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Effect of short-term nutritional supplementation on hormone concentrations in ovarian follicular fluid and steroid regulating gene mRNA abundances in granulosa cells of ewes

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ABSTRACT

This study was conducted to investigate effects of short-term nutritional supplementation on concentrations of reproductive hormones in follicular fluid and on mRNA abundance in granulosa cells (GCs) during the luteal phase of ewes. Eighteen ewes were randomly assigned to treatment or control groups ($n = 9$, each group). All the ewes were subjected to estrous synchronization using a Controlled Intravaginal Releasing Device (CIDR). From the second to the eleventh day of estrous synchronization, ewes were fed a diet with a relatively greater (treatment group) or a maintenance (control group) energy content. Samples of follicular fluid and GCs were collected from antral follicles of estrous ewes after CIDR removal. The results indicate mean FSH concentration of fluid in small follicles and mean LH concentrations of fluid in large follicles of the ewes in the treatment group were greater ($P < 0.05$) than that of ewes in the control group. Follicular fluid E_2 concentrations were directly related ($P < 0.05$) to follicular diameter. The ewes of the treatment group had greater ($P < 0.05$), compared with the control group, abundances of *Follicle Stimulating Hormone Receptor* (*FSHR*) in small and medium follicles, and ($P < 0.05$) *Luteinizing Hormone Receptor (LHR), Steroid Acute Regulatory Protein (STAR), cytochrome P450 (CYP17A1, CYP19A1)* enzyme and *Estrogen Receptor (ESR1)* in large follicles. The results of this study provide evidence for a potential mechanism by which short-term nutritional supplementation improves follicular development possibly because of increased expression of steroid synthesis-regulating genes in large follicles.

1. Introduction

Nutritional intake is important in regulating the reproductive performance of ewes. Nutritional supplementation has a stimulatory effect on the selection of dominant follicles during follicular development and on follicular growth, as well as improving the quality of oocytes, with the magnitude of these effects being dependent on the amount and duration of supplementation (Gallet et al., 2011; Senosy et al., 2013; Vlckova et al., 2014; Chavarro and Schlaff, 2018). Short-term nutritional supplementation resulted in an increase in the concentrations of glucose, insulin and leptin (Scaramuzzi et al., 2010; Guo et al., 2019), and relatively greater concentrations of glucose, insulin and leptin are associated with a greater ovulation rate (Vinoles et al., 2010). In ewes, greater nutritional intake can

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lead to greater circulating concentrations of FSH and ovarian steroids and increased ovulation rates (Vinoles et al., 2010). There are functions of both FSH and 17β-estradiol (E_2) in recruitment, selection and development of follicles from which ovulation occurs (Kosior-Korzecka and Bobowiec, 2003). Alternatively, and increased intake of dietary energy 4 to 10 days before ovulation results in an increase in production of glucose, insulin and IGF-1 within the ovary (Kosior-Korzecka and Bobowiec, 2003; Habibizad et al., 2015; Nogueira et al., 2017) and these substrates and hormones stimulate synthesis of FSH receptors (Hand, 2011), thus, leading to an increased ovulation rate without any increase in FSH concentrations *per se*. In contrast, short-term negative energy balance inhibits follicular growth, possibly by reducing local production of reproductive hormones via reduced intra-follicular expression of steroidogenic genes including those for the cytochrome enzymes (*CYP17A1*, *CYP19A1*; Ying et al., 2013).

Steroidogenesis has an important function in the development of follicles, partly because ovarian steroids affect production of LH and FSH either by modifying the secretion of GnRH from the hypothalamus or by direct effects on gonadotropin secreting cells in the anterior pituitary gland (Nett et al., 2002). Steroidogenic acute regulatory protein (*STAR*) is the rate limiting enzyme for synthesis of steroids, with the final substrate conversion in the steroidogenic pathway being production of E_2 involving the cytochrome P450 aromatase (*CYP19A1*) and an androgen-regulated process for induction of the *CYP19A1* gene in granulosa cells (GCs; Miller and Auchus, 2011). Short-term supplementation of ewes with lipids during the late luteal phase, however, has also led to improvements in lambing rates without affecting the steroidogenic capacity of GCs (Hashem and El-Zarkouny, 2014).

An increase in pre-mating dietary energy supply to sheep positively affects ovulation rate and this practice in livestock systems is known as nutritional “flushing”. The mechanism by which short-term nutritional supplementation affects follicle development during the luteal phase, however, has not been fully elucidated. The aim of the present study was to investigate the effects of short-term nutritional supplementation of sheep on follicular fluid concentrations of key reproductive hormones and on expression of important genes, as estimated by mRNA abundances, in GCs during an artificially induced luteal phase.

2. Materials and methods

All experimental procedures were authorized by the Guide for Animal Care and Use of Laboratory Animals of the Institutional Animal Care and Use Committee of Hebei Agricultural University (Hebei, P.R. China; permit number DK596).

2.1. Animals and treatments

The experiment was performed at the Hengshui Shunyao Sheep Farm (Hebei, P.R. China) and 18 Dorset × Small Tail Han crossbred ewes (9 ± 0.5 months of age; 39 ± 2.18 kg bodyweight) were used to conduct this study. Ewes were randomly allocated to a treatment or control group (n = 9 per group) and were housed in individual pens with a natural photoperiod, with free access to mineral blocks and water.

All ewes were administered an intramuscular injection of 1 ml of a prostaglandin F2α solution (PGF2α, 0.1 mg/ml; Ningbo Dier Hormone Factory, Jiangsu, China) and stage of the estrous cycle was synchronized using intravaginal progesterone-release devices (300 mg progesterone; Eazi-Breed CIDR, New Zealand) which were inserted for 12 days (Fig. 1). The day of CIDR insertion was designated as Day 0. At the time of CIDR removal (Day 12) a second injection of prostaglandin F2α (as above) was administered (Fig. 1). From Day 2–11, ewes in the treatment group were fed a diet with relatively greater energy content than the control group which was fed a diet for maintenance of bodyweight during the study (Table 1). Estrous behavior was monitored on Day 13 using two vasectomized rams at 0900 h and 1600 h. Ewes that were mounted by the rams were considered to be in estrus.

2.2. Collection of follicular fluid and GCs

The procedures for collecting and dissecting ovaries were described previously (Sullivan et al., 2013) with minor modifications. Nine estrous ewes of each group were killed using humane electro-narcosis stunning procedures followed by exsanguination, which was performed by severing the carotid and jugular arteries, and ovaries (18 in total) were obtained within 5 min of death on Day 13. Atretic follicles were removed based on the previously reported descriptions (Moor et al., 1978). Follicular diameter was determined

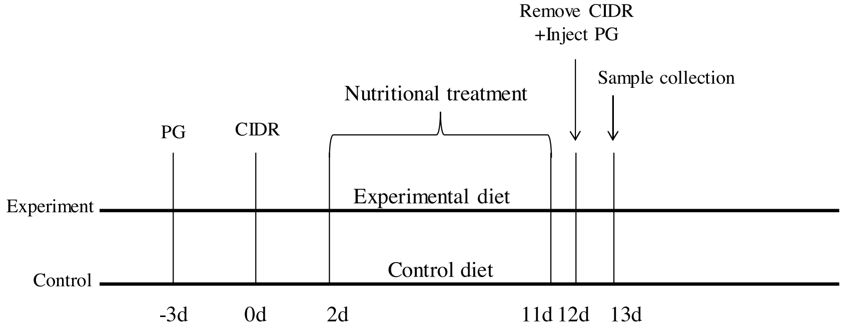

Fig. 1. Schematic representation of the experimental design and treatment timeline.

Table 1
Dietary and chemical composition of diets fed to ewes with the aim of providing the energy required for maintenance (Control) and supplemental treatment (Experiment).

Items	Control	Experiment
Ingredient (%)		
Corn straw silage	58.38	45.29
Corn	19.65	37.67
Corn meal	10.98	8.52
Soybean meal	3.47	2.69
Wheat bran	4.62	3.59
$CaHPO_4$	0.58	0.45
$NaHCO_3$	0.29	0.41
Salt	0.41	0.27
Limestone	0.49	0.27
Premix[a]	1.16	0.90
Diet composition		
DE(MJ/day)	11.72	18.75
DP(g/day)	79.71	108.44
Ca(g/day)	6.19	6.36
P(g/day)	3.29	4.21

[a] The premix provides following per kg diet: 10260 IU of VA, 301 IU of VE, 2200 IU of VD, 57.86 mg of Fe, 42.73 mg of Zn, 33.65 mg of Mn, 9.34 mg of Cu, 0.19 mg of Se, 0.76 mg of I, 0.23 mg of Co.

using a ruler. Visible follicles on the surface of each ovary were aspirated using a 26-gauge needle attached to a 1.5 mL disposable syringes containing 1 mL of phosphate-buffered saline solution. There was collection of follicular contents using a 1.5 mL syringe containing 1 mL PBS from each follicle. After removal of follicular fluid, each follicle was flushed three times with PBS to collect the GCs. The follicular fluid and PBS was then pipetted into the 96-well culture plate based on diameter (small, ≤ 3.0 mm; medium, 3.0–5.0 mm; large, ≥ 5.0 mm) of the follicles from which the fluid was obtained. The GCs were obtained by sorting from other cells using a stereoscopic microscope, after which the aspirates were pooled in centrifuge tubes according to follicular diameter (small, medium, large). The pooled aspirates were immediately centrifuged at 12,000 *g* for 10 min to provide a cell-free supernatant of follicular fluid. Quantification of hormone concentrations (subsequently described) were conducted on these cell-free supernatants and mRNA relative abundance determinations were conducted on the centrifuged pellets which contained GCs.

2.3. Reproductive hormone quantifications

The FSH concentration of follicular fluid was quantified using a commercial radioimmunoassay (RIA) kit (HY-10024, Institute of Beijing Sino-uk Biological Technology, Beijing, China). This assay had a sensitivity of 0.05 ug/L and the intra- and inter-assay coefficients of variation (CVs) were < 5% and 10%, respectively. The concentration of LH was quantified using a double antibody RIA kit (HY-10025, Institute of Beijing Sino-uk Biological Technology, Beijing, China) which had a sensitivity of 0.2–5.0 mIU/mL and intra- and an inter-assay CVs of < 2.0%–2.4% and < 4.2%–7.5%, respectively. The E_2 was quantified using a RIA kit (HY-10029, Institute of Beijing Sino-uk Biological Technology, Beijing, China) with the assay sensitivity being < 5 pg/mL and the intra- and inter-assay CVs being < 10% and 15.2%, respectively. Concentration of progesterone (P_4) was quantified using an RIA kit (HY-031, Institute of Beijing Sino-uk Biological Technology, Beijing, China) with the assay sensitivity being < 0.01 ng/mL and the intra- and inter-assay CVs being < 5% and 10%, respectively.

2.4. Analysis of mRNA abundances

Total RNA was isolated from GCs in the pellets obtained from the centrifuged follicular fluid that had been pooled according to diameter of follicles from which the fluid was obtained. For both groups of ewes, the numbers of isolates analyzed were: 20 for follicles with diameters < 3.0 mm, 13 for those with diameters 3.0 to 5.0 mm, and 11 for those > 5.0 mm in diameter for each group. Relative abundances of mRNA were determined using the High pure RNA Tissue Kit (Takara, Dalian, China) according to the manufacturer′s instructions. Samples with an OD 260/280 value between 1.8 and 2.0 were selected for further analysis. Reverse transcription reagent kits (Takara, Dalian, China) were used to remove genomic DNA (gDNA Eraser, up to 1 ug/reaction, 2 min, 42 °C) and to reverse transcribe (Master Mix, 37 °C for 15 min, 85 °C for 5 s) the RNA samples.

Quantitative real-time PCR (qRT-PCR) was performed to detect relative abundance of mRNA. Primers were designed using Oligo software 6.0 (Oligo 6.0). The qRT-PCR was conducted in triplicate using the Light Cycler 480 SYBR Green I Master Kit (Roche Applied Science, Penzberg, Germany) in a 20 u L reaction on an ABI 7300 Sequence Detection System (Applied Biosystems Inc., Foster City, CA, USA), using the following program: 95 °C for 10 min; 34 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s; and 72 °C for 6 min). The relative abundances of mRNA for the target genes were normalized based on the mRNA transcript abundance of the house-keeping gene, *GAPDH*, relative quantification of target-gene mRNA abundances was determined using the $2^{-\triangle\triangle Ct}$ method.

Table 2
Primer sequences for q-PCR.

Target genes	Primer sequence(5′-3′)	Product Size(bp)	Accession number
GAPDH	F:CAAGTTCCACGGCACAGTCA R: CTCAGCACCAGCATCACCC	156 bp	NC_019460.2
FSHR	F:AATGATGTTTTCCAGGGAGC R:TGCTGCTTGCTTTTTAGTCC	140 bp	L12767
LHR	F:ATCCAGAGCTGATGGCTACC R:GCAGCTGAGATGGCAAAGAA	115 bp	L36329
ESR1	F:GACAGGGAGCTGGTACACAT R:TCTAGCCAGGCACATTCCAA	104 bp	AY033393
STAR	F:GGAAGTCCCTCAAGGACCAA R:CGAGAGGACCTGGTTGATGA	106 bp	NM_174189
CYP17A1	F:GCATTGGCGACCTTACCATT R:GCTGCCACTCCTTCTCATTG	115 bp	NM_001009483.1
CYP19A1	F:TCGTCCTGGTCACCCTTCTG R:CGGTCTCTGGTCTCGTCTGG	115 bp	AJ012153

The sequence and GenBank accession numbers of the primers sets used for amplification of the target genes (*FSHR*, *LHR*, *ESR*, *CYP17A1, CYP19A1* and *STAR*) are presented in Table 2.

2.5. Statistical analysis

Statistical analyses were conducted using the statistical software package SAS 9.1 (Statistical Analysis Systems Institute, Cary, NC). The linear mixed model was used to analyze the repeated measurements of follicular hormones (FSH, LH, E_2, P_4) concentration. The model included the fixed effects of treatment, diameter and the interactions. The model was

$$Y_{ijk}=\mu+T_i+M_j+TM_{ij}+C(T)_{ik}+\varepsilon_{ijk}$$

μ is the average effect; T is treatment (i = 1,2), Fixed effect; M is follicular diameter (j = 1, 2, 3; small, medium, large), Fixed effect; C is sheep (k = 1…..18), Random effects; ε is residual. An analysis of variance (ANOVA) was used to compare the effects of treatment on the follicular hormone concentrations of follicles ≤3.0 mm, 3.0–5.0 mm and ≥5.0 mm in diameter. The LSD test was used to further analyze differences between the treatment and control group. The relative abundances of mRNA in GCs were analyzed for each follicular diameter size (small, ≤3.0 mm; medium, 3.0–5.0 mm; large, ≥5.0 mm) using an one-way ANOVA. Effects of the nutritional treatment were determined using the LSD for the relative abundances of mRNA transcripts. When the fold change of differential mRNA abundances was > 1.5 or < 0.67, the difference was significant.

3. Results

3.1. FSH concentrations

The mean concentration of FSH was greater ($P < 0.05$) in fluid from small follicles of ovaries from the ewes of the treatment than the control group (Table 3). There was no effect on follicular fluid FSH concentration of follicular diameter and there was no interaction between nutritional treatment and follicle diameter ($P > 0.05$; Table 3).

3.2. LH concentrations

The nutritional supplementation resulted in a greater mean follicular fluid LH concentration of large follicles in comparison with follicular fluid from the ewes of the control group ($P < 0.05$) and there was an interaction for LH concentration between nutritional treatment and follicular diameter ($P < 0.05$), but there was no effect of follicular diameter *per se* ($P > 0.05$; Table 3).

3.3. Estradiol and progesterone concentrations

Follicular fluid concentrations of 17β-estradiol (E_2) and progesterone (P_4) were not affected ($P > 0.05$) by nutritional treatment of the ewes (Table 3). In the case of E_2, there was only an effect ($P > 0.05$) of follicle diameter, with the greatest concentrations in fluid of the large follicles, and there was no treatment by follicle diameter interactions ($P > 0.05$; Table 3).

3.4. Relative abundances of FSHR mRNA in GCs

The relative abundance of *FSHR* mRNA was greater in GCs from small and medium follicles for ewes in the treatment than control group ($P < 0.05$), and the relative abundance of this mRNA transcript was less in cells from large follicles ($P > 0.05$). In the treatment group, ewes had a greater relative abundance of *FSHR* mRNA in small and medium follicles than large follicles ($P < 0.05$),

Table 3
Effects of short-term nutritional supplementation of ewes on ovarian reproductive hormone concentrations.

Conc. in follicular fluid	Control	Experiment	*P*-value		
			Treatment	Diameter	Treatment × Diameter
FSH(mIU/mL)					
Small follicles (n = 20)	3.73 ± 0.26^{a}	4.48 ± 0.13^{b}		0.163	0.115
Medium follicles (n = 13)	3.54 ± 0.16	3.97 ± 0.13	0.021		
Large follicles (n = 11)	3.90 ± 0.04	3.86 ± 0.26			
LH(mIU/mL)					
Small follicles (n = 20)	2.74 ± 0.14	3.51 ± 0.06		0.362	0.001
Medium follicles (n = 13)	3.04 ± 0.19	2.72 ± 0.07	0.001		
Large follicles (n = 11)	2.74 ± 0.04^{a}	3.57 ± 0.08^{b}			
E_2(pg/mL)					
Small follicles (n = 20)	13.93 ± 1.23	14.45 ± 0.44		< 0.001	0.844
Medium follicles (n = 13)	13.64 ± 0.90	13.04 ± 0.69	0.678		
Large follicles (n = 11)	129.42 ± 5.96	122.13 ± 1.23			
P_4(ng/mL)					
Small follicles (n = 20)	0.136 ± 0.01	0.126 ± 0.00		0.177	0.547
Medium follicles (n = 13)	0.166 ± 0.01	0.115 ± 0.00	0.075		
Large follicles (n = 11)	0.177 ± 0.04	0.152 ± 0.01			

Values with different superscripts within rows differed ($P < 0.05$).

but there was no difference between small- and medium-sized follicles ($P > 0.05$; Fig. 2A).

3.5. Relative abundances of LHR and ESR1 mRNAs in GCs

The relative abundance of *LHR* mRNA was less in GCs from small follicles ($P < 0.05$) of ewes in the treatment group than the control group, however, the relative abundance of this mRNA transcript was greater in large follicles ($P < 0.05$) of the treatment than the control group and there were no differences in abundances of mRNA transcripts in medium follicles ($P > 0.05$; Fig. 2B) of the two groups. The relative abundance of *LHR* mRNA was greater in large follicles than small and medium follicles ($P < 0.05$) and greater in medium sized than small follicles ($P < 0.05$). Relative abundance of *ESR1* mRNA transcript (Fig. 2C) was greater ($P < 0.05$) in GCs from large follicles of ewes in the treatment than control group, but was comparatively less ($P < 0.05$) for GCs from small and medium follicles. Relative abundance of *ESR1* mRNA was greater in large follicles than small and medium follicles

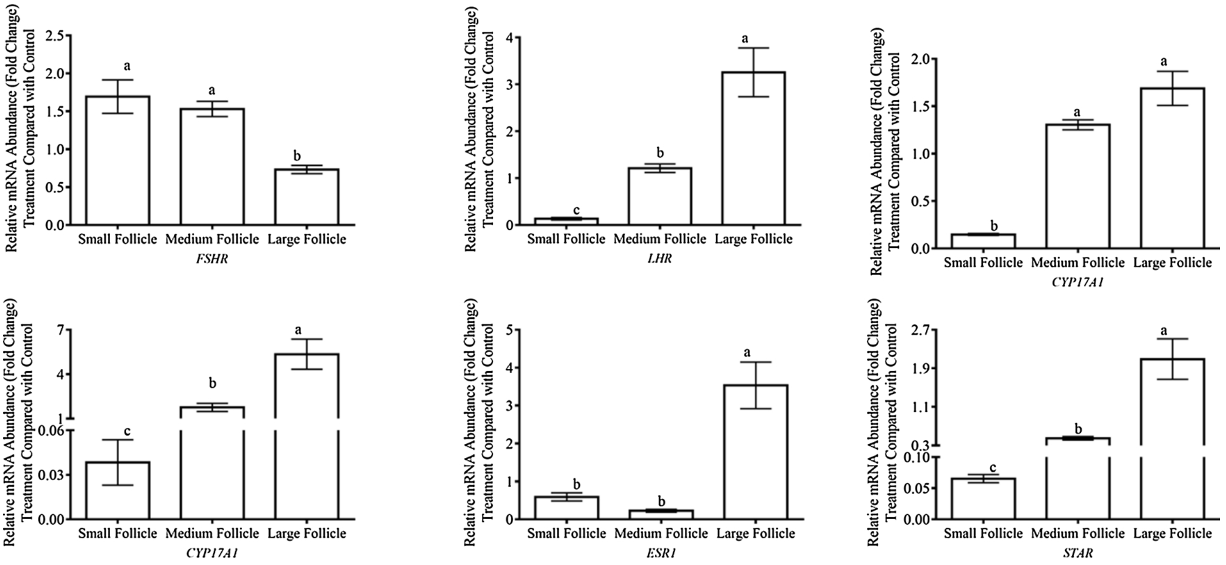

Fig. 2. Relative abundance of mRNA for genes that encode proteins related to development of follicles of different sizes; GCs were collected by centrifugation from follicular fluid aspirated from small (≤3.0 mm, n = 20), medium (3.0–5.0 mm, n = 13), large (≥ 5.0 mm, n = 11) from each group; Results from real time PCR were expressed as least squares means of GAPDH mRNA abundance ($2^{-\triangle\triangle Ct}$; arbitrary units) ± SEM; Bars within graphs with differing superscripts were different ($P < 0.05$).

($P < 0.05$), but there was no difference between small and medium follicles ($P > 0.05$).

3.6. Relative abundance of STAR mRNA transcript in GCs

Relative abundance of *STAR* mRNA (Fig. 2D) was less ($P < 0.05$) in GCs of small and medium follicles from ewes of the treatment than control group, however, was greater ($P < 0.05$) in GCs from large follicles of ewes in the treatment as compared with control group. The relative abundance of *STAR* mRNA was greater in large follicles than small and medium follicles ($P < 0.05$) and greater in medium than small follicles ($P < 0.05$).

3.7. Relative abundance of CYP17A1 and CYP19A1 mRNA transcripts in GCs

Relative abundance of *CYP17A1* (Fig. 2E) and *CYP19A1* (Fig. 2F) mRNA was greater ($P < 0.05$) in medium and large follicles, however, was less in GCs from small follicles ($P < 0.05$) in the ewes from the treatment than control group. The relative abundance of *CYP17A1* mRNA was less in small follicles than medium and large follicles ($P < 0.05$), however, there was no difference between medium and large follicles in abundances of this mRNA transcript ($P > 0.05$). The relative abundance of *CYP19A1* mRNA was greater in large follicles than small and medium follicles ($P < 0.05$) and greater in medium follicles than small follicles ($P < 0.05$).

4. Discussion

Results of this study indicate a short-term nutritional supplementation of ewes during an artificially induced luteal phase alters the concentrations of reproductive hormones in ovarian follicular fluid and affects relevant abundances of mRNA transcripts for these hormones in GCs. In addition, nutritional supplementation in combination with estrous synchronization enhanced the number of larger follicles and ovulation rate by 43% but did not affect plasma concentration of LH (Nogueira et al., 2016), FSH and 17β-estradiol (Somchit et al., 2007). The energy content of diets may affect follicular development through direct actions of nutrients on the ovarian microenvironment (Guo et al., 2017; Grazul-Bilska et al., 2018) and development of gonadotropin receptors (Ying et al., 2013). Short-term feeding of diets with greater energy content to ewes altered this microenvironment (Ying et al., 2011) and results of other studies indicated that supplementary nutrition results in modulation of ovarian functions involved in modifying follicular responses to LH and FSH (Scaramuzzi et al., 2006). As follicles develop beyond the primordial stages, there is a progressive increase in sensitivity to FSH and LH (Webb and Campbell, 2007). In the latter stages of follicular development, the reduction in circulating concentrations of FSH is thought to induce atresia of small follicles, whereas there is not atresia of dominant follicles because of changes in developmental dependence from FSH to LH (Scaramuzzi et al., 2010). Results of the present study indicate there is an enhanced response to the immediate actions of dietary nutrents on to increase the FSH concentration in the fluid of small follicles and the LH concentration in fluid of large follicles, thereby enhancing the normal progression of changes and possibly explaining the resultant enhanced follicular development. Follicular distribution in the control compared with the dietary-supplemented group for small follicles was 17.11 compared with 6.78, medium follicles 3.2 compared with 4.16, large follicles 0.88 compared with1.61 in a previous study (Guo et al., 2019). An increase in concentration of LH, therefore, in the ewes of the treatment group of the present study may be related to an increase in the number of follicles greater than 5 mm follicles as a result of short-term dietary supplementation during an induced luteal phase.

Steroid hormones (E_2 and progesterone) are involved in the regulation of recruitment, selection and development of ovulatory follicles (Fernandez et al., 2018) and E_2 signaling may modulate coupling of reproductive functions with energy metabolism (Della et al., 2011). In the present study, the nutritional supplementation had no effect on E_2 and progesterone concentrations during the follicular phase of the estrous cycle, however, the concentration of E_2 was markedly increased in follicles greater than 5.0 mm in diameter as compared with small and medium follicles. The concentrations of E_2 in follicular fluid in the present study were closely related to follicular diameter and this is consistent with findings of relatively greater concentrations of E_2 in follicular fluid of dominant and preovulatory follicles (Fortune et al., 2004). This hormonal milieu promotes mitosis and mitotic spindle formation in GCs, as well as oocyte maturation (Swinbourne et al., 2014). The mean follicular fluid concentration of progesterone was less in follicles less than 3.5 mm in diameter and concentrations of 17β-estradiol were not different as a result of short-term nutritional supplementation in a previous study (Somchit et al., 2007). Short-term nutritional supplementation of ewes resulted in decreased E_2 concentrations in follicular fluid (≥ 2.5 mm) but did not affect the concentrations of progesterone (Ying et al., 2013). A similar effect of nutrition on E_2 concentration was reported by (Somchit-Assavacheep et al., 2013) but was limited to the follicular phase of the estrous cycle, whereas Gallet et al. (2011), using glucose infusion, it was reported there was an acute (within 28 h) reduction of E_2 concentrations that was sustained during the luteal phase of the estrous cycle. The concentration of progesterone was affected in goat does as a result of imposing an estrous synchronization treatment, however there was no affect of dietary supplementation. The inconsistent results in some previous studies about the effects of nutritional supplementation were not borne out by findings in the present study with effects in the present study being trends rather than being statistically significant. It, however, is noteworthy that the relationship between the hormone concentrations and follicular size was observed in the present study.

The *FSHR* and *LHR* are G protein-coupled membrane receptors have a central function in the control of follicular growth. There is activation by FSH of the hormone-sensitive adenylate cyclase (cAMP) signaling pathway by binding to the FSHR of small and medium follicles, leading to an increase in intracellular cAMP (Jacobus and Walker, 2012; Ayoub et al., 2016). In the present study, nutritional supplementation increased the abundance of the *FSHR* mRNA transcript of in GCs of small and medium follicles during the luteal phase, potentially promoting the differentiation into larger, more mature follicles.

In the ovary, the LHRs are present in theca cells where there are functions of this protein in regulation of production of androgen production, and in luteinization of GCs and, thus, regulation of progesterone production (Huhtaniemi et al., 2002). There was detection of an increase in abundance of *LHR* mRNA transcript before the development of dominant follicles (Chen et al., 2018) and this mRNA was in greatest abundance as there was advancement of development to near the time of ovulation (Beg et al., 2001). The newly selected dominant follicles had a greater abundance of LH receptor mRNA transcript in both theca and granulosa cells compared with other follicles that were a part of the recruitment cohort in a wave of ovarian follicular development (Bao and Garverick, 1998). The results of the present study indicate nutritional supplementation resulted in an increase in abundance of *LHR* mRNA transcript in the large follicles and the relative abundances were associated with follicular diameter, as previously described by Ni et al. (2011). These observations support the concept that nutritional effects resulting from short-term dietary supplementation on growth of follicles, especially large follicles are mediated via the LH receptor.

In mammals, E_2 functions through a receptor (ESR1) and has marked modulatory actions in regulation of various reproductive functions. Rovani et al. (2014) reported that FSH functioned to promote maturation of subordinate follicles into dominant follicles as a result of increased abundances of *ESR1* mRNA which is assumed to be associated with an increase in ESR1 protein. The present findings of increased follicular fluid E_2 concentrations and an increased abundance of *ESR1* mRNA transcript in large follicles is consistent with these previous findings. Nutritional supplementations may improve follicular growth through enhanced actions of *ESR1* on an intra-follicular feedback loop in large follicles (Ying et al., 2013). Estrogen secretion is regulated by many factors and there has been reported to be a positive correlation between 17β-estradiol and glucose concentrations (Ying et al., 2011). The lack of an effect of ewe nutritional supplementation on E_2 and progesterone follicular fluid concentrations in the present and a previous study (Somchit et al., 2007) is rationale that there be some caution in reaching any conclusion about the effects of nutritional supplementation on ovarian follicles.

There were effects of ewe nutritional supplementation on expression of steroid synthesis-regulating genes in ovarian follicular GCs. Transcription of *STAR*, *CYP17A1* and *CYP19A1* are tightly controlled processes in the steroidogenic pathway (Lavoie and King, 2009) which is important for both follicular development and atresia. The STAR protein facilitates the translocation of cholesterol from the outer to the inner mitochondrial membrane which is a rate-limiting process is conversion of cholesterol into androgens as a results of actions of the CYP17A1 protein. The *CYP17A1* gene is co-expressed with *STAR* in mouse ovaries (Wang et al., 2017) following an LH-induced signaling (Nimz et al., 2010) and together these two proteins function in regulation of E_2 secretion. Nutritional supplementation of ewes during the luteal phase led to a greater abundance of *STAR* and *CYP17A1* mRNAs in follicles greater than 2.5 mm in diameter in GCs of Hu sheep (Ying et al., 2013) and in GCs from large follicles of the crossbred sheep in the present study. Abundance of both *STAR* and *CYP17A1* mRNA transcripts was greater in the large follicles in the present study. This observation indicates that nutritional supplementation could improve cholesterol uptake into GCs for subsequent androgen synthesis during the luteal phase. Follicular sensitivity to stimulation by FSH may be augmented by the presence of androgens having actions the androgen receptors (Nielsen et al., 2011). Also, androgens which may be primarily synthesized by thecal cells, and can activate the androgen receptor in GCs to stimulate production of enzymes in the estrogen synthesis pathway, as well as in providing the substrate for estrogen synthesis (Nielsen et al., 2011). This latter steroid conversion in the estrogen synthesis pathway is catalyzed by cytochrome P450 aromatase (*CYP19A1*; Miller and Auchus, 2011). Results of the present study have provided evidence that these important conversions in the estrogen synthetic pathway are enhanced in response to nutritional supplementation of ewes during the luteal phase.

5. Conclusion

Nutritional supplementation of ewes shortly before ovulation promotes ovarian follicular development by increasing the concentrations of FSH in small follicles and LH in large follicles. The potential mechanism by which such treatments enhance follicular development may involve increases in expression of *STAR, CYP17A1, CYP19A1, LHR* and *ESR1* genes as indicated by the increased abundance of mRNAs encoded by these genes in large follicles that is associated with an increase in FSH and LH concentrations in the follicular fluid.

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Declaration of Competing Interest

No potential conflict of interest was reported by the authors. We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled, “Effect of short-term nutritional supplementation on hormone concentrations in ovarian follicular fluid and steroid regulating gene mRNA abundances in granulosa cells of ewes”.

References

Ayoub, M.A., Yvinec, R., Jégot, G., Dias, J.A., Poli, S.M., Poupon, A., Crépieux, P., Reiter, E., 2016. Profiling of *FSHR* negative allosteric modulators on LH/CGR reveals biased antagonism with implications in steroidogenesis. Mol. Cell. Endocrinol. 436, 10–22. https://doi.org/10.1016/j.mce.2016.07.013.

Beg, M.A., Bergfelt, D.R., Kot, K., Wiltbank, M.C., Ginther, O.J., 2001. Follicular-fluid factors and granulosa-cell gene expression associated with follicle deviation in cattle. Biol. Reprod. 64, 432–441. https://dio.org/10.1095/biolreprod64.2.432.

Bao, B., Garverick, H.A., 1998. Expression of steroidogenic enzyme and gonadotropin receptor genes in bovine follicles during ovarian follicular waves: a review. J. Anim. Sci. 176, 1903–1921. https://dio.org/10.1051/gse:19980406.

Chavarro, J.E., Schlaff, W.D., 2018. Introduction: impact of nutrition on reproduction: an overview. Fertil. Steril. 110, 557–559. https://dio.org/10.1016/j.fertnstert.2018.07.023.

Chen, H., Cui, Y., Yu, S., 2018. Expression and localization of *FSHR*, *GHR* and *LHR* in different tissues and reproductive organs of female yaks. Folia Morphol. 77, 301–309. https://dio.org/10.5603/FM.a2016.0095.

Della, T.S., Rando, G., Meda, C., Stell, A., Chambon, P., Krust, A., Ibarra, C., Magni, P., Ciana, P., Maggi, A., 2011. Amino acid-dependent activation of liver estrogen receptor alpha integrates metabolic and reproductive functions via IGF-1. Cell Metab. 13, 205–214. https://dio.org/10.1016/j.cmet.2011.01.002.

Fernandez, J., Bruno-Galarraga, M.M., Soto, A.T., de la Sota, R.L., Cueto, M.I., Lacau, I.M., Gibbons, A.E., 2018. Hormonal therapeutic strategy on the induction of accessory corpora lutea in relation to follicle size and on the increase of progesterone in sheep. Theriogenology 105, 184–188. https://dio.org/10.1016/j.theriogenology.2017.09.020.

Fortune, J.E., Rivera, G.M., Yang, M.Y., 2004. Follicular development: the role of the follicular microenvironment in selection of the dominant follicle. Anim. Reprod. Sci. 82–83, 109–126. https://dio.org/10.1016/j.anireprosci.2004.04.031.

Gallet, C., Dupont, J., Campbell, B.K., Monniaux, D., Guillaume, D., Scaramuzzi, R.J., 2011. The infusion of glucose in ewes during the luteal phase increases the number of follicles but reduces oestradiol production and some correlates of metabolic function in the large follicles. Anim. Reprod. Sci. 127, 154–163. https://dio.org/10.1016/j.anireprosci.2011.07.017.

Grazul-Bilska, A.T., Bass, C.S., Kaminski, S.L., Ebel, K.K., Leke, E., Thammasiri, J., Kraisoon, A., Navanukraw, C., Holst, M., Shelton, M., Dorsam, S.T., Redmer, D.A., 2018. Effects of plane of nutrition and arginine on ovarian follicles in non-pregnant sheep: cell proliferation, and expression of endothelial nitric oxide and its receptor. Acta Histochem. 121, 189–197. https://dio.org/10.1016/j.acthis.2018.12.009.

Guo, Y.X., Nie, H.T., Sun, L.W., Zhang, G.M., Deng, K.P., Fan, Y.X., Wang, F., 2017. Effects of diet and arginine treatment during the luteal phase on ovarian NO/PGC-1α signaling in ewes. Theriogenology 96, 76–84. https://dio.org/10.1016/j.theriogenology.2017.03.028.

Guo, Y.X., Song, J., Liu, Y.Q., Zhang, Y.J., Duan, C.H., 2019. Effect of short-term nutritional supplementation of follicular development in Dorset × Han crossbred sheep. Chin. J. Vet. Sci. 39, 575–583. https://dio.org/:10.16303/j.cnki.1005-4545.2019.03.33.

Habibizad, J., Riasi, A., Kohram, H., 2015. Effect of long-term or short-term supplementation of high energy or high energy-protein diets on ovarian follicles and blood metabolites and hormones in ewes. Small Rumin. Res. 132, 37–43. https://dio.org/10.1016/j.smallrumres.2015.10.004.

Hand, J.M., 2011. The flushing effect and expression of follicle stimulating hormone receptor variants in sheep. Master Thesis. Kansas State University Manhattan, Kansas. http://hdl.handle.net/2097/12025.

Hashem, N.M., El-Zarkouny, S.Z., 2014. Effect of short-term supplementation with rumen-protected fat during the late luteal phase on reproduction and metabolism of ewes. J. Anim. Physiol. An. N. 98, 65–71. https://dio.org/10.1111/jpn.12032.

Huhtaniemi, I., Zhang, P.F., Kero, J., Hämäläinen, T., Poutanen, M., 2002. Transgenic and knockout mouse models for the study of luteinizing hormone and luteinizing hormone receptor function. Mol. Cell. Endocrinol. 187, 49–56. https://dio.org/10.1016/S0303-7207(01)00698-0.

Jacobus, A., Walker, W.H., 2012. FSH regulation of sertoli cell proliferation and differentiation via raf kinases. Biol. Reprod. 87, 580–585. https://doi.org/10.1093/biolreprod/87.s1.580.

Kosior-Korzecka, U., Bobowiec, R., 2003. Changes in the level of endogenous leptin, FSH, 17b-oestradiol and metabolites during lupin-induced increase in ovulation rate in ewes. J. Vet. Med. A Physiol. Pathol. Clin. Med. 50, 343–349. https://doi.org/10.1046/j.1439-0442.2003.00558.x.

Lavoie, H.A., King, S.R., 2009. Transcriptional regulation of steroidogenic genes: *STARD1*, *CYP11A1* and *HSD3B*. Exp. Biol. Med. 234, 880–907. https://dio.org/10.3181/0903-MR-97.

Miller, W.L., Auchus, R.J., 2011. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. Endocr. Rev. 32, 81–151. https://dio.org/10.1210/er.2010-0013.

Moor, R.M., Hay, M.F., Dott, H.M., Cran, D.G., 1978. Macrocopic identification and steroidogenic function of atretic follicles in sheep. J. Endocrinol. 77, 309–318. https://dio.org/10.1677/joe.0.0770309.

Nett, T.M., Turzillo, A.M., Baratta, M., Rispoli, L.A., 2002. Pituitary effects of steroid hormones on secretion of follicle-stimulating hormone and luteinizing hormone. Domest. Anim. Endocrin. 23, 33. https://dio.org/10.1016/S0739-7240(02)00143-1.

Ni, H.M., Zhang, H.B., Liu, Y.H., Lu, T.G., Deng, G.X., Guo, Y., 2011. Distribution of FSH receptor on the ovaries among different sheep breeds by different efficiency of superovulation. Sci. Agr. Sin. 44, 1232–1238. https://dio:10.3864/j.issn.0578-1752.2011.06.019.

Nielsen, M.E., Rasmussen, I.A., Kristensen, S.G., Christensen, S.T., Wreford, A.E., Byskov, A.G., Andersen, C.Y., 2011. In human granulosa cells from small antral follicles, androgen receptor mRNA and androgen levels in follicular fluid correlate with FSH receptor mRNA. Mol. Hum. Reprod. 17, 63–70. https://dio.org/10.1093/molehr/gaq073.

Nimz, M., Spitschak, M., Furbass, R., Vanselow, J., 2010. The pre-ovulatory luteinizing hormone surge is followed by down-regulation of *CYP19A1*, *HSD3B1*, and *CYP17A1* and chromatin condensation of the corresponding promoters in bovine follicles. Mol. Reprod. Dev. 77, 1040–1048. https://dio.org/10.1002/mrd.21257.

Nogueira, D.M., Cavalieri, J., Fitzpatrick, L.A., Gummow, B., Blache, D., Parker, A.J., 2016. Effect of hormonal synchronisation and/or short-term supplementation with maize on follicular dynamics and hormone profiles in goats during the non-breeding season. Anim. Reprod. Sci. 171, 87–97. https://dio.org/10.1016/j.anireprosci.2016.06.003.

Nogueira, D.M., Eshtaeba, A., Cavalieri, J., Fitzpatrick, L.A., Gummow, B., Blache, D., Parker, A.J., 2017. Short-term supplementation with maize increases ovulation rate in goats when dietary metabolizable energy provides requirements for both maintenance and 1.5 times maintenance. Theriogenology 89, 97–105. https://dio.org/10.1016/j.theriogenology.2016.10.014.

Rovani, M.T., Gasperin, B.G., Ilha, G.F., Ferreira, R., Bohrer, R.C., Duggavathi, R., Bordignon, V., Gonçalves, P.B.D., 2014. Expression and molecular consequences of inhibition of estrogen receptors in granulosa cells of bovine follicles. J. Ovarian Res. 7, 1–20. https://dio.org/10.1186/s13048-014-0096-0.

Scaramuzzi, R.J., Brown, H.M., Dupont, J., 2010. Nutritional and metabolic mechanisms in the ovary and their role in mediating the effects of diet on folliculogenesis: a perspective. Reprod. Domest. Anim. 45, 32–41. https://dio.org/10.1111/j.1439-0531.2010.01662.x.

Scaramuzzi, R., Campbell, B., Downing, J., Kendall, N.R., Khalid, M., Muoz-Gutiérrez, M., Somchit, A., 2006. A review of the effects of supplementary nutrition in the ewe on the concentrations of reproductive and metabolic hormones and the mechanisms that regulate folliculogenesis and ovulation rate. Reprod. Nutr. Dev. 46, 339–354. https://dio.org/10.1051/rnd:2006016.

Senosy, W., Abdel-raheem, S.M., Abd-allah, M., Fahmy, S., Hassan, E.H., Derar, R.I., 2013. Effect of transient high-energy diets just after ovulation on ovarian performance and metabolic status in cyclic ewes. Small Rumin. Res. 109, 152–155. https://dio.org/10.1016/j.smallrumres.2012.07.007.

Somchit, A., Campbell, B.K., Khalid, M., Kendall, N.R., Scaramuzzi, R.J., 2007. The effect of short-term nutritional supplementation of ewes with lupin grain (*Lupinus luteus*), during the luteal phase of the estrous cycle on the number of ovarian follicles and the concentrations of hormones and glucose in plasma and follicular fluid. Theriogenology 68, 1037–1046. https://dio.org/10.1016/j.theriogenology.2007.08.001.

Somchit-Assavacheep, A., Campbell, B.K., Khalid, M., Kendall, N.R., Scaramuzzi, R.J., 2013. The effect of short-term nutritional supplementation of ewes with lupin grain (*Lupinus luteus*) on folliculogenesis, the concentrations of hormones and glucose in plasma and follicular fluid and the follicular levels of P450 aromatase and IRS-1, -2 and -4. Reproduction 145, 319–333. https://dio.org/10.1530/REP-12-0135.

Sullivan, R.R., Faris, B.R., Eborn, D., Grieger, D.M., Cino-Ozuna, A.G., Rozell, T.G., 2013. Follicular expression of follicle stimulating hormone receptor variants in the ewe. Reprod. Biol. Endocrinol. 11, 1–8. https://dio.org/10.1186/1477-7827-11-113.

Swinbourne, A.M., Kelly, J.M., Kind, K.L., Kennaway, D.J., van Wettere, W.H., 2014. The effects of season and moderate nutritional restriction on ovarian function and oocyte nuclear maturation in cycling gilts. Theriogenology 82, 1303–1309. https://dio.org/10.1016/j.theriogenology.2014.08.016.

Vinoles, C., Paganoni, B., Glover, K.M.M., Milton, J.T.B., Blache, D., Blackberry, M.A., Martin, G.B., 2010. The use of a "first-wave" model to study the effect of nutrition on ovarian follicular dynamics and ovulation rate in the sheep. Reproduction 140, 865–874. https://dio.org/10.1530/REP-10-0196.

Vlckova, R., Posivak, J., Valocky, I., Kravcova, Z., Eibenova, A., Sopkova, D., 2014. The effect of short-term lupin (Lupinus angustifolius) feed supplementation on serum steroid hormones, insulin-like growth factor I, and ovarian follicular development and atresia in Merino ewes. Turk. J. Vet. Anim Sci. 38, 686–692. https://dio.org/10.3906/vet-1404-23.

Wang, C.Y., Pan, D., Zhang, Z.Y., Fan, K.K., Li, T., Du, C.G., 2017. Expression of steroidogenic factor in mice ovary and brain. Chin. J. Vet. Med. 53, 29–34. https://dio.org/0529-6005(2017)02-0029-04.

Webb, R., Campbell, B.K., 2007. Development of the dominant follicle: mechanisms of selection and maintenance of oocyte quality. Soc. Reprod. Fertil. Suppl. 64, 141–163. https://dio.org/10.5661/RDR-VI-141.

Ying, S.J., Xiao, S.H., Wang, C.L., Zhong, B.S., Zhang, G.M., Wang, Z.Y., He, D.Y., Ding, X.L., Xing, H.J., Wang, F., 2013. Effect of nutrition on plasma lipid profile and mRNA levels of ovarian genes involved in steroid hormone synthesis in Hu sheep during luteal phase. J. Anim. Sci. 91, 5229–5239. https://dio.org/10.2527/jas.2013-6450.

Ying, S.J., Wang, Z.Y., Wang, C.L., Nie, H.T., He, D.Y., Jia, R.X., Wu, Y.C., Wan, Y.J., Zhou, Z.R., Yan, Y.B.M., Zhang, Y.L., Wang, F., 2011. Effect of different levels of short-term feed intake on folliculogenesis and follicular fluid and plasma concentrations of lactate dehydrogenase, glucose, and hormones in Hu sheep during the luteal phase. Reproduction 142, 699–710. https://dio.org/10.1530/REP-11-0229.