doi: 10.17221/15/2017-CJAS

The Study of Endometrium at Gestational Days 5 and 15 in Dairy Goats (*Capra hircus*)

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ABSTRACT

Zhang L., Liu X.-R., Han P., Zhou Z.-Q., Cao B.-Y., Song Y.-X. (2017): **The study of endometrium at gestational days 5 and 15 in dairy goats (*Capra hircus*)**. Czech J. Anim. Sci., 62, 357–368.

Endometrium receptivity, an absolutely necessary part of successful embryo implantation, and several morphological and biochemical endometrial receptivity biomarkers have already been studied and proposed in some animals. However, to our knowledge, no such a study has as yet been undertaken in dairy goat. In the present study, the serum and uterus of dairy goat were studied at gestational days 5 and 15, the estrogen (E2) and progesterone (P4) concentrations in serum were determined by electrochemiluminescence immunoassay, the surface topography of endometrium was observed by scanning electron microscopy. Furthermore, the estrogen receptors (*ER*), prolactin (*PRL*), some marker genes of receptive endometrium, and cell proliferation and apoptosis in the uterus were also detected. Well-formed pinopodes were found on the surface of endometrium at day 15 with higher E2 and P4 concentrations in the serum and higher estrogen receptors *ERα* and *ERβ* expression levels and a lower *PRL* level in the endometrium. Moreover, some expression levels of marker genes of receptive endometrium (*OPN, VEGF, LIF, PRLR*) were increased at day 15 compared to day 5, but no significant differences were observed in the cell proliferation and apoptosis in the uterus. The results showed that the endometrium reached the receptive state at gestational day 15 in dairy goats.

Keywords: endometrial receptivity; pinopodes; gestational day 15 (D15)

List of abbreviations: E2 = estrogen, P4 = progesterone, D = gestational day (D0, D1, D5, D15), *ER* = estrogen receptor, *ERα* = E2 receptor α, *ERβ* = E2 receptor β, *PR* = P4 receptor, *PRL* = prolactin, *PRLR* = prolactin receptor, PCNA = proliferating cell nuclear antigen, *GHR* = growth hormone receptor, *IFG1R* = insulin-like growth factor 1 receptor, *OPN* = osteopontin, *VEGF* = vascular endothelial growth factor, *LIF* = leukaemia-inhibitory factor, RT-qPCR = quantitative real-time polymerase chain reaction, MPA = medroxyprogesterone acetate, FSH = follicle-stimulating hormone, PMSG = pregnant mare's serum gonadotropin, SEM = scanning electron microscopy, PA = paraformaldehyde, PBS = phosphate-buffered saline, ECLI = electrochemiluminescence immunoassay, HE = hematoxylin/eosin, IHC = immunohistochemistry, BSA = bull serum albumin, HRP = horseradish peroxidase, DAB = diaminobenzidine, NC = negative control, TUNEL = terminal deoxyribonucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling, LE = luminal epithelium, GE = glandular epithelium, SC = stromal cell, VE = vascular endothelial cell, IOD = integrated optical density, WOI = window of implantation, EEC = endometrial epithelial cells, ESC = endometrial stromal cells

Supported by Shaanxi Science and Technology Innovation Project Plan (2013KTZBO2-02-01 and 2015KTCQ03-08).

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doi: 10.17221/15/2017-CJAS

The embryo implant into the uterus is a key step for female reproduction in all mammalian (Xia et al. 2014), requiring the preparation of uterus to receptive state (uterine receptivity) (Dey et al. 2004). Uterine receptivity, also known as the window of implantation, lasts for a limited period during which blastocysts can normally implant. The uterine receptivity and endometrial function were accepted to be major limiting factors in the establishment of pregnancy in human and animals (Strowitzki et al. 2006).

Moreover, the formation of endometrial receptivity is a tissue remodelling process, which requires the concerted effort of numerous cell types, involving cell migration, proliferation, differentiation, and apoptosis (Zhang et al. 2013). After implantation, an appropriate balance of apoptosis and cell proliferation is also essential for the successful maintenance of pregnancy (Demir et al. 2002). Several studies dealt with the genomic analysis of endometrial receptivity in natural cycles (Carson et al. 2002), and our previous study revealed the first microRNA (miRNA) profile related to the biology of the goat receptive endometrium during embryo implantation, and the results suggested that a subset of miRNAs might play important roles in the formation of endometrial receptivity (Song et al. 2015). In addition, a total of 3255 different gene expressions were discovered in the period between the pre-receptive endometrium and the receptive endometrium, which not only reveals new information regarding the development of dairy goat endometrial receptivity, but also provides a broad and novel vision for future research at the molecular level in dairy goats (Zhang et al. 2015).

Endometrial receptivity has become one of the research hotspots in recent years, and accumulated evidence suggests that hormones (Young 2013), osteopontin (*OPN*) (Talbi et al. 2006), vascular endothelial growth factor (*VEGF*) (Lai et al. 2015), leukaemia-inhibitory factor (*LIF*) (Miravet-Valenciano et al. 2015), prolactin (*PRL*) (Kautz et al. 2015) and other factors participate in the preparation of endometrial receptivity. In addition, intensive research work has been performed to better understand the regulation of the endometrium and its clinical implications to improve implantation in human, and many genes have been reported to influence endometrial development (Strowitzki et al. 2006). However, the situation of endometrial development is a very complicated process, not a "gold standard" marker of endometrial receptivity to embryo implantation that would allow evaluation of endometrial function outside of a conception cycle under natural conditions. The mechanisms regulating receptive endometrium are not well understood and, to the best of our knowledge, no previous studies have investigated this process in dairy goat. The objective of the study was to detect the serum E2/P4 levels, and the surface topography of endometrium at days 5 and 15. Furthermore, hormone related genes, some gene markers of endometrial receptivity, and uterine cell proliferation and apoptosis at gestational days 5 and 15 in dairy goats were studied.

MATERIAL AND METHODS

All animals in this study were maintained according to the No. 5 Proclamation of the Ministry of Agriculture, P.R. China. And animal protocols were approved by the Review Committee for the Use of Animal Subjects of the Northwest A&F University.

Collection and preparation of uterine tissues. A total of 14 healthy, 24-month-old multiparous dairy goats (Xinong Saanen) were induced to oestrous synchronization for this study. The protocols were applied to goats as follows: each goat was injected with 0.2 mg prostaglandin F2α (Ningbo Pharmaceutical Co., Ltd., China). On the same day (day 1), goats were administered intravaginal sponges containing 60 mg medroxyprogesterone acetate (MPA). On day 10, each goat was twice injected with 20 IU of follicle-stimulating hormone (FSH) (Ningbo Pharmaceutical Co., Ltd.) (at 8.00 and 19.00 h). On the day of sponge removal (day 11), each goat received an intramuscular injection of 0.1 mg prostaglandin F2α and 200 IU pregnant mare's serum gonadotropin (PMSG) (Ningbo Pharmaceutical Co., Ltd.). The experimental goats were observed three times daily to ascertain the estrous sign, and mated naturally twice in estrus. The first day of mating was considered to be day 0 (D0) of pregnancy. Then the goats were euthanized following intravenous injection of a barbiturate (30 mg/kg) on gestational days 5 (D5, n = 7) and 15 (D15, n = 7). Immediately after animals had been killed, uterine tissue samples were collected and kept at −70°C. Tissues were fixed in 2.5% glutaraldehyde for scanning electron microscopy (SEM), and in

doi: 10.17221/15/2017-CJAS

4% paraformaldehyde (PA) in phosphate-buffered saline (PBS) for immunohistochemical studies.

Blood collection and hormone assays. Jugular vein blood samples (5 ml) were recovered from each dairy goat at D1, D5, and D15. The samples were centrifuged at 1400 *g* for 15 min within 2 h of collection and the serum was decanted and stored at –20°C until subsequently assayed for the P4 and E2 concentrations. The serum concentrations of E2 (pg/ml) and P4 (ng/ml) were determined by electrochemiluminescence immunoassay (ECLI).

Scanning electron microscopy. Glutaraldehyde (2.5%)-fixed samples were thoroughly washed with PBS buffer, dehydrated in a graded ethanol, placed in 2% isoamyl alcohol for 3 h, and underwent critical point drying. The samples were attached to the sample stage for observation with the surface (endometrial cavity surface) up and painted with silver conductive plastic using a vacuum coating apparatus for metal samples coating. Then, the samples were observed under a JSM-6330F scanning electron microscope (JEOL, Japan). All results are descriptive, not quantified.

Hematoxylin-eosin staining. Tissue samples were fixed in 4% PA for 1 h, washed several times with ethanol (70–100%) for 5.5 h and xylol (2.5 h), and embedded into paraffin (2 h). Hematoxylin/eosin (HE) staining of 5-mm tissue sections was performed by automation (Shandon Veristain Gemini slide stainer; GMI, Inc., USA) following deparaffinization with xylol for 10 min, washing with ethanol and water. The tissue sections were stained with hematoxylin for 3 min and eosin for 20 s. The sections were dehydrated through an ethanol series into xylene after the colour reaction. The thickness and morphology of endometrium were observed and measured under semi-automatic inverted biological microscope with imaging system (DMI4000B; Leica, Germany).

RT-qPCR analysis. Total RNA from endometrium was extracted using TRIzol reagent (TaKaRa, China), and converted to cDNA using the Prime Script™ RT reagent Kit with gDNA Eraser (TaKaRa). The quantitative real-time PCR (RT-qPCR) was performed using a CFX 96 Real Time Detection System (Bio-Rad Laboratories, Inc., USA) and

Table 1. qRT-PCR primers used in the present study

Gene	GenBank Acc. No.	Primer sequences (5'→3')	Amplicon length (bp)	T_m (°C)
GADPH	AF_030943.1	F: ACTTTGGCATCGTGGAGG R: GAAGAGTGAGTGTCGCTGTTG	379	54
OPN	KJ789112.1	F: ACGGAAAGCACAAATGAT R: GTCCTCCTCTGTGGCATC	111	60
VEGF	KJ789112.1	F: TTGCTGCTCTACCTTCACC R: ATGTCCACCAGGGTCTCA	140	59
LIF	XM_005691625.3	F: CTTCCCCAACAACCTGGA R: GCGATGATGCGATACAGC	150	55
PRL	NM_001285547.1	F: TCAACAGCTGCCATACCTCC R: TTTCATACCCCGCACCTCTG	155	59
ERα	NM_001001443.1	F: CCTCAGGCTACCATTACG R: CTCCTCTTCGGTCTTTCC	210	54
ERβ	NM_001285688.1	F: CATCCATTGCCAGTCGTC R: CCGTTCCTCTTGGTTTTG	477	57
PR	NM_001009204.1	F: TTCTGAATGCTTCCCTGTA R: AGAAATATGCCACCCACA	88	54
PRLR	XM_005695703.2	F: AGGTGTTGGAGTGCTTTGAGA R: GCTTGACGTTGCTGACGC	408	57
IGF1R	XM_018065947.1	F: CTGCCGCCACTACTACTACG R: TTCACGTAGCCTGTCACCAC	417	61
GHR	NM_001285648.1	F: CATAGTGCGGTCTGCTTCCA R: GAGATCCATTTGACTCTCACCCT	182	62

T_m = annealing temperature

doi: 10.17221/15/2017-CJAS

SYBR® Green PCR Master Mix (TaKaRa) in a 20 ml reaction mixture according to the manufacturer's instruction. *GAPDH* was used as the reference, the relative expression levels of the mRNAs were calculated using the equation

$$N = 2^{-\Delta\Delta Ct}$$

$$\Delta\Delta Ct = (Ct_{mRNA} - Ct_{GAPDH})_{D15} - (Ct_{mRNA} - Ct_{GAPDH})_{D5}$$

The primers are listed in Table 1.

Immunohistochemistry (IHC). The tissue sections were deparaffinized in xylene for 15 min (twice), and rehydrated in descending concentrations of ethanol (anhydrous ethanol for 5 min, 95% ethanol for 5 min, 85% ethanol for 5 min, 75% ethanol for 5 min, rinsed with distilled water). Antigen retrieval in sodium citrate buffer (pH = 9.0) for 10 min in a microwave oven at 100°C, then the tissue sections were placed in PBS (pH = 7.4) and washed three times (5 min each time) in a decolouring shaking bed. Endogenous peroxidase was inhibited by incubation with 3% hydrogen peroxide for 25 min at room temperature. The tissue sections were blocked in 3% bull serum albumin (BSA) for 30 min and incubated with a primary antibody at 4°C overnight, and then washed three times (5 min each time) in a decolouring shaking bed. After incubation with the primary antibody (as shown in Table 2), the tissue sections were incubated with a horseradish peroxidase (HRP)-labelled secondary antibody for 30 min at 37°C. After washing three times in PBS, the colour reaction was developed with the substrate diaminobenzidine (DAB) according to the manufacturer's instructions, the slides were washed under running tap water for 5 min, counterstained to hematoxylin (VWR International, USA). After re-dying the nucleus with hematoxylin and ammonia, finally the slides were dehydrated with concentrations of ethanol and sealed with xylene for 5 min. The sample treated with only secondary antibody without primary antibody was used as negative control (NC).

Table 2. Antibodies used in the present study

Antibody name	Manufacturer	Product No.
OPN	Boster Co., China	BM0032
VEGF	Boster Co., China	BA0407
LIF	Boster Co., China	BA1239-2
Prolactin (PRL)	Boster Co., China	BA14521
PRLR	Boster Co., China	BA3818
ERα	Boster Co., China	BA0345
ERβ	Abnova, Taiwan	PAB14421
PR	Boster Co., China	PB0077
IGF1R	Boster Co., China	BA0498
GHR	Boster Co., China	BA1619
PCNA	Boster Co., China	BM0104

TUNEL stain for detection of apoptotic cells. Cells apoptosis was detected using the terminal deoxyribonucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling (TUNEL) method. Apoptotic indices in luminal epithelium (LE), glandular epithelium (GE), stromal cell (SC), and vascular endothelial cell (VE) were detected by an OLYMPUS BX41 microscope (Olympus Corp., Japan) equipped with a digital camera. The relative density of positive cells (density/area) in each slide was analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, USA). The sample treated with only secondary antibody without primary antibody was used as NC.

Statistical analysis. All the data were processed using SPSS software (Version 17.0, 2008), one-way ANOVA (Single Factor Analysis of Variance) was used, and homogeneity of variance was evaluated using the Levene's test statistic, and only when the *P*-value was lower than 0.05, the Least Significant Difference (LSD) Multiple Comparison procedure and the Independent *t*-test were used to further analyze the statistical differences (considered significant at $P < 0.05$ and very significant at $P < 0.01$).

RESULTS

Uterus structure of dairy goats at days 5 and 15. The surface topography of endometrium at D5 and D15 (Figure 1) indicates rich microvilli on the surface of epithelial cells in the endometrium, but no pinopodes were found at D5 in dairy goats. And well-formed pinopodes were found on the surface of endometrium at D15, suggesting the endometrium was prepared for embryo implantation. At the same time, the result of HE staining showed that the endometrial thickness was also significantly increased at D15 compared to D5 (1 383 vs 780 μm, Figure 2).

E2 and P4 concentration in serum. The serum concentrations of E2 (pg/ml) and P4 (ng/ml) in dairy goats were determined (Table 3), and the

doi: 10.17221/15/2017-CJAS

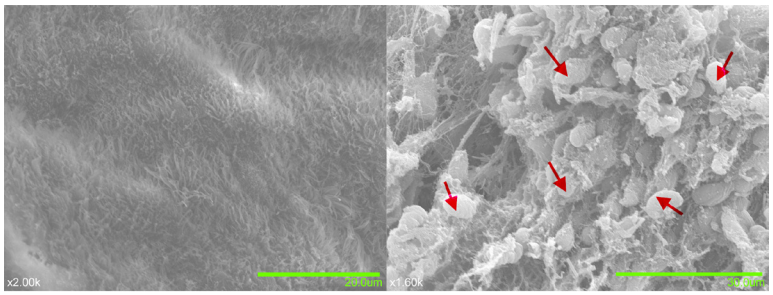

Figure 1. Surface topography images of dairy goat endometrium at gestational days 5 and 15 revealed by scanning electron microscopy
red arrows are pinopodes, green lines are scale bars, original magnifications ×2000 (day 5) and ×1600 (day 15)

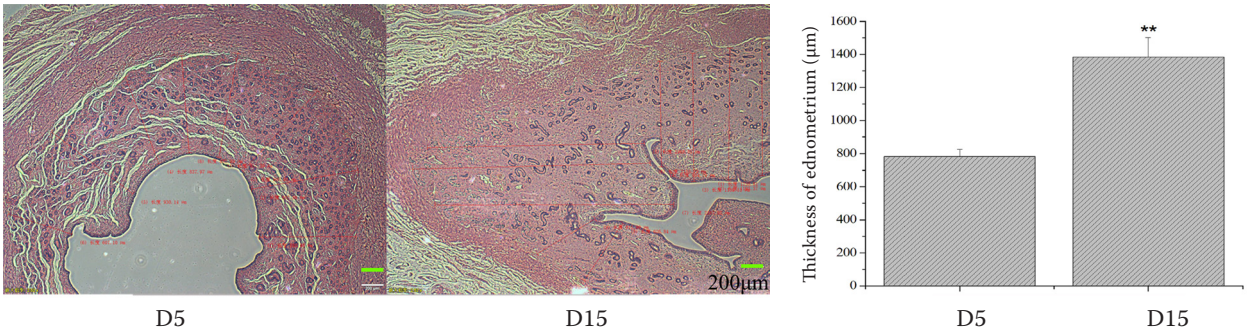

Figure 2. Thickness of dairy goat endometrium at gestational days 5 and 15
hematoxylin/eosin staining of representative uterus tissue sections; the *t*-test was used to compare the differences of endometrial thickness, **very significant differences ($P < 0.01$) between day 15 and day 5
scale bars = 200 μm (green lines), original magnification ×40

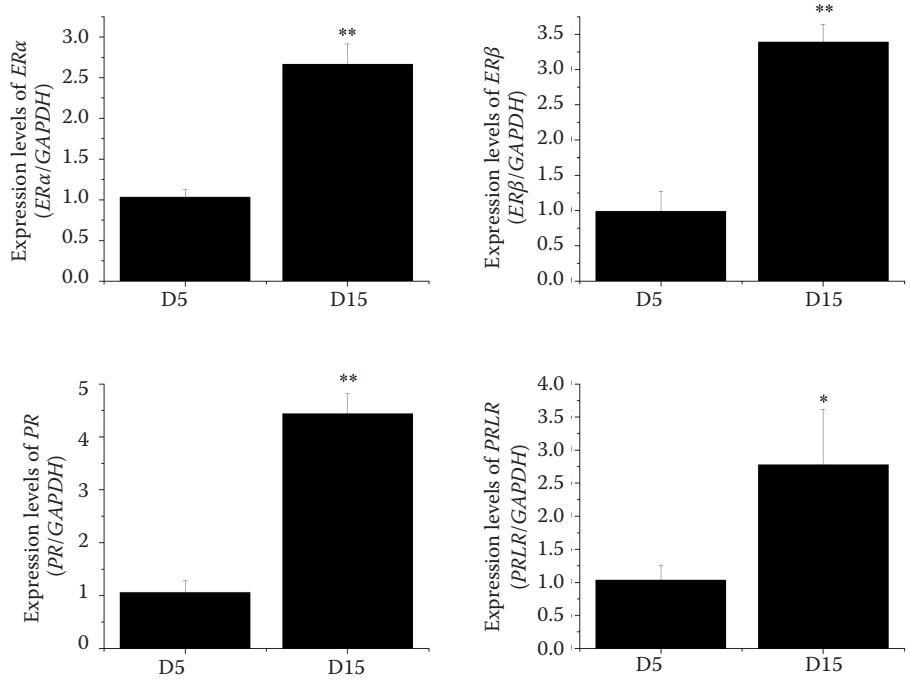

Figure 3. The mRNA levels of *ER*, *PR*, and *PRLR* genes in dairy goat endometrium at gestational days 5 and 15
values are means ± SD at day 15 ($n = 3$) that were relative to day 5 ($n = 3$), all values of day 5 = 1 because of the $2^{-\Delta\Delta Ct}$ method, $\Delta\Delta Ct = (Ct_{\text{target gene of D15}} - Ct_{\text{GAPDH of D15}}) - (Ct_{\text{target gene of D5}} - Ct_{\text{GAPDH of D5}})$; *GAPDH* was used as internal control gene for normalization; the *t*-test was used to compare the differences of mRNA levels in the same tissue
**very significant differences ($P < 0.01$), *significant differences ($P < 0.05$) between day 15 and day 5 values

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results showed that the E2 level changed at D5 after mating as compared to D1, but significantly increased at D15 (P = 0.003). As to the serum concentrations of P4, these increased significantly from 1.32 ng/ml (D0) to 3.38 ng/ml (D5, P = 0.03), and to 8.83 ng/ml (D15, P = 0.00).

ER and PR expression levels in endometrium. It is to note that a significant up-regulation of the *ERα* ($P < 0.01$), *ERβ* ($P < 0.01$), *PR* ($P < 0.01$), and *PRLR* ($P < 0.05$) mRNA levels was found in the uterus of dairy goats at D15 compared to D5 (Figure 3).

As to the protein, ERα and ERβ proteins were found in the cell nuclei and cytoplasm of all LE, GE, SC, and VE, without obvious changes between D15 and D5 in the expression intensity (Figure 4A–D). However, considering the number

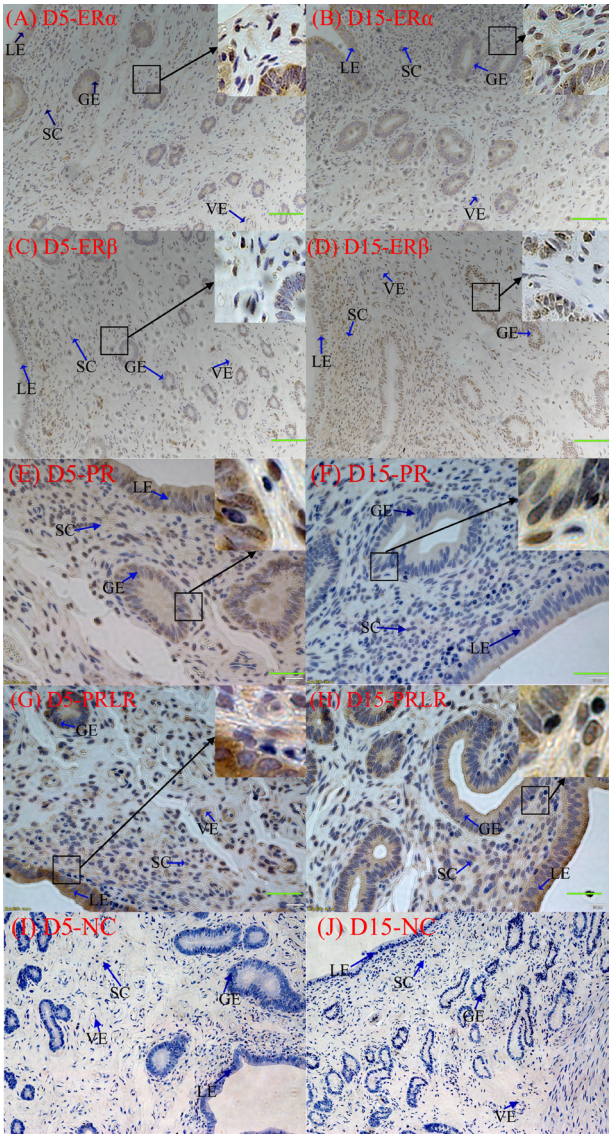

Figure 4. Expression patterns of ER, PR, and PRLR proteins in dairy goat endometrium at gestational days 5 and 15

immunohistochemistry for ERα, ERβ, PR, PRLR proteins was performed in endometrium at day 5 (**A**, **C**, **E**, **G**) and day 15 (**B**, **D**, **F**, **H**); negative controls (NC) were (**I**) for day 5 and (**J**) for day 15

LE = luminal epithelium, GE = glandular epithelium, SC = stroma cell, VE = vascular endothelial cell

scale bars = 40 μm (green lines), original magnification ×400

Table 3. Estrogen (E2) and progesterone (P4) concentrations in serum detected by electrochemiluminescence immunoassay

	E2 (pg/ml)	P4 (ng/ml)
Day 1	52.71 ± 6.02^{A}	1.32 ± 0.11^{A}
Day 5	50.63 ± 3.33^{A}	3.38 ± 0.05^{B}
Day 15	92.66 ± 5.71^{B}	8.84 ± 0.44^{C}

results are means ± SEM, n = 7

$^{A–C}$means in the same row with different superscripts differ significantly at $P < 0.01$

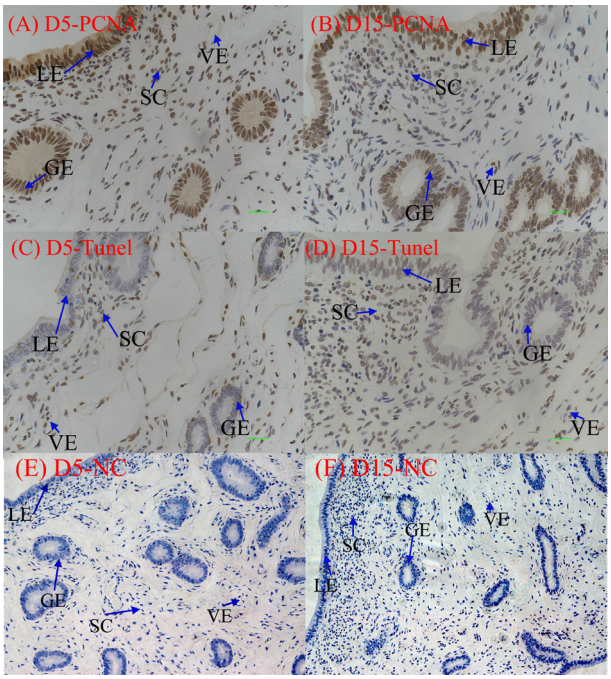

Figure 5. Cell proliferation and apoptosis in the uterus of dairy goats

proliferating cell nuclear antigen staining at gestational day 5 (**A**) and day 15 (**B**), terminal deoxyribonucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling staining at day 5 (**C**) and day 15 (**D**); negative controls (NC) were (**E**) for day 5 and (**F**) for day 15

LE = luminal epithelium, GE = glandular epithelium, SC = stroma cell, VE = vascular endothelial cell

scale bars = 40 μm (green lines), original magnification ×400

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Table 4. Cell proliferation and apoptosis in the uterus of dairy goats

Index		PCNA		TUNEL	
		day 5	day 15	day 5	day 15
Area	mean	474.91	687.74	337.84	450.90
	sum	343 832	70 495	449 331	1 776 985
Density	mean	0.57	0.48	0.41	0.29
	sum	411.13	488.10	544.38	1 157.77
IOD	mean	276.02	343.49	144.44	136.90
	sum	199 840	350 364	192 104	539 539

PCNA = proliferating cell nuclear antigen, TUNEL = terminal deoxyribonucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling, IOD = integrated optical density

and density of cells, all the protein levels of ERα and ERβ proteins at D15 were clearly higher than those at D5. PR-protein was also expressed in the cell nuclei and cytoplasm of LE, GE, SC, and VE. On D5, the PR-protein mainly was detected in the cytoplasm of LE and GE, and some in the cell nuclei of SC. As to D15, the cell nuclei and cytoplasm of LE, GE, and SC were all stained with PR-antibody (Figure 4E, F). In a word, the expression level of PR-protein was lower at D15 than at D5, especially in the cell nuclei of SC. The PRLR-protein expressed in the cytoplasm and all LE, GE, SC, and VE were stained, but no difference was found between D5 and D15 (Figure 4G, H).

Cell proliferation and apoptosis in the uterus of dairy goats. Proliferating cell nuclear antigen (PCNA) staining is shown in Figure 5A, B. A larger number of cells stained for PCNA were in the LE and GE than in the SC. It is especially noteworthy that there were more PCNA-stained cells and a higher integrated optical density (IOD) on D15 compared to D5 (Table 4). Small particle SC were detected by TUNEL in the uterus of dairy goats at D5 and D15 (Figure 5C, D), but hardly any differences of apoptotic SC were found between D5 and D15. Further analyses showed that the sum of IOD at D15 was higher than that at D5 (Table 4), and this result may be due to the greater cell density in the uterus at gestational day 15.

There were no statistically significant differences in the *IGF1R* and *GHR* mRNA levels between D5 and D15 (Figure 6). IGF1R-protein was expressed in the cytomembrane of all LE, GE, SC, and VE, and no difference was found between D5 and D15 (Figure 7A, B). GHR-protein was also expressed in the cytomembrane, and all LE, GE, SC, and VE stained, but no difference was found between D5 and D15 (Figure 7C, D).

Expression levels of some marker genes in the endometrium. Endometrial receptivity is the result

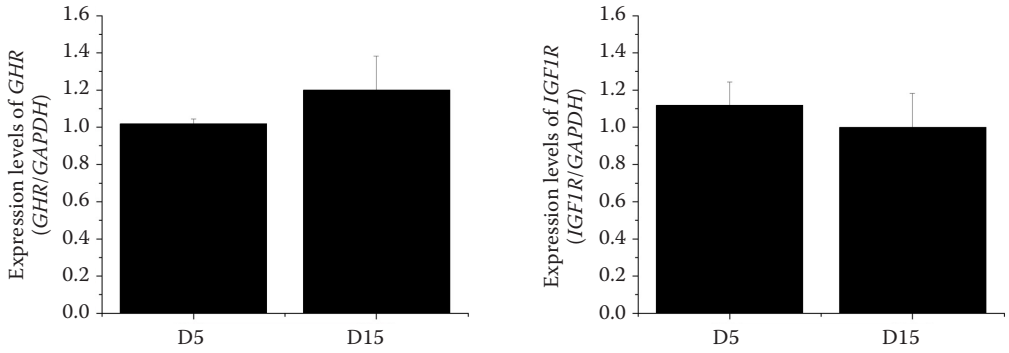

Figure 6. The mRNA levels of *GHR* and *IGF1R* genes in dairy goat endometrium at gestational days 5 and 15
GAPDH was used as internal control gene for normalization; values are means ± SD at day 15 (n = 3) that are relative to day 5 (n = 3), all values of day 5 = 1 because of the $2^{-\Delta\Delta Ct}$ method, $\Delta\Delta Ct = (Ct_{\text{target gene of D15}} - Ct_{\text{GAPDH of D15}}) - (Ct_{\text{target gene of D5}} - Ct_{\text{GAPDH of D5}})$, the t-test was used to compare the differences of mRNA levels in the same tissue

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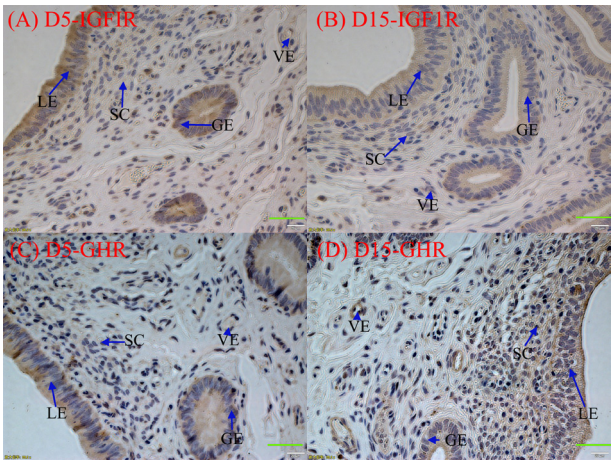

Figure 7. Expression patterns of IGF1R and GHR proteins in dairy goat endometrium at gestational days 5 and 15
immunohistochemistry for the IGF1R and GHR proteins was performed in endometrium at day 5 (**A**, **C**) and day 15 (**B**, **D**)
LE = luminal epithelium, GE = glandular epithelium, SC = stroma cell, VE = vascular endothelial cell
scale bars = 40 μm (green lines), original magnification ×400

of the synchronized and integrated interaction among ovarian hormones, growth and transcription factors, lipid mediators, and cytokines with paracrine signalling. Ovarian steroid hormones E2 and P4 are the primary regulators of this process. More recently several morphological and biochemical endometrial receptivity biomarkers have been proposed, including *OPN, VEGF, LIF, PRL*, and *PRLR* during window of implantation (WOI). Therefore, the mRNA level changes of *ER*, *PR*, and some marker genes were detected between D5 and D15 with RT-qPCR (Figure 8). The mRNA level of *OPN* in the endometrium tissue increased at D15 compared to D5, and the difference was significant ($P < 0.01$). *VEGF* also raised its mRNA level by some 50% at D15 compared to D5, and the difference was significant ($P < 0.05$). As to *LIF* and *PRL*, their mRNA levels did not change between D5 and D15.

In the present study it was also observed that the luminal and glandular epithelia, vascular endothelial and smooth muscle cells in the endometrium displayed a strong cytoplasmic and cytomembrane distribution pattern for OPN (Figure 9A, B), and this OPN immunoreaction intensity increased at D15 compared to D5. VEGF-protein was expressed in the cytoplasm of all stained LE, GE, and SC cells. It was noteworthy that VEGF-protein was highly expressed in vascular endothelial cells, where the

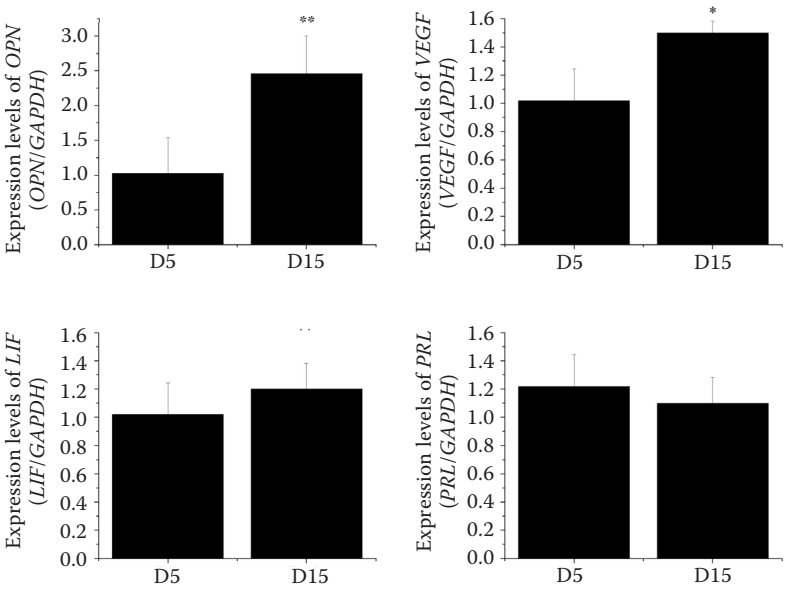

Figure 8. The mRNA expression levels of some gene markers used to detect endometrial receptivity in dairy goat at gestational days 5 and 15
GAPDH was used as internal control gene for normalization; values are means ± SD at day 15 ($n = 3$) that are relative to day 5 ($n = 3$), all values of day 5 = 1 because of the $2^{-\Delta\Delta Ct}$ method, $\Delta\Delta Ct = (Ct_{\text{target gene of D15}} - Ct_{\text{GAPDH of D15}}) - (Ct_{\text{target gene of D5}} - Ct_{\text{GAPDH of D5}})$; the *t*-test was used to compare the differences of mRNA levels in the same tissue
**very significant differences ($P < 0.01$), *significant differences ($P < 0.05$) between day 15 and day 5 values

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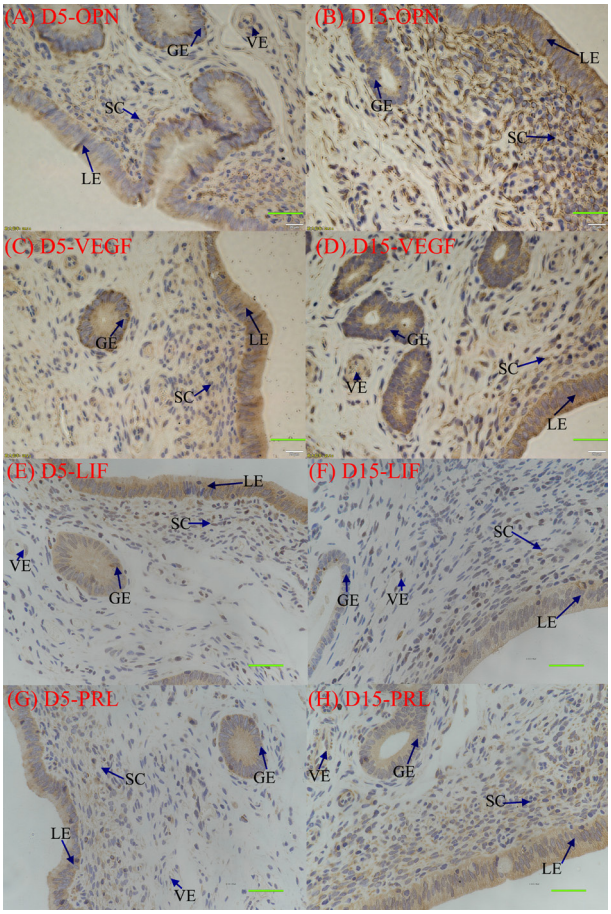

Figure 9. Immunohistochemistry for representative protein biomarkers of endometrial receptivity in dairy goat at gestational days 5 and 15

immunohistochemistry for OPN, VEGF, LIF, PRL proteins was performed in endometrium at day 5 (**A**, **C**, **E**, **G**) and day 15 (**B**, **D**, **F**, **H**)

LE = luminal epithelium, GE = glandular epithelium, SC = stroma cell, VE = vascular endothelial cell

scale bars = 40 μm (green lines), original magnification ×400

reaction intensity increased at D15 compared to D5 (Figure 9C, D). LIF-protein, perhaps unsurprisingly, decreased at D15 compared to D5 in this study (Figure 9E, F). PRL-protein was mainly expressed in the cytoplasm of LE and GE, and in a small amount of SC. Notably, there were no differences in LE and GE, but a remarkable increase was found in SC on D15 compared to D5 (Figure 9G, H).

Briefly, the increase of *OPN*, *VEGF*, and *PRL* reactivity observed in the endometrium of the goat uterus on D5 may be associated with the development of endometrial receptivity in dairy goats.

DISCUSSION

Uterine epithelium (endometrial epithelial cells, EEC) is the first cell-layer to have physical and physiological contact with the blastocyst trophectoderm during WOI (Murphy 2004). Morphological changes of the luminal epithelium include the apical microvilli retraction and emergence of large apical protrusions (pinopodes) (Paria et al. 1992), which were observed on the surface of endometrium at D15 in dairy goats in the present study. At the same time, the endometrial thickness was significantly increased at D15 compared to D5. All these results show that the endometrium undergoes pronounced structural and functional changes preparing it to be receptive to adhesion and subsequent invasion by the embryo.

EECs undergo structural and functional changes to establish uterine receptivity under the coordination of E2 and P4 before the blastocyst comes to the uterus in animals. The endometrial receptivity to embryo implantation is time-limited and this timing is driven by the time of the P4 exposure after a sufficient exposure to E2 (Young 2013). Moreover, the experience with assisted reproduction suggests that despite the diverse set of hormones produced by the ovary in a cycle-dependent fashion, E2 and P4 are sufficient to prepare a highly receptive endometrium in humans (Young 2013). In the present study, serum E2 concentrations significantly increased at D15 ($P = 0.003$) compared to D5 and D0 ($P < 0.01$), P4 concentrations significantly increased with increasing time ($P < 0.01$), which is to some extent consistent with previous studies (Zhang et al. 2013). Given the critical and fundamental role that E2 and P4 play in the establishment of receptivity (Young 2013), a deep understanding of the action of these steroid hormones on the goat endometrium will allow clear insight into the mechanisms determining endometrial receptivity.

Both E2 and P4 act through high-affinity specific receptors to directly regulate expression of a large number of genes. There are two nuclear E2 receptors – E2 receptor α (*ERα*) and E2 receptor β (*ERβ*) showing a high sequence homology. In this study, both *ERα* and *ERβ* mRNA levels increased significantly at D15 compared to D5 ($P < 0.01$), and IHC results showed that the protein levels of ERα-protein in the endometrium at gestational day 15 was higher than that at gestational D5, but no palpable shift was observed in the ERβ-protein levels.

doi: 10.17221/15/2017-CJAS

In the present study, the mRNA levels of *PR* remarkably increased in the endometrium at gestational D15 compared with D5. Furthermore, obvious changes of the PR-protein levels were detected in the endometrium at gestational D5 and D15. *PR* localized in the cell nucleus and cytoplasm, and all LE, GE, SC, and VE were stained. On D5, the PR-protein mainly was detected in the cytoplasm of LE and GE, and some in the cell nuclei of SC. As to D15, the cell nuclei and cytoplasm of LE, GE, and SC were all stained with PR-antibody. In a word, the expression level of PR-protein was lower at D15 than at D5, especially in the cell nuclei of SC (Figure 7). Given the temporally specific process of WOI, it was not surprising that the expression and localization of PR-protein varied markedly in the endometrium.

The homeostasis of the endometrial tissue achieved by E2/P4-dependent processes involving endometrial growth and regression under physiological conditions in a cyclical pattern plays an important role in the preparation of the uterus for each new oestrous cycle or for supporting pregnancy (Roberto da Costa et al. 2007). Several studies performed in humans and different animal species, such as horses, dogs, rats and rabbits, have shown that the cyclic pattern of ovarian secretion of E2 and P4 affects cell proliferation and apoptosis in the uterine endometrium. It is well documented that the rodent uterine epithelium around the embryo undergoes apoptosis in response to the presence of the blastocyst (Parr et al. 1987).

Some studies have been performed with the purpose of evaluating cell proliferation and apoptosis in the endometrium during the oestrous cycle e.g. in sow, canine, rabbi, and women. However, to our knowledge, no such a study has so far been undertaken in the dairy goat. In dairy goats, a larger number of cells were stained for PCNA in LE and GE than in the SC. It is especially noteworthy that there were more PCNA-stained cells on D15 compared to D5. The identification of the actual number of endometrial cells undergoing this process could be somewhat difficult because the rate of replacement of apoptotic cells is rapid and the occurrence of programmed cell death is low (Bursch 1990). Small particle SC was detected by TUNEL in the uterus, but hardly any differences in apoptotic SC were found between D5 and D15 in the present study.

Osteopontin (*OPN*) has an arginine–glycine–aspartic acid (RGD)-binding site that can bind to the transiently expressed αvβ3 and α4β1 integrin heterodimers present during the WOI (Lessey et al. 1994). It has been detected during the mid- to late-secretory phase in glandular epithelial cells and in uterine secretions from the secretory phase (von Wolff et al. 2001). Consolidating its role in endometrial receptivity, microarrays analysing showed that *OPN* is consistently up-regulated during the WOI when compared with both the late proliferative phase and the early secretory phase (Horcajadas et al. 2005). In this study, the mRNA level of *OPN* was higher on D15 than on D5, and the IHC analysis further revealed OPN-protein to be expressed in the cytoplasm of LE, GE, and SC, and the expression intensity increased on D15.

The rate of endometrial blood flow during the normal female reproductive cycle has been correlated with an increased expression of angiogenic factors (El-Zenneni et al. 2015). The vascular endothelial growth factor (*VEGF*) is an angiogenic factor involved in angiogenesis in different parts of the body (Clauss and Breier 1997), and it is a member of a family of heparin binding proteins that act directly on the endothelial cells (Gordon et al. 1995). It stimulates endothelial cell proliferation, permeability, migration, and assembly into capillary tubes (Okada et al. 2014). And the temporal and spatial distribution of *VEGF* expression in the three components of human endometrium during the menstrual cycle suggests the functional role of angiogenesis in the remodelling process of endometrial tissue (Lai et al. 2015).

Furthermore, *VEGF* is expressed in the endometrium and probably participates in the increased angiogenesis and vascular permeability necessary for implantation. It appears that the expression of *VEGF* is highly regulated in a temporal and spatial manner at the early stage of implantation (Demir et al. 2010). In dairy goat, higher levels of *VEGF* mRNA were detected in the endometrium at D15 compared to D5, and VEGF-protein was expressed in the cytoplasm of LE, GE, and the SC. It is noteworthy that VEGF-protein was highly expressed in vascular endothelial cells, where the reaction intensity increased at D15 compared to D5. This, together with the concomitantly highly upregulated expression of VEGF-protein, confirms our previous findings on the higher mRNA levels at D15, and points towards species-specific regulatory mechanisms.

doi: 10.17221/15/2017-CJAS

The leukemia inhibitory factor *(LIF*), which binds to the *LIF* receptor as a common signal-transduction partner with other cytokines, is critical for implantation (Stewart et al. 1992). *LIF*-deficient female mice showed implantation failure that can be rescued by supplementing with exogenous *LIF*. However, the potential mechanism underlying how *LIF* executes its effects on implantation is not clear. In humans, *LIF* is expressed at a high level in the glandular epithelium of the secretory endometrium (Rashid et al. 2011). Based on the above studies, *LIF* has important roles in the attachment reaction and it is crucial for successful implantation. In the present study, LIF-protein was expressed in the cell nuclei and cytoplasm of all LE, GE, and SC, but the expression intensity was lower on D15 compared to D5. And there was no significant change in the *LIF* mRNA level between D5 and D15. At the same time, it appears plausible that the strongly elevated levels of LIF-protein could compensate for low *LIF* mRNA expression levels, locally increasing its relative biological availability.

The endometrium starts to remodel to be prepared for embryo implantation during WOI. Specifically, the endometrial stromal cells (ESC) undergo a marked rearrangement of the intracellular architecture and begin to accumulate glycogen, initiating the secretion of various proteins, growth factors, and cytokines, such as *PRL*, which are induced in ESC during decidualization and are therefore recognized as a marker of decidualization (Gellersen and Brosens 2003). In contrast to human and rodent models, however, canine decidualized cells exhibited very low expression of *PRL* (Kautz et al. 2015). PRL-protein was mainly expressed in the cytoplasm of LE and GE, and in a small amount in SC in dairy goats, notably, there was no difference in LE and GE, but a remarkable increase was found in SC on D15 compared to D5. However, there were no significant changes in the *PRL* mRNA and PRLR protein levels between D5 and D15 in dairy goats.

CONCLUSION

In dairy goats, well-formed pinopodes were found on the surface of endometrium at gestational day 15 (D15) with higher E2 and P4 concentrations in the serum and *ERα*, *ERβ*, and *PR* concentrations in the endometrium. Moreover, some expression levels of marker genes of receptive endometrium increased at D15 compared to D5, but no significant differences were observed in the cell proliferation and apoptosis in the uterus. Based on the results of this study it can be safely concluded that the endometrium developed into the receptive state at gestational D15 in dairy goats.

Acknowledgement. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Received: 2017–02–16
Accepted after corrections: 2017–05–31