig. 7B).

Disscussion

It is necessary to restore postpartum ovarian activity as soon as possible in order to achieve normal fertility and shorten calving interval [18]. The resumption of ovarian activity in postpartum cows depends upon a fully functional hypothalamo-pituitary- ovarian (HPO) axis interaction [19]. Previous studies have shown that the LH depletion/redepletion cycle in the anterior pituitary is the main limiting factor for early postpartum recovery at 2–4 weeks after calving [20]. The LH is essential for follicular development and

oocyte maturation [21]. In addition, it is related to the increased sensitivity of hypothalamus to the positive feedback effect of estradiol [20]. Usually, estrogens act via two types of receptors (ERα and ERβ), which is selectively stimulated or inhibited, depending upon a balance between ERα and ERβ activities in target organs [22]. Also, the growth rate of antral follicles is inhibited during pregnancy even after calving. This inhibition is often lasted for about 20 days after calving and reduced the frequency of ovulation on the same side of the uterine horn [23]. In the present study, the higher plasma concentrations of E2, P4, LH, and the mRNA and protein levels of gonadotrophin response gene (FSHR, LHR) and oestradiol synthesis related genes (ERα, ERβ) in the ovaries of the cows in the RPG group compared to CT group on the 14 d postpartum suggested that RPG addition stimulates the secretion of reproductive hormones, the expressions of hormone receptors in the ovaries of early postpartum dairy cows. Furthermore, glucose is taken up by the ovary depending on insulin during the oestrous cycle [24]. Our previous study had shown that the plasma insulin level can be enhanced in postpartum dairy cows fed RPG supplementation [10]. Moreover, feeding dietary starch that promotes glucose levels favours an early resumption of the postpartum ovulation [25]. These results suggested that feeding a glucogenic diet could stimulate the ovary to utilize glucose, improving somatotropic axis synergies with the gonadotropins (FSH, LH, and P4, etc.) in post-partum dairy cows [26].

Moreover, ovarian steroid hormones regulate follicular growth and atresia, which are synthesized by the supply of cholesterol substrates to ovarian theca and steroidogenic enzymes, such as CYP19A1, CYP17A1, and CYP11A1 [27]. The expressions of 17β-HSD and CYP17Al (the key gene of P4 and androstenedione secretion [28] were upregulated in RPG-fed cows compared with CT cows in this study, although the expressions of 3β-HSD and StAR were downregulated and CYP11Al and CYP19A1 were not affected. These results suggested that the synthesis of gonadal hormone might be accelerated and the expressions of steroidogenic factors were stimulated in the cows of the RPG group compared with cows in the CT group.

Early resumption of the postpartum ovarian activity refers to oocyte growth and follicle development as well as early ovulation, which depends on the regulation of pathways involved in cell proliferation, cell survival and cell cycle regulation, such as the PI3K/AKT/mTOR signaling pathway [29, 30]. Indeed, it has been suggested that LH simulates AKT phosphorylation and the activation of PI3K/AKT is involved in CYP17A1 mRNA expression in cultured bovine theca cells. Also, LH is involved in mTOR activation in bovine granulosa cells [31]. It has been also demonstrated that FSH can activate the PI3K/AKT pathway in granulosa cells and cultured follicles, and this pathway plays a key role in folliculogenesis, including the activation of follicles and maturation [32]. In the present study, the *p*-AKT/AKT and *p*-mTOR/mTOR expression levels were upregulated in ovaries of cows fed with RPG supplementation diets versus cows fed with CT diets. Although the percentage of primordial follicles was not changed, an incremental trend in the proportion of growing follicles was observed in RPG-fed cows. This result was consistent with a previous report that rumen by pass starch decreased the maximum number of small follicles and improves ovarian function of high-yielding dairy cows in early laction [33]. This response might depend

upon insulin supply being adequate for small follicle recruitment and movement of follicles to larger [33]. Furthermore, glucose is taken up by the ovary depending on insulin during the oestrous cycle [24]. Our previous study had shown that the plasma insulin level can be enhanced in postpartum dairy cows fed RPG supplementation [10]. These results indicated that the AKT/mTOR pathway was activated in the ovaries of RPG-fed cows, and the dietary supplementation of RPG might contribute to the promoted oocyte growth and follicle development in postpartum dairy cows.

Due to the condition limitation of the experimental farm where this study was conducted, only 12 cows were selected in this study. The number of animals is a limitation of the results of this study, especially for the dairy industry that manages large populations of cows under different management. A study with more animal number is needed to further explore the effects of RPG on a larger scale.

But we also calculated the statistical power by using *GPower* software. Base on the current scenario with 12 cows, the power of E2, P4, FSH, and LH are 0.992, 0.802, 0.999, and 0.964 (Supplementary Fig. 1), respectively, indicating the statistical power in 12 cows was credible.

Conclusions

In summary, our results revealed that dietary RPG supplementation regulated the secretion of gonadal hormones, and stimulated the expressions of hormone receptors and the mTOR/AKT pathway in the ovaries of early postpartum dairy cows (Fig. 8).

Materials And Methods

Ethics approval. The experiment procedures followed guidelines for the care and use of agricultural animals and were approved by the Animal Welfare Committee (Permit No. ISA000257), Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha, China. Experiments also comply with the ARRIVE guidelines (PLoS Bio 8(6), e1000412,2010).

Animals and Experimental Procedures. Twelve healthy Holstein cows of age (4–5 years), body weight (515 ± 42 kg), milk yield (16.1 ± 3.7 kg/d), parity (2.8 ± 0.4), and expected calving date (June 12 ± 2.6 d) were randomly assigned to 2 treatments (n = 6/group): (1) control diet (CT) group; (2) control diet plus rumen-protected glucose (RPG, 200 g/d per cow) group. The RPG (Yahe Nutrition Technology Co., Ltd., Beijing, China) is manufactured by a patented technique, which contains 45% glucose coated with fat (45%). Considering the possible effect of coating fat in RPG, equally quantified coating fat was supplemented in the CT group with 90 g per cow daily. Twelve cows were housed in 16-row free-stall barns with deep recycled manure bedding in Hunan Province, a typical subtropical region in southern China. In addition, pregnant animals in the prepartum pen were frequently monitored by on-farm personnel for imminent signs of parturition, at which time they were moved into contiguous maternity pens at calving. All cows received the same close-up diets from d -21 to expected parturition, and lactation diets from parturition through d 20. The RPG and coating fat were both top-dressed on the Total

Mixed Rations (TMR) and fed to all treatments from d -7 ± 2 to d 14 twice daily in equal quantities at the 7:15 and 14:15 h during summer (June to July). The basal diets were fed twice daily at 7:30 and 14:30 h for ad libitum. The ingredients and chemical composition of the diets are shown in Supplementary Table 1. All cows had free access to drink water throughout the experimental period. The environment temperature was 34 ± 0.35°C, and the daily average humidity (g/kg) was 940 ± 12.5.

Sample collection. Blood samples were collected from each cow before the morning feeding using heparinized tubes (18 U/mL) on d 1, 7 and 14 postpartum, and immediately stored on ice. Plasma was separated via centrifugation (3000 × g, 15 min) and stored at -80°C. The cows approximately on d 14 during the post-partum period were slaughtered using electrical stunning followed by exsanguination according to the usual practices of the China beef industry [34]. The ovaries (bilateral) were collected from cows within 20 min after slaughter, then cleaned up fat and connective tissue, rinsed in RNase-free phosphate buffer, and the wet weight of the ovaries were measured. The ovary index was calculated according to the following formula: ovarian index = ovarian wet weight (g)/body weight (kg) × 100%. The unilateral ovary of each cow was divided into quarters, and two quarters were immediately frozen in liquid nitrogen, and stored at -80°C for RNA isolation and protein extraction. Another two quarters of the ovary were fixed in 10% buffered formalin for histology and immunohistochemistry analyses. Also, the operator who took the ovary samples was completely blinded to the purpose of the cow treatment.

Blood hormonal Analysis. The concentrations of FSH, LH, E2, P4 in the plasma of cows were determined by Bovine specific Enzyme Linked Immimosorbent Assay (ELISA) kits (Cusabio, Wuhan, China) according to the manufacturers' protocols. The product codes of FSH, LH, E2, P4 ELISA kits are CSB-E15856B, CSB-E12826B, CSB-E08173b, and CSB-E08172b, respectively. These kits are validated for plasma of cows. The detection ranges of these FSH, LH, E2, and P4 assay kits are 2-800 mIU/mL, 1.25–100 mIU/mL, 40-1000 pg/mL, and 0.15-70 ng/ mL, respectively. The sensitivity of these FSH, LH, E2, and P4 assay kits are 2 mIU/mL, 1.56 mIU/mL, 40 pg/mL, and 0.2 ng/mL, respectively. Each plasma sample was analyzed in duplicate. Plasma samples of all cows in all time points were measured in one run. The intra- and inter-assay coefficients of variation for E2, P4, FSH, and LH were 5.5 and 6.1, 6.1 and 7. 2, 5.5 and 6.6, 5.1 and 6.1%, respectively.

Gene Expression Analysis. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) as described previously [11, 35]. The total RNA concentration and quality were assessed according to Wang et al.[11]. To eliminate traces of genome DNA, 1 μg total RNA was treated with 5 × gDNA Eraser Buffer, 2.0 μl Eraser Buffer, 1.0 μl g DNA Eraser, and RNase-free DNase I (TaKaRa, Dalian, China) for 2 min at 42°C. Thereafter, cDNA was synthesized using a PrimeScriptTM RT reagent Kit (TaKaRa, Dalian, China) according to manufacturer's instructions, and stored at -20°C until analysis.

Relative mRNA expression was conducted using a LightCycler480 system (Roche, Basel, Switzerland) with the SYBR® Premix EX TaqTM (TaKaRa, Dalian, China). The target and internal reference (GAPDH) genes primer sequences are detailed in Table 1 and were obtained from previously published papers [36]. Quantitative polymerase chain reaction (qPCR) parameters were as follows: denaturation 30 s at 95°C,

followed by 40 cycles of denaturation 5 s at 95°C, and annealing 20 s at 60°C. The specificity of the PCR reaction was confirmed through a melting curve analysis. The efficiency of PCR amplification for each gene was checked with the dilutions of the samples. The relative expression ratios of targeted genes were calculated by R = 2^-ΔΔCt method [37], with the CT group used as the calibrator.

Table 1
The primer sequences of the target genes

Target genes[a]	Primer sequence (5'→3')	Product length, bp	Accession No.[b]
GAPDH	Forward (F): ACCCAGAAGACTGTGGATGG	178	NM_001034034
	Reverse (R): CAACAGACACGTTGGGAGTG		
FSHR	F: GCCAGCCTCACCTACCCCAGC	75	NM_174061
	R: AATTGGATGAAGGTCAGAGGTTTGCC		
LHR	F: ATTGCCTCAGTCGATGCCCAGACC	92	NM_174381
	R: AAAAAGCCAGCCGCGCTGC		
ERα	F: TCAGGCTACCATTACGGAGTTT	120	NM_001001443
	R: TTCTGATCCTGCTGTTGAGAAA		
ERβ	F: CTTCGTGGAGCTCAGCCTGT	241	NM_174051
	R: GTTTTTATCAATCGTGCACTGG		
mPRα	F: CCGGCGGTCCATCTATGA	159	NM_001038553
	R: CCACCCCCTTCACTGAGTCTT		
mPRβ	F: TGCCCCTGCTCGTCTATGTC	120	NM_001101135
	R: CCCACGTAGTCCACGAAGTAGAA		
StAR	F: TTTTTTCCTGGGTCCTGACAGCGTC	103	NM_174189
	R: ACAACCTGATCCTTGGGTTCTGCACC		
3β-HSD	F:GCCACCTAGTGACTCTTTCCAACAGCG	111	NM_174343
	R: TGGTTTTCTGCTTGGCTTCCTCCC		
17β-HSD	F: CGCATATTGGTGACCGGGAGCATA	108	NM_001102365
	R: AATCGCCAGACTCTCGCACAAACC		
CYP11A1	F: CAGTGTCCCTCTGCTCAACGTCC	99	NM_176644
	R: TTATTGAAAATTGTGTCCCATGCGG		
CYP19A1	F: CGCCACTGAGTTGATTTTTGCTGAGA	301	NM_174305
	R: TAAGGCTTTGCGCATGACCAGGTC		
CYP17A1	F: GACAAAGGCACAGACGTTGTGGTCA	301	NM_174304
	R: TGATCTGCAAGACGAGACTGGCATG		

Western Blot Analysis. Total protein was extracted using RIPA solution (Cell Signaling Technology, Inc., Danvers, MA), with the protease inhibitor cocktail (Roche, Penz-berg, Germany) and phosphatase inhibitor cocktail (Roche, Penz-berg, Germany) as described previously [11]. The protein concentration was determined with an enhanced BCA protein assay kit (Beyotime Biotechnology, Shanghai, China). Protein samples were mixed with 5 × loading buffer, denatured (100°C, 5 min), and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The isolated protein was transferred into Polyvinylidene Fluoride membranes (Immobilon-FL membrane, Millipore, Danvers, MA) with 0.45 μm apertures at a constant 200 mA for 1.5 h at 4°C. Nonspecific binding sites of membranes were blocked in 5% bovine serum albumin (BSA) and 0.1% Tris-Buffered-Saline with Tween (TBST) at room temperature for 2 h. Then, membranes were washed and subsequently incubated at 4°C overnight with the specific primary antibodies (Table 2). After several washes in TBST, membranes were incubated with corresponding secondary antibodies (Table 2) for 2 h at room temperature. Finally, the blot was washed 3 times for 10 min with wash buffer, and bands were detected by enhanced chemiluminescence (ECL) using Luminata Classico Western HRP Substrate (WBLUC0100, Millipore, Danvers, MA). The ECL signals were recorded using an imaging system (Bio-Rad, California, USA) and quantified using Quantity One software (Bio-Rad, USA) normalized to GADPH.

Histological Examination. Ovaries (n = 3 per group) were fixed in 10% buffered formalin, processed routinely through ascending alcohols and xylene, embedded in paraffin, serially sectioned at 5 μm, and stained with hematoxylin and eosin (HE) [38]. Follicle classification as primordial or growing was performed on HE-stained sections under an Eclipse TE2000U inverted microscope with a twin CCD camera (Nikon, Tokyo, Japan). Three sections of each tissue for each cow were detected [39]. In brief, to avoid counting a follicle twice, only follicles with a visible oocyte nucleus were counted. Primordial follicle was defined as an oocyte surrounded by a single layer of flattened pre-granulosa cells. Growing follicle included follicles in all developing stages. No atretic follicles examined in this study. The number of follicles in each category was counted by two experimenters without knowing the groups. Finally, the percentage of the number of follicles (primordial or growing) on total number of oocytes was calculated.

Immunohistochemical Analysis. Sections of each ovary sample (slides: three for every groups) were made for Immunohistochemical (IHC) examinations of FSHR and LHR using a previously described method[40]. The primary antibody for each protein was diluted in PBS at the following dilutions: FSHR (Biosynthesis Biotechnology, Beijing, China) 1:400; LHR (Biosynthesis Biotechnology, Beijing, China) 1:400 and incubated for overnight at 4°C. On the following day, the sections were cleaned 3 times in PBS for 5 min, incubated with biotinylated anti-rabbit IgG antibody (for FSHR and LHR, Birmingham, USA) diluted 1:200 in PAV buffer for 50 min at 4°C. Thereafter, the sections were washed 3 times in PBS for 5 min, and visualized using diaminobenzidine tetrahydrochloride (DAB). Next, they were rinsed and counterstained with Mayer's hematoxylin. Then, the sections were dehydrated and covered with a mounting medium (DPX; POCh, Gliwice, Poland). Finally, the sections were examined by two independent observers using an optical microscope. Semi-quant was assessed to estimate the expression level of LHR and FSHR in ovary for every groups taking advantage of software Image-pro plus 6.0 (Media Cybernetics,

Washington, USA) [41]. Approximately, 3 fields were examined per slide and 3 slides were examined per group. Ovary tissue exposed to secondary antibody only was used as negative control.

Statistical Analysis. All data were analyzed by using SPSS 21.0 (Chicago, IL, USA). For post-partum plasma hormones concentrations, the data were presented as Box and Whisker Plots, with an overlap of scatter plot. Two-way analysis of variance was utilized to evaluate the statistical significance. The fixed effects were the RPG treatment, collection time, and RPG treatment by collection time interactions. The collection time was considered a repeated measure. Mann–Whitney U test was used to assess the difference between the CT and RPG groups in each time point. For the ovarian weight, ovary index, gene and protein expressions of the ovary, the data were presented as Box and Whisker Plots, with an overlap of scatter plot. Mann-Whitney U test was used to assess the difference between the CT and RPG groups. For the histology and immunohistochemistry of the ovary, the data were presented as the mean ± standard error of the mean (SEM). Mann-Whitney U test was used to assess the difference between the means. G power analysis of hormones was performed *Gpower* software (version 3.0.10). Statistical significance was considered significant when $P < 0.05$, and $0.05 < P < 0.1$ was considered a tendency.

Table 2
Antibodies, suppliers and dilutions used for western blot analysis.

Items	Type	Suppliers	Dilution
Primary antibodies			
p-AKT	Rabbit Polyclonal 4060	Cell Signaling Technology, Danvers, MA, USA	1/2000
AKT	Rabbit Polyclonal 9272	Cell Signaling Technology, Danvers, MA, USA	1/1000
p-mTOR	Rabbit Polyclonal 2971	Cell Signaling Technology, Danvers, MA, USA	1/1000
mTOR	Rabbit Polyclonal ab2732	Abcam, Cambridge, UK	1/1500
p-PI3K	Rabbit Polyclonal bs-5582R	Biosynthesis Biotechnology, Beijing, China	1/200
PI3K	Rabbit Polyclonal	Biorbyt, Cambridge, UK	1/300
ERα	Rabbit Polyclonal ab3575	Abcam, Cambridge, UK	1/500
ERβ	Rabbit Polyclonal ab3577	Abcam, Cambridge, UK	1/500
FSHR	Rabbit Polyclonal bs-0895R	Biosynthesis Biotechnology, Beijing, China	1/500
LHR	Rabbit Polyclonal ab179780	Abcam, Cambridge, UK	1/500
StAR	Rabbit Polyclonal Ab96637	Abcam, Cambridge, UK	1/1000
CYP17A1	Rabbit Polyclonal orb5948	Biorbyt, Cambridge, UK	1/300
17β-HSD	Rabbit Polyclonal bs-6603R	Biosynthesis Biotechnology, Beijing, China	1/500
GAPDH	Mouse Monoclonal 60004-1-lg	Proteintech, USA	1/5000

Items	Type	Suppliers	Dilution
Secondary antibodies			
Goat anti-Rabbit IgG	SA00001-2	Proteintech, USA	1/3000
Donkey Anti-Mouse	SA00001-8	Proteintech, USA	1/3000

Declarations

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Author contributions statement.

YW: conceptualization, conducted the experiment, laboratory analysis, writing-original draft, and formal analysis. ZW and JK: review and editing. ZT and XH: conceptualization, review and editing, investigation, supervision, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

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Competing interests

The authors declare no competing interests.

Data Availability Statement

All the data and materials applied in the study could be available from the corresponding author only on academic or other non-business requests.

Supporting information

Supplementary Figure 1. The statistical power of E2 (A), P4 (B), FSH (C), and LH (D) in 12 cows were calculated by *GPower* software

Supplementary Table 1. Ingredients and nutrient composition of the diets.

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Figures

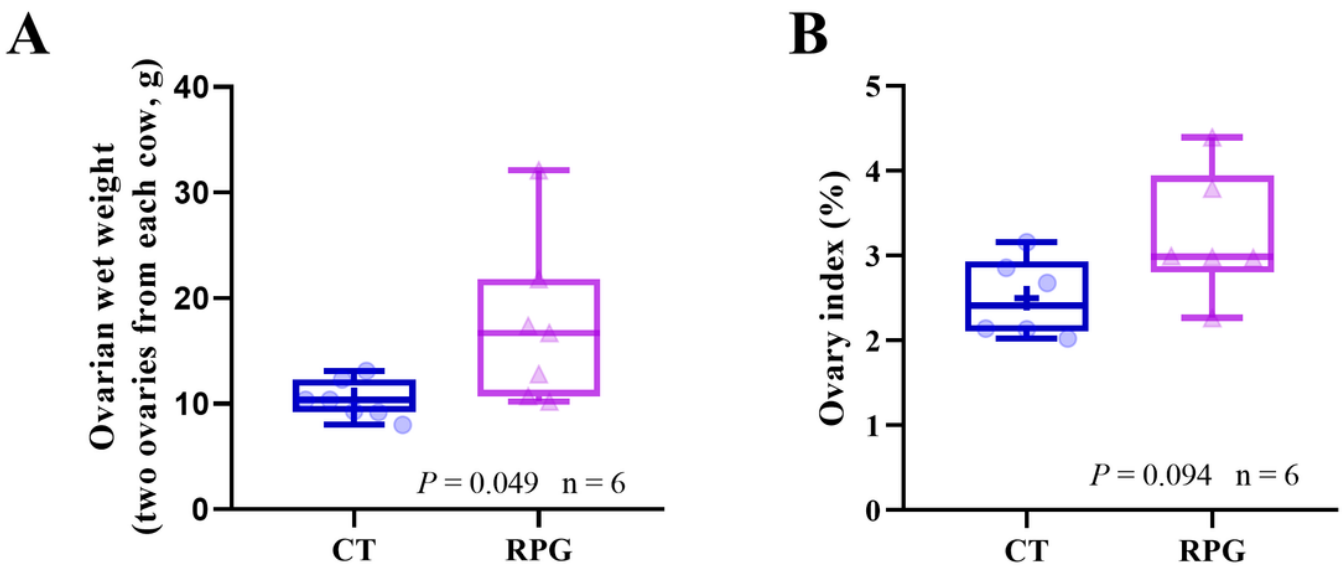

Figure 1

Box and whisker plots of the ovarian weight (A) and ovary index (B) from postpartum dairy cows that show significant ($P < 0.05$) or a tendency ($0.05 < P < 0.01$) between control diet and rumen-protected glucose diet.

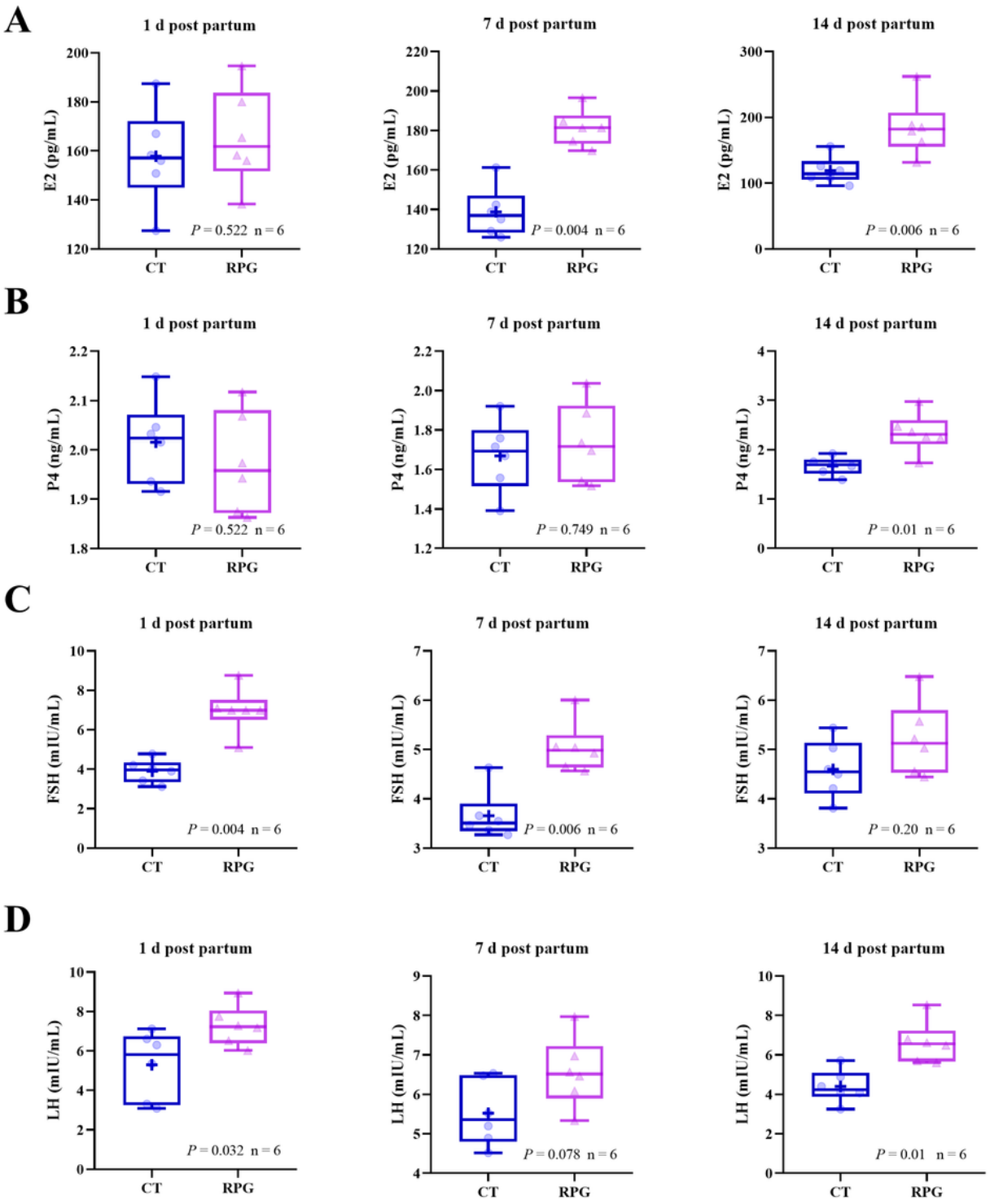

Figure 2

Box and whisker plots of the plasma concentrations of (A) E2, (B) P4, (C) FSH, and (D) LH from postpartum dairy cows that show significant ($P < 0.05$) or a tendency ($0.05 < P < 0.1$) between control diet and rumen-protected glucose diet. E2: $P_{Time} = 0.562$, $P_{Group} = 0.000$, and $P_{Time \times Group} = 0.17$; P4: $P_{Time} = 0.002$, $P_{Group} = 0.003$, and $P_{Time \times Group} = 0.001$; FSH: $P_{Time} = 0.003$, $P_{Group} = 0.000$, and $P_{Time \times Group} = 0.001$; LH: $P_{Time} = 0.228$, $P_{Group} = 0.000$, and $P_{Time \times Group} = 0.394$.

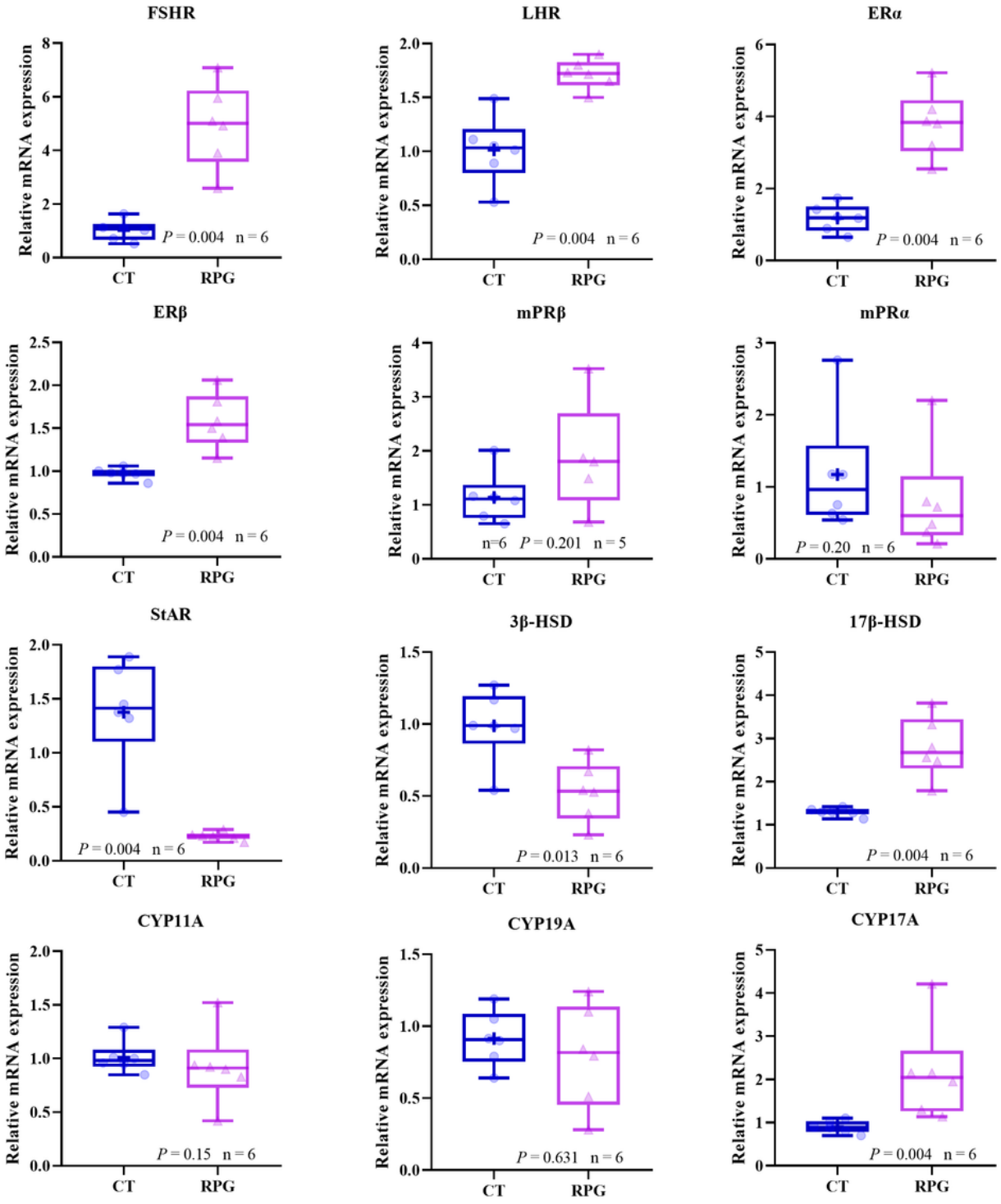

Figure 3

Box and whisker plots of the FSHR, LHR, ERα, ERβ, mPRα, mPRβ, StAR, 3β-HSD, 17β-HSD, CYP11A1, CYP19A1 and CYP17A1 gene expression in ovarian tissue from postpartum dairy cows that show significant ($P < 0.05$) between control diet and rumen-protected glucose diet.

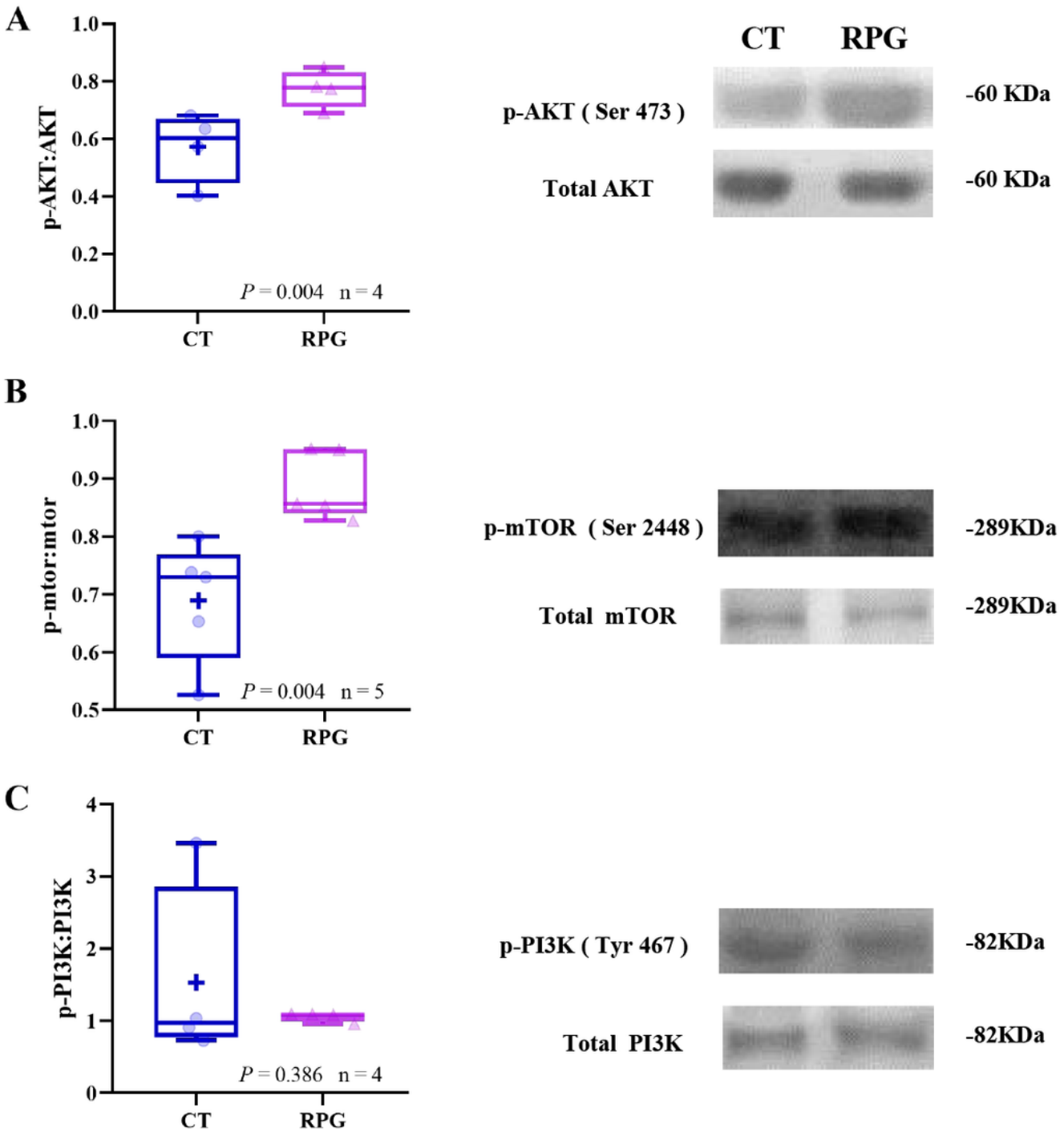

Figure 4

Box and whisker plots of the protein expressionratio of (A) p-AKT (Ser473): AKT, (B) p-mTOR (Ser2448): mTOR, and (C) p-PI3K (Tyr 467): PI3K proteins in ovarian tissue from postpartum dairy cows that show significant ($P < 0.01$) between control diet and rumen-protected glucose diet. Figure 4B and 4C original blots/gels are presented in Supplementary Figure 2A and 2B.

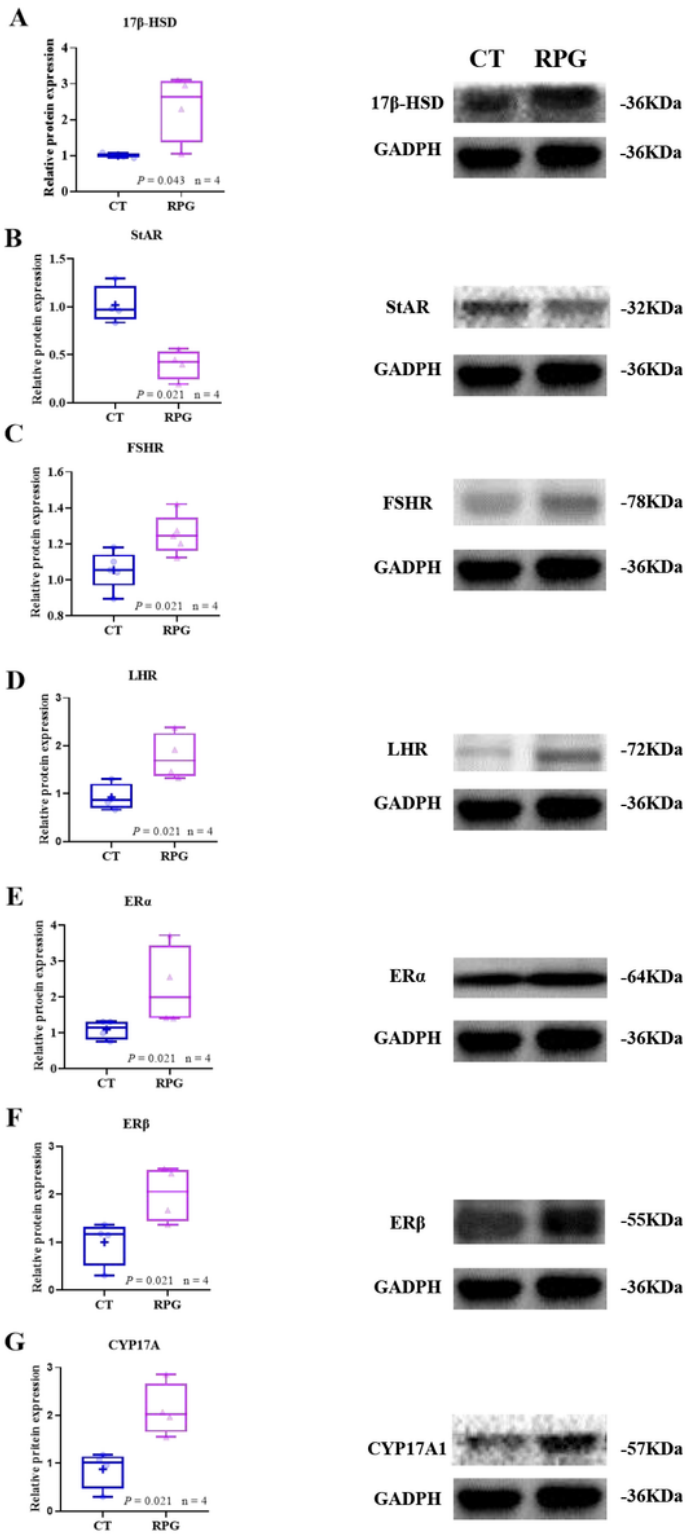

Figure 5

Box and whisker plots of the protein expression of (A) 17 β-HSD, (B) StAR, (C) FSHR, (D) LHR, (E) ERα, (F) ERβ, and (G) CYP17A1 in ovarian tissue from postpartum dairy cows show significant ($P < 0.05$) between control diet and rumen-protected glucose diet.

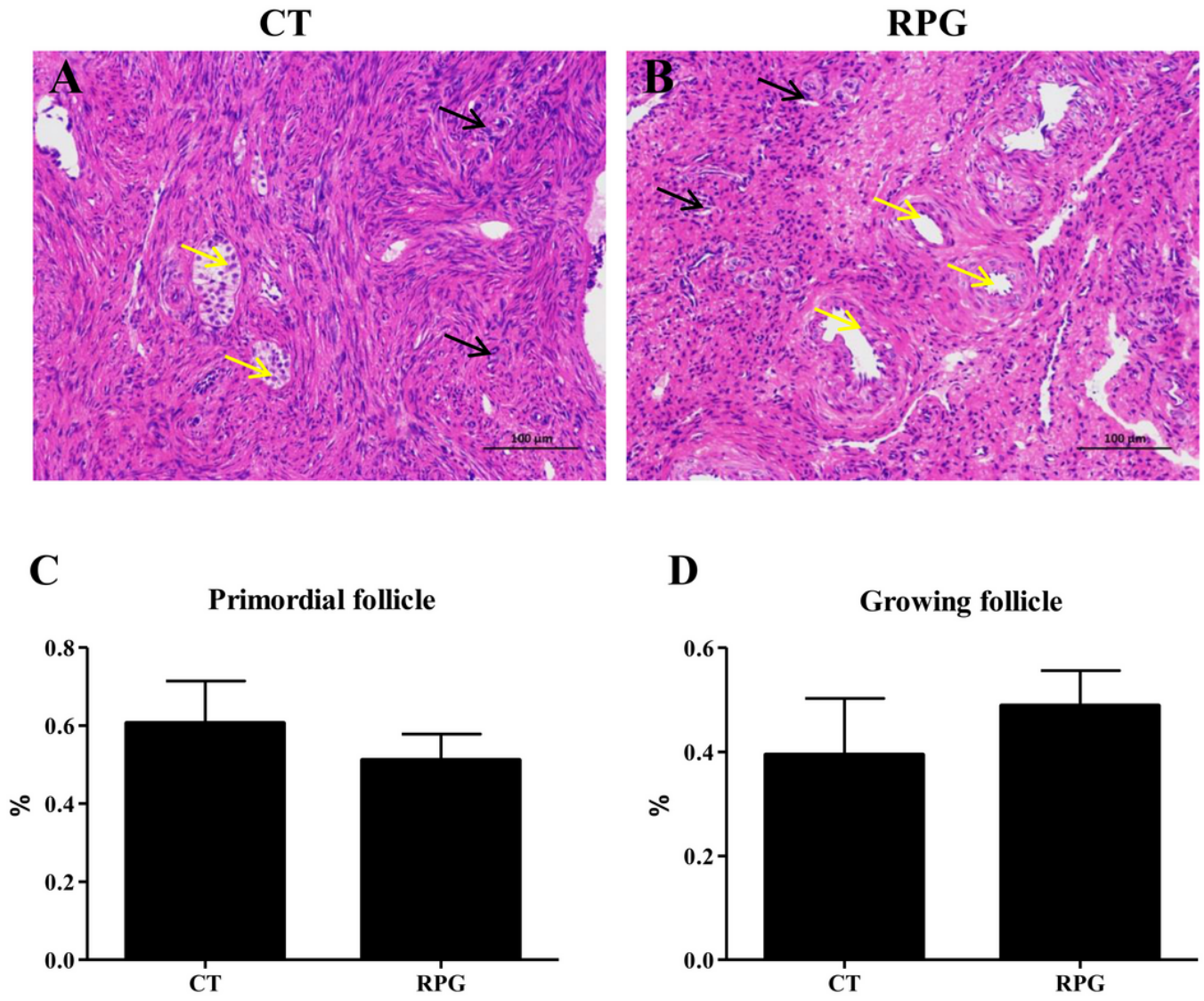

Figure 6

Effects of RPG and CT supplementation on ovarian follicle development. (A) Ovary of a CT dairy cows. (B) The ovary with increasing growing follicles after RPG treatment. (C) The percentage of primordial follicle in two groups. (D) The percentage of growing follicle in two groups. Black arrows represent primordial follicle. Yellow arrows represent growing follicles. Scale bars = 100 μm. Data are presented as the means ± SEM.

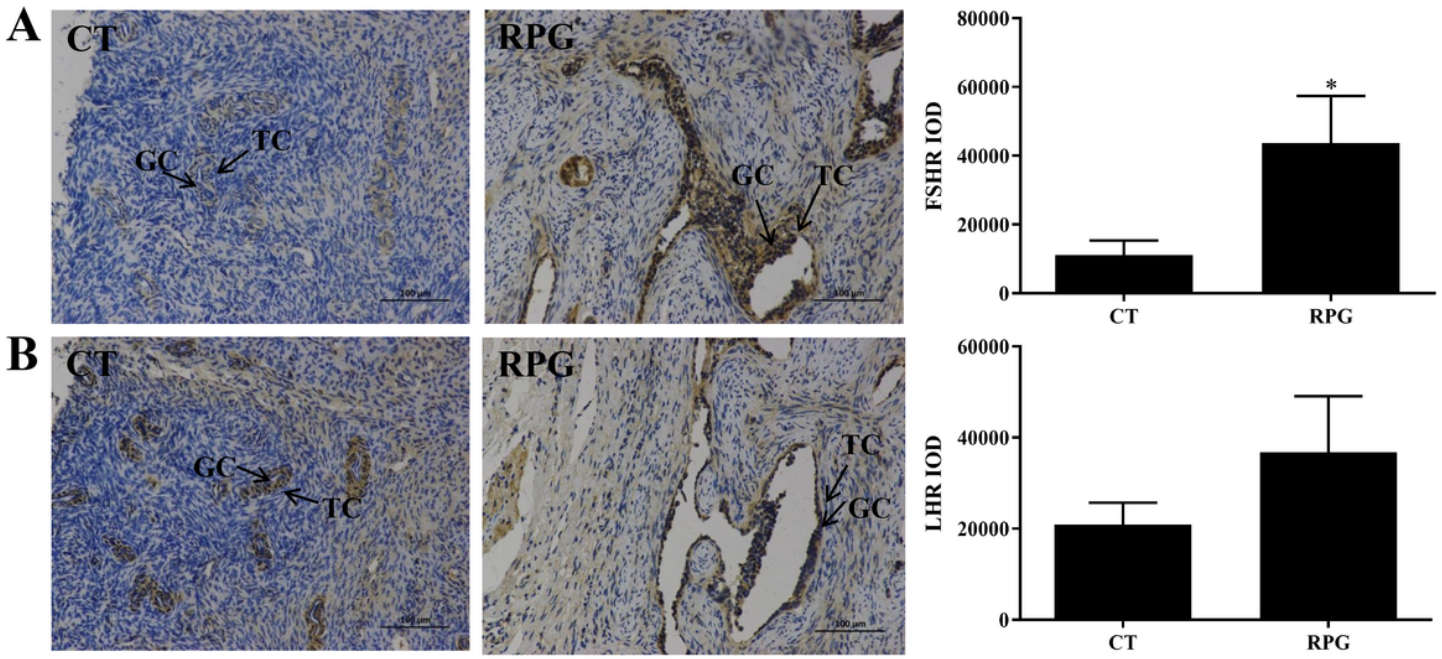

Figure 7

Effects of RPG and CT supplementation on FSHR and LHR expression level in ovarian tissue from postpartum dairy cows. (A) Representative micrographs of positive FSHR staining in ovarian tissues. (B) Representative micrographs of positive LHR staining in ovarian tissues. The integral optical density (IOD) of FSHR and LHR was calculated by Image-Pro Plus 6.0. B. IC, interstitial cell; TC, theca cell; GC, granulosa cell. Data are presented as the means ± SEM. Compared to control diet: *$P < 0.05$.

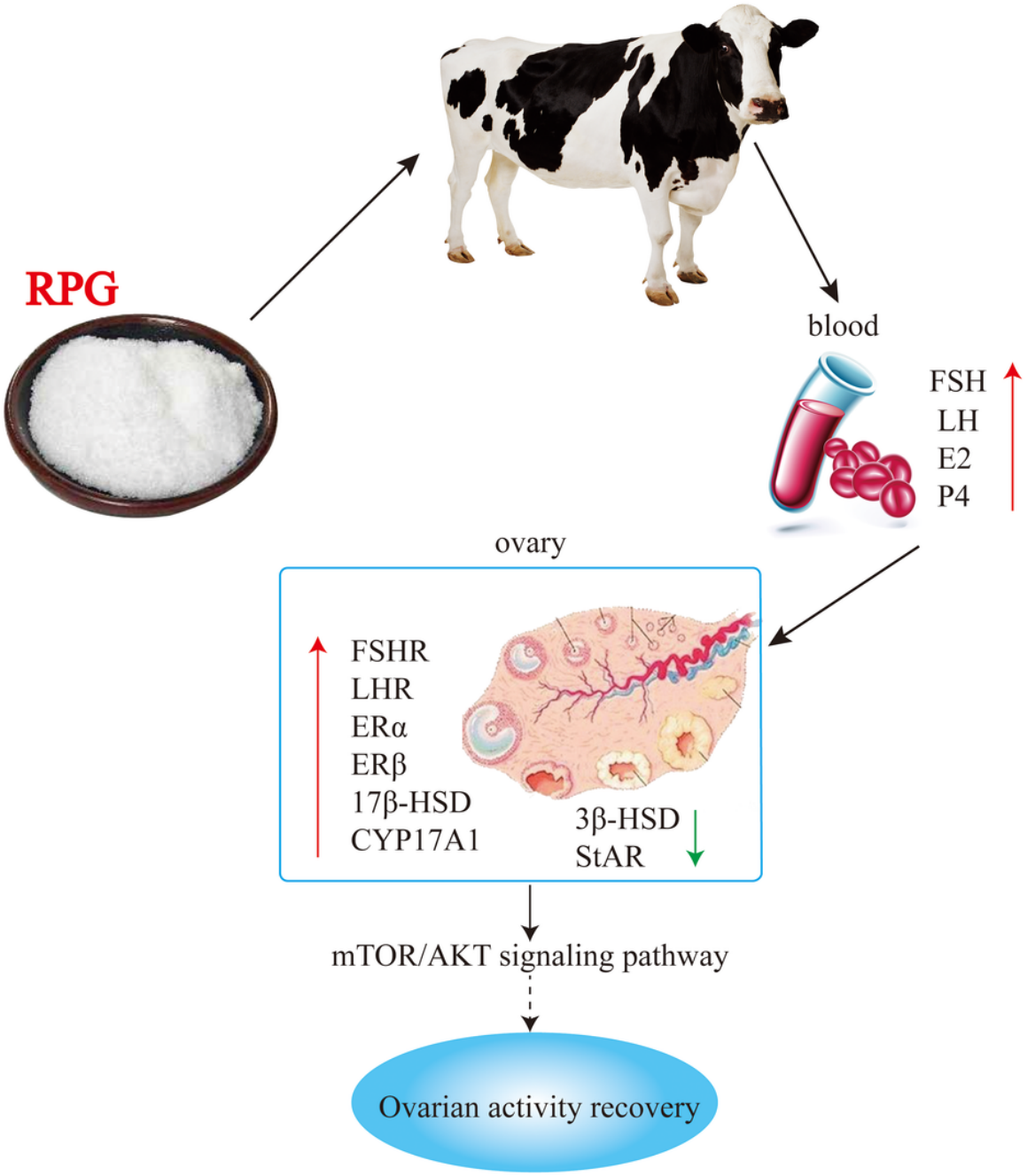

Figure 8

Schematic diagram for RPG improving the ovarian activity recovery in post-natal dairy cows. RPG addition might promote FSH, LH, E2, and P4 binding to their respective receptors, thereby activating the mTOR/AKT signaling pathway and accelerating varian activity recovery in the post-calving dairy cows.

Supplementary Files

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- Supplementaryinformation.docx