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Modulation of Expression, Localization of FSHR and Uterine Development by GnRH Agonist Active Immunization in Ewes

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Key words: Gonadotropin-releasing hormone agonist; FSH receptor; protein expression; microstructure; immunohistochemistry; uterus; ewe

1 SUMMARY

The present study was designed to investigate the effects of gonadotropin-releasing hormone (GnRH) agonist immunization on the expression of follicle stimulating hormone receptor (FSHR) mRNA in the pituitary and FSHR protein in the uteri, also to confirm the efficacy on uterine development. 42 ewes, 5 to 6 months of age, were assigned to 6 experimental groups (EG, n=7 per group). Animals in EG-Ⅰ, EG-Ⅱ and EG-Ⅲ were subcutaneously injected with 200 μg, 300 μg and 400 μg alarelin antigens twice (on day 0, 28 and 14), respectively. Animals in EG-Ⅳ and EG-Ⅴ were subcutaneously injected with 200 μg and 300 μg alarelin antigen, four times (on day 0, 7, 14 and 21), respectively. Animals in the Control Group (CG) were subcutaneously injected with 2.0 mL of GnRH agonist solvent twice (on day 0 and 14). The pituitary and uterine horns were dissected aseptically on day 70 and weighed immediately using an electronic balance. The expression of FSHR protein in the uteri was detected using Western blotting. Fluorescence quantitative RT-PCR was implemented to measure the expression of FSHR mRNA in the pituitary. Tissue slices were observed under light and electron microscopes and photographed. The results showed that alarelin active immunization can promote the expression of FSHR protein in the uteri, increase the localization of FSHR in uteri, and inhibit the expression of FSHR mRNA in the pituitary gland. It can also lessen endometrial epithelium thickness and uterine wall thickness, decrease the uterine weights, similarly affect the microstructure and ultrastructure of uteri, resulting in the inhibition of uterine development dose-dependently.

2 INTRODUCTION

Gonadotropin-releasing hormone analogues (GnRH-A) can increase the binding affinity to GnRH receptors (GnRHR) (Guo and Gao, 2005). GnRH-A have been shown to increase the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in the pituitary gland, and improve the pregnancy rate by 12% to 15% in sheep (Schneider *et al.,* 2006). Sang (2005) reported a quicker estrus and ovulation, a shorter uterine involution and calving intervals in dairy cows after GnRH-A administration. However, the heterogeneous effects are caused by large

doses. The long-lasting treatment with GnRH agonist induces modifications in the pituitary secretion in small ruminants (Gharb *et al*., 2012). Administration of a higher dose of GnRH-A inhibited gonad development, resulting in a contraceptive effect (Leung *et al.,* 2003; Jiang *et al.,* 2010). It is not clear on the exact effects and the mechanism of GnRH analogue on reproductive performance in animals (Bertschinger *et al.,* 2006). Its mechanism is still unclear (Hosseini *et al.,* 2010). GnRH agonist (GnRHa) has been widely used for treating endometriosis, uterine fibroids and infertility (Li and Xu, 2008; Sullivan *et al.,* 2006). Early studies indicated that GnRHa treatment could significantly reduce the uterine growth (Chia *et al.,* 2006; Sun *et al.,* 2007). The uterine volume was reduced by 36% at 12 weeks and 45% at 24 weeks following subcutaneous injection of 3.75 mg of GnRHa, once every 4 weeks, for 24 weeks (Friedman *et al.,* 1991). However, there was no significant difference between degrees of uterine volume reduction following administration of 3.75 mg leuprolide acetate (Jasonni *et al.,* 2001). The uterine reduction was not associated with the doses and times of administrating GnRH agonist (Chia *et al.,* 2006). Alarelin, a nonapeptide GnRH agonist, was applied in gynecological clinics and animal practice for adjusting reproduction because of its easy synthesis and lower cost. Its strength of interaction with GnRHR is 15-20 times higher than the native substance (Wei, 2006). Alarelin active immunization inhibited obviously uterine growth and development, especially the EEC and uterine gland. The effects were dose-dependent (Wei *et al.,* 2012a). However, the quantitative effects of GnRHa (especially alarelin acetate) immunization in the uterine development of ewes at different doses and durations remain still undecided (Chia *et al.,* 2006; Wei *et al.,* 2011). Distribution and the localization of GnRH have been identified in numerous animals such as the chicken, horse, cattle, monkey and pig, using immunohistochemistry (Xia and Huang, 2001). A distinct pattern of localization of gonadotropin subpopulations has been reported within the pituitary gland of sheep (McNeilly *et al.,* 2003). Our previous experiments showed that alarelin active immunization in female rabbits could increase serum FSH and LH levels in experimental groups, and inhibit meaningful expression of GnRHR and FSH-β mRNA in the pituitary gland, when compared to the control group (Wei *et al.,* 2010, 2012a). Furthermore, alarelin active immunization can suppress the expression of GnRHR, FSHR and LHR mRNAs in the pituitary gland of the mice (Wei *et al.,* 2012a). Meanwhile, little is known regarding the uterine localization of FSHR in the sheep (Schirman-Hildesheim *et al.,* 2005; Leonardo *et al.,* 2006). It is indeterminate whether a GnRH agonist influences the expression level of FSHR mRNA and FSHR protein in the uterus of ewes (Crawford *et al.,* 2009). On the basis of previous studies in connection with the immuno-regulations on female rabbits (Gong *et al.,* 2010; Wei *et al.,* 2010; Wei *et al.,* 2011a), the present study aimed to explore the effects of the GnRH agonist immunization on the expression of FSHR mRNA in the pituitary and FSHR protein in the uteri, to investigate the relation between the expressions levels of FSHR mRNA in pituitary and FSHR protein in the uteri, also to confirm the efficacy of the uterine histological structure and development of uteri in ewes, to expound the mechanisms of the GnRH agonist in modulating the uterine development, so as to offer the scientific basis for applying GnRH-A to treat uterine diseases and improve the reproduction functions in ewes.

3 MATERIALS AND METHODS

3.1 Animals and experimental design: 42 pre-pubertal ewes (5 to 6 months-old and body weight of 24.21±2.51 kg), the first generation of the domestic sheep in the Yuzhong country of China hybridized with small-tail sheep, were randomly assigned to six groups (*n=7* per group), namely the experimental group 1 (EG-Ⅰ), experimental group 2 (EG-Ⅱ), experimental group 3 (EG-Ⅲ), experimental group 4 (EG-Ⅳ), experimental group 5 (EG-Ⅴ) and the

control group (CG). Ewes were determined healthy through a thorough physical examination and serum biochemical and hematologic analyses. Throughout the study period, health status of the ewes was monitored daily by observation of their behavior and feed intake. Preparation of the alarelin antigen emulsion was performed according to the previous report (Wei and Zhang, 2008). The concentration of alarelin was 100μg/mL. Animals in EG-Ⅰ, EG-Ⅱ and EG-Ⅲ were subcutaneously injected with 200 μg, 300 μg or 400 μg alarelin antigens twice (on day 0 and 14), respectively. Ewes in EG-Ⅳ and EG-Ⅴ were subcutaneously injected with 200 μg and 300 μg alarelin antigen four times (on day 0, 7, 14 and 21). Animals in the CG were subcutaneously injected with 2.0 mL of a solvent twice (on day 0 and 14). The experiment was conducted over a period of 70 days on the basis of GnRH antibody duration in a previous study (Wei and Zhang, 2008). Animals were fed hay and a commercial concentrate diet, ad libitum. All experimental procedures on animals were conducted according to the Regulations for the Administration of Affairs Concerning Experimental Animals in China. The solvent was also prepared (only excluding alarelin acetate) as the same method described above.

3.2 Collection of samples: Blood samples were obtained from the jugular vein on day 0, 7, 14, 21, 28, 35, 45, 60 and 70 following the first airline antigen injection. Serum for measurement of FSH concentration was separated through centrifugation and stored at -20 ˚C until analysis. The animals were heavily sedated by injecting 0.2mg/kg xylazine intramuscularly on day 70, then euthanatized by exsanguination from the common carotid for each ewe. The pituitary gland and uterine horns were harvested aseptically from each ewe. After bilateral uterine horns were harvested aseptically, then each sample in each ewe was immediately weighed using an electronic balance, and the uterine index was calculated. The uterine index in each ewe was equal to the average weight of both right and left uterus divided by her body weight on day 70. The pituitary gland and uterine horns in each ewe was separated into three parts. Two parts were fixed in 10% formaldehyde (Yuexin Company, Guangdong, China) and 3% glutaric dialdehyde (Xinweihua company, Jiangsu, China), respectively. The third part was stored in liquid nitrogen.

3.3 Western blotting analysis of FSHR protein in uteri: To evaluate the FSHR expression of the uteri of ewes in protein level after alarelin active immunization, the Western blotting was performed. Briefly, the ovary samples were lysed in buffer (0.5% Nonidet P (NP) 40, 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM ethylene diamine tetra-acetic acid [EDTA], 1 mM Na3VO4) containing protease inhibitor (1 mM phenylmethylsulfonyl fluoride [PMSF]). Proteins were loaded on a 10% SDS–PAGE, then transferred to polyvinylidene fluoride (PVDF) membranes and blocked in 5% non-fat milk in 10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% (w/v) Tween 20 for 2 h. Rabbit anti-sheep FSHR polyclonal antibodies (Sigma, USA, 1: 200) and β-actin polyclonal antibody (1:1000) were diluted and incubated at 4˚C overnight, followed by 1 h incubation with the appropriate secondary antibody (1:500). Anti-β-actin mouse monoclonal antibody was diluted in 1:10000 for sample loading control. Blots were further developed using the chemiluminiscence reagent (SuperSignal West Pico, Rockford, IL). The integral optical density (IOD) of the scanned band images was done by using Quantity One software (Bio-Rad Company, USA). The relative contents of FSHR proteins were presented as the ratio between gray values of FSHR divided by that of β-actin. The experiments were repeated three times.

3.4 Fluorescence quantitative RT-PCR (FQ-PCR)

3.4.1 Primer design: Primers of GAPDH (GenBank accession number: HM-043737.1) and FSHR (GenBank accession number: NM-001009289) were designed using Primer Premier 5.0 software. The concentrations of primers (100 nM, 200 nM, 300 nM and 500 nM) were evaluated, and formation of primer-dimers was assessed using the melting curve analysis. Thus, only those concentrations of primers which showed dimer-free reactions were used for the final analysis. Primers and probes were synthesized by Takara Bio, Dalian, China (Table 1).

Table 1: Primers of FHSR and GAPDH mRNAs for FQ-PCR

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Gene	Primer Sequence (5'-3')	Tm	bp
FSHR	Forward TCTTTGCTTTTGCAGTTGCC	59.1	126
	Reverse GCACAAGGAGGGACATAACATAG	58.4	
GAPDH	Forward CTTCAACAGCGACACTCACTCT	57.1	152
	Reverse CCACCACCCTGTTGCTGTA	57.0	

3.4.2 RNA extraction and cDNA synthesis: About 100 mg of pituitary gland in each ewe was used for total RNA extraction using the TRIzol reagent (Invitrogen, Beijing, China), according to the manufacturer's instructions. Total RNA was treated with the gDNA wipeout buffer, supplied with the QuantiTect reverse transcription kit (Qiangen, Beijing China) to remove traces of genomic DNA contamination. Assessment of RNA quality was performed using a 1.2% agarose gel containing ethidium bromide (EB) and photographed with the Bio-BEST 140E imaging system (SIM company, USA). RNA samples were quantified using a Nanodrop spectrophotometer (Zhiyan Company, Shanghai, China). The absorbance ratios of 260/280 nm in all samples were more than 1.9, indicating the high RNA purity. cDNA was synthesized with the superscript™ III first-strand synthesis system for RT-PCR (Invitrogen, Beijing, China), according to the manufacturer's instructions. The resulting single stranded cDNA products were quantified using a Nanodrop spectrophotometer (Zhiyan Company, Shanghai, China), and then diluted 50-fold with deionized water prior to use them as templates for the FQ-PCR reactions.

3.4.3 Fluorescence quantitative RT-PCR (FQ-PCR): The expression levels of FSHR mRNA were determined using fluorescence quantitative RT-PCR (FQ-PCR). Gene amplifications by FQ-PCR were performed using a SLAN thermocycler (Hongshi, Shanghai, China). Each 25 µL reaction volume in a 96-well plate was comprised of 4 µL of a 50× diluted cDNA template, 1 µL of each primer pair at 10 µM, 0.25 µL probe at 10 µM, buffer, dNTP and SYBR® Green II (Promega, Beijing, China). Plates were sealed with adhesive optical film (Promega, Beijing, China). After an initial denaturation step of 15 min at 95 °C, 44 cycles of amplification were performed on the basis of the following thermo cycling profiles: denaturation for 30 s at 95 °C, annealing for 20 s at 60 °C and extension for 20 s at 72 °C. Fluorescence data were acquired during the last step. A dissociation protocol with a gradient from 65 °C to 97 °C was used to investigate the specificity of the FQ-PCR reaction and the presence of primer dimers. Gene expression levels were recorded as the threshold cycle (C_T) values that corresponded to the number of cycles at which the fluorescence signal could be detected above the threshold value - arbitrarily set to 0.3. GAPDH was used as an endogenous control. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative values of mRNA abundances, compared to the CG. The samples were run triplicate.

3.4.4 Immunohistochemistry (IHC) and immunostaining evaluation : *IHC* was utilized to localize the FSHR in the uteri and confirm the presence of the FSHR in those cell types corresponding to FSHR. Tissue samples fixed in 10% formaldehyde were embedded with paraffin wax and sliced with a slicer. Paraffin-embedded sections (5 µm) of the uteri were immunostained for visualization of the FSHR. IHC was performed utilizing sheep polyclonal anti-FSHR antibody (diluted 1:200). After deparaffinized and hydrating the sections, they were stained as follows: Sections were incubated in H_2O_2 (1:10 in methanol) for 15 min to remove endogenous peroxidase prior to block them in 0.5% BSA-PBS/TBS for 20 min. Specimens were incubated with the primary antibody (1:100 in PBS) for 105 min, following incubation with a rabbit anti-sheep horseradish peroxidase conjugate (1:300 in PBS) for 30 min. Staining was administered by incubation with diaminobenzidine (DAB) for approximately 20 min. In each incubation step, the slides were washed twice in PBS; each for 5 min. Hematoxylin was used as a counterstain. Positive staining was defined microscopically by visual identification of brown particles. Negative specimens were processed in the absence of the primary antibody.

When the brown particles were observed in the slices under the optical microscope, FSHR-immunostained cells were recognized to be positive. Based on Wibur's method regarding the estrogen and progesterone receptor detection in paraffin embedded tissue from breast carcinomas (Wibur *et al.,* 1992), the expressive intensity was determined. Five slides for each ewe were microscopically evaluated and pictures photographed (×100 and ×400). Labeling intensities

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of the immunostained cells were scored as absent (-), weak (+), moderate (++) or strong (+++).

3.4.5 Histological observation and image measurement of uteri： The tissue samples fixed in 10% formaldehyde were embedded with paraffin wax, sliced (5 μm) and stained with hematoxylin and eosin (H&E). The sections were observed under the light microscope (Leica, Japan) and electron microscope (JEOL, Japan). Microscopic images of the uteri were photographed. Five sites in each section (4 sections in every group, totaling 140 sites for each group) were measured. The data of uterine wall thickness (UWT) and endometrial epithelium thickness (EET) were measured using Images Advanced 3.2 and Image ProPlus 2.0 (MOTIC Company, Hong Kong, China). The correlations between the averages of left uterus weights (g), right uterus weights (g), UWT (μm), EET (μm), and expression of FSHR were analyzed using the Pearson's model of SPSS 18.0, respectively.

3.4.6 Statistical analysis： The results were presented as the means ± SEM. Statistical analysis was performed using SPSS v. 18.0 (SPSS Inc. Chicago, IL, USA). After a square root transformation of the data, all variables complied with the assumptions for a one-way ANOVA. Pearson's model was used to analyze the correlations between FSHR expression and uterine growth. When significant differences were identified, supplementary Tukey's post-hoc tests were performed to investigate pairwise differences. $P<0.05$ was considered to be significant.

4 RESULTS

4.1 Expression of FSHR protein in the uteri of ewes: FSHR was detected at the protein level by Western blotting in all samples of the uteri of ewes following alarelin active immunization. Expression levels of FSHR protein in five experimental groups (EGs) tended to increase along with the alarelin immunization dosages compared to CG. Meanwhile, expression levels of FSHR protein in EGs were higher than that of CG ($P<0.05$ or $P<0.01$). Expression levels of FSHR protein in EG-Ⅲ and EG-Ⅴ were higher than ($P<0.01$) that in CG (Fig. 1). This illustrated that alarelin active immunization can promote the expression of FSHR proteins in uteri of ewes.

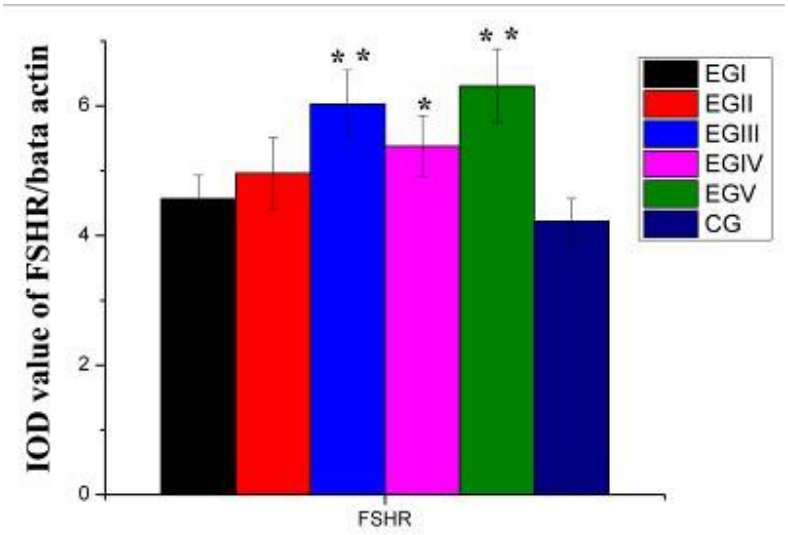

Fig. 1: Expression of FSHR protein in the uteri of ewes following immunization with alarelin, * indicates the difference was significant ($P<0.05$) when compared to the control group (CG); ** indicates the difference was highly significant ($P<0.01$) when compared to the control group (CG).

Expression of FSHR mRNA in the pituitary: The expression levels of FSHR mRNA in five EGs decreased. FSHR mRNA expressions were significantly reduced by 58% ($P<0.05$), 88% ($P<0.01$) and 91% ($P<0.01$) in EG-Ⅰ, EG-Ⅱ and EG-Ⅲ, compared to the CG (Fig. 2). Meanwhile, FSHR mRNA expressions in EG-Ⅳ and EG-Ⅴ were

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reduced by 64.29% (P<0.05) and 10.11 (P<0.01), compared to the EG-Ⅰ and EG-Ⅱ (Fig. 2).

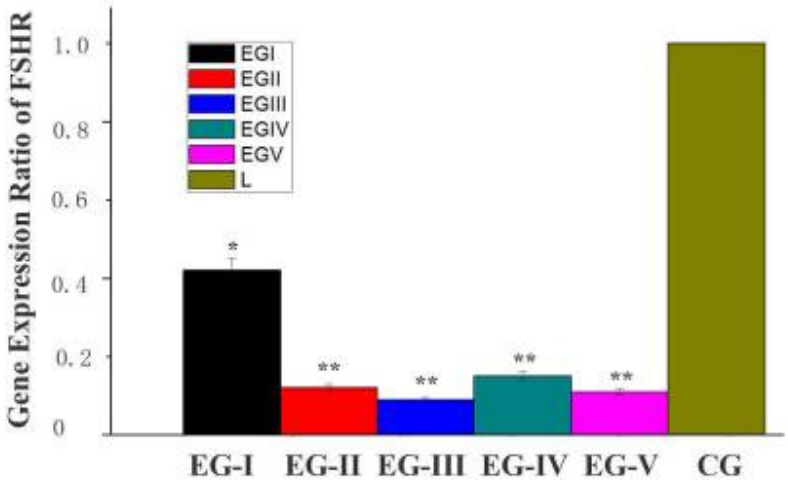

Fig. 2: Expression of FSHR mRNA in the pituitary gland of ewes
* indicates the difference was significant (P＜0. 05) when compared to the control group (CG); ** indicates the difference was highly significant (P＜0. 01) when compared to the control group (CG).

The expression level of FSHR mRNA in EG-Ⅰ was higher than that in EG-Ⅱ and EG-Ⅲ (P<0.05). Furthermore, FSHR expression level in EG-Ⅱ was higher than that in EG-Ⅲ. The results showed a dose-dependent ability of alarelin to inhibit the expression of FSHR mRNA in the pituitary gland of ewes. Immunolocalization of FSHR in uteri of ewes: Immunohistochemistry showed the positive immunostaining for FSHR were present in the ewe's uteri and distributed predominantly in the cytoplasm and nuclei of the uterine endometrial cells (UEC) and glandular epithelial cells (GEC) that had distinct immunostaining intensities (Fig. 3). Little difference was recorded regarding the immunostains between slides in the same animals. The negative control slides were lacked of immunostaining.

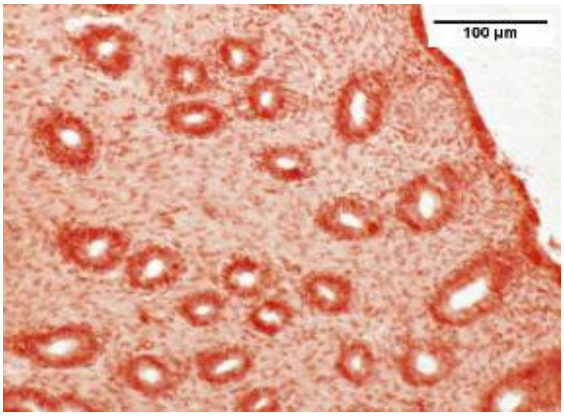

Fig. 3: Immunohistochemstry of FSHR in the uteri of ewes following alarelin immunization

The moderate (++) immunostains in EG-Ⅰ were observed in the glandular epithelium cells (GEC). Uterine endometrial cells (UEC) were immunostained weakly (+). The cytoplasm and nuclei of UEC and GEC in EG-Ⅱ and EG-Ⅳ were FSHR-immunostained moderately (++). The immunostaining intensities in EG-Ⅳ were slightly more than that in EG-Ⅱ. UEC and GEC in EG-Ⅲ

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and EG-Ⅴ were FSHR-immunostained strongly (+++). The cytomembrane was immunoreacted moderately (++). Meanwhile, the immunostaining intensities in EG-Ⅴ were more than that in EG-Ⅲ. The cytoplasm and nuclei of UEC and GEC in the control group (CG) were FSHR-immunostained weakly (+). The results demonstrated that the maximum immunostains were observed in the uterine slides of EG-Ⅲ and EG-Ⅴ. Alarelin active immunization can enhance distribution and expression of FSHR in uteri of ewes.

4.2 Uterine microstructure changes in ewes：

4.2.1 Control group: The uterine endometrium in CG was smooth and flat. The simple columnar epithelia arranged regularly with the sparse epithelial cells. The microvilli were not seen. The stromal cells proliferated. There were fewer glands and blood vessels. There was no secretion in the gland lumen. However, a lot of spiral arteries were observed. (Fig. 4 F)

4.2.2 EG-Ⅰ: Compared to the control group, the uterine lumens became narrow slightly. The uterine wall and endometrium were thinned as well. The cytoplasm of endometrial epithelial cells decreased slightly. The uterine glands increased and the mucous ruffled. (Fig. 4 A)

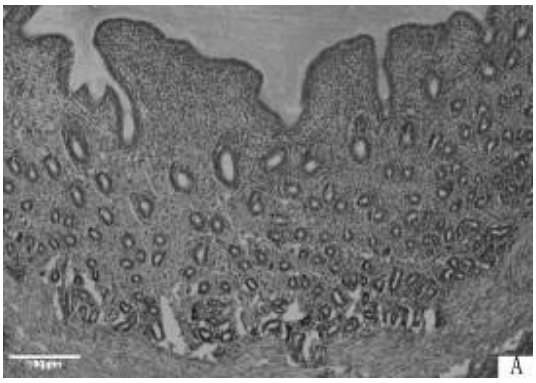
4.2.3 EG-Ⅱ: The uterine section reduced. The uterine wall and endometrial epithelium were thinned obviously. The amount of endometrial ruffles lessened. The cytoplasm of endometrial epithelial cells reduced. The cavities of uterine glands in EG-Ⅱ were smaller than that in CG. (Fig. 4 B)

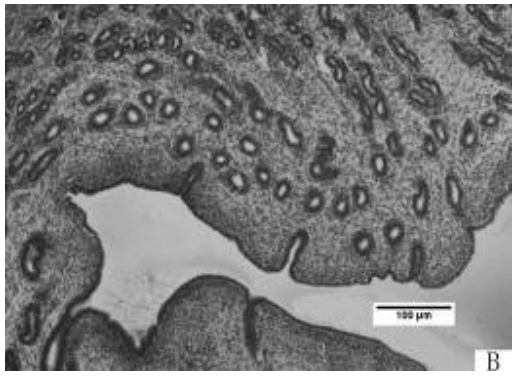
4.2.4 EG-Ⅲ: In comparison with the EG-Ⅱ, the uterine wall was thinner, the number of uterine glands reduced. The glandular cavity contracted apparently. The endometrial epithelium was thinned obviously. Less epithelial cells were observed in EG-Ⅲ. The cytoplasm of endometrial epithelial cells decreased remarkably. The endometrial plica disappeared nearly. The smooth muscle cells and their nuclei were smaller. Uterine gland ducts wrinkled and the ruffles reduced considerably. (Fig. 4 C)

4.2.5 EG-Ⅳ: The lumen of the uteri and their glands contracted. The number of uterine glands and endometrial plicas reduced. No secretion was found. (Fig. 4 D)

4.2.6 EG-Ⅴ: The uterine wall thinned obviously, especially seen in the endometrium. The endometrium had irregular structure, the epithelium also ranged irregularly with few glands. The number of endometrial plica reduced apparently. The uterine cavity contracted. The smooth muscle cells and their nuclei were smaller sharply than that in CG and EG-Ⅲ. (Fig. 4 E)

Evidences testified that alarelin immunization may shrink obviously the uterine wall, especially the endometrial epithelium, reduce the number of uterine glands and reduce the glandular cavity. Such it affected the histological structure of experimental groups, so as to inhibit the development and growth of the uteri of ewes.





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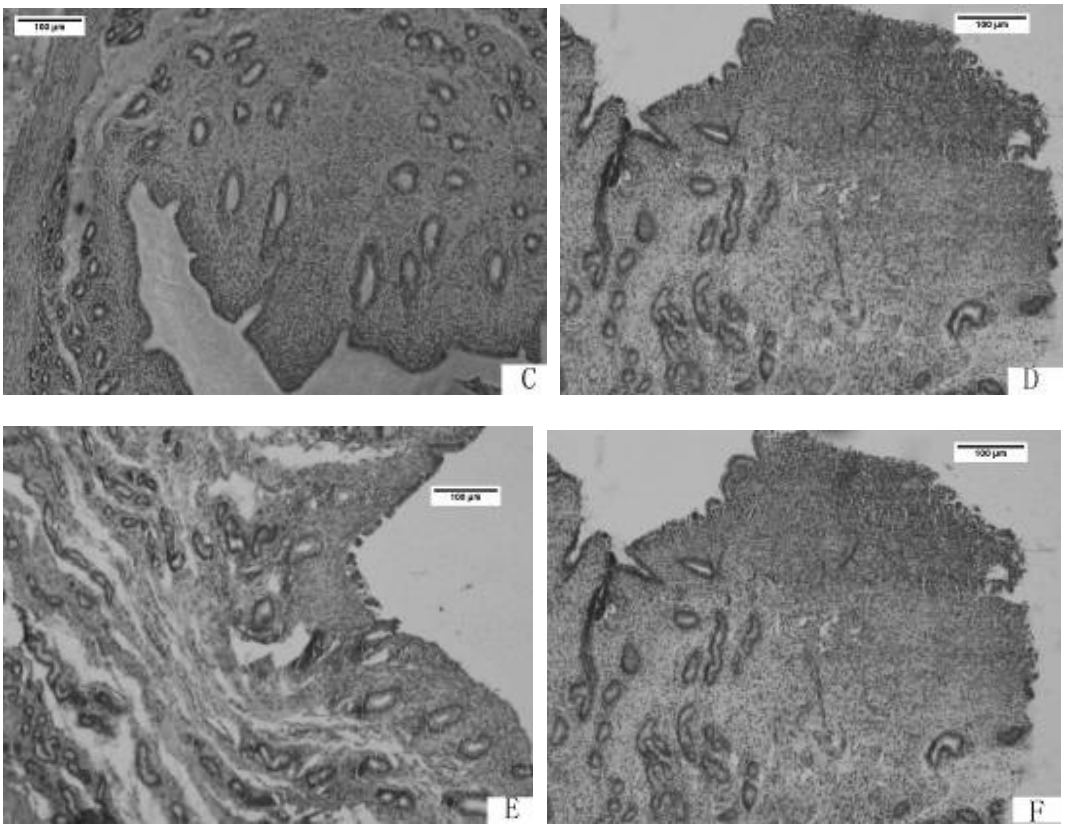

Fig. 4: Histological changes of uteri under optic microscope in ewes (×400)
A, B, C D, E and F represent EG-Ⅰ, EG-Ⅱ, EG-Ⅲ, EG-Ⅳ, EG-Ⅴ and CG groups, respectively.

4.3 Ultrastructure changes of the uteri of ewes：

4.3.1 CG: The epithelial cells were secretary cells. The columnar cells of uterine glands were arranged regularly. The rich organelles, secretory granules and glycogen granules were observed. Few vacuoles were visible in the slides. The structures of mitochondria, mitochondrial crista and microvilli were clear. The karyotheca and nucleoli were also seen prominently.

4.3.2 EG-Ⅰ: Compared to CG, The organelles, secretory granules and glycogen granules were observed too. The Golgi body developed insufficiently. The microvilli shortened and chromatins became sparse.

4.3.3 EG-Ⅱ: The chromatins became apparently sparse. The secretory granules and glycogen granules scattered. The lysosomes contained numerous vacuoles were distributed sporadically. The mitochondria, mitochondrial crista and rough endoplasmic reticulum (RER) became less. A few karyothecas invaginated slightly and the microvilli shortened.

4.3.4 EG-Ⅲ: The columnar epithelial cells arranged irregularly and organelles became less. The mitochondria and mitochondrial cristae reduced obviously and microvilli shortened. The RER decreased. The secretory granules and glycogen granules nearly disappeared. The structure of the critique was clear.

4.3.5 EG-Ⅳ: Compared to EG-Ⅰ, less secretory granules and scarcer chromatins were observed. A lot of vacuoles existed in cytoplasm. The mitochondria and mitochondrial cristae reduced and microvilli shortened further.

4.3.6 EG-Ⅴ: Compared to EG-Ⅱ, the chromatins became sparser. The lysosomes contained numerous vacuoles were distributed sporadically. The mitochondria and RERs were reduced further. The mitochondrial crista disappeared nearly. The microvilli shortened still more. The findings showed that alarelin antigen immunization can lead to uterine ultrastructure changes obviously, decrease the quantities of mitochondria, mitochondrial crista and

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microvilli, also shorten the microvilli, thereby it inhibited the uterine development of ewes.

4.4 Measurements of uterine weights, UWT and EET in ewes: In comparison with the CG, the bilateral uterine weights, EET and UWT in all EGs reduced remarkably along with the alarelin doses and frequencies (Table 2). Left uterine weights in EG-Ⅰ, EG-Ⅱ and EG-Ⅲ reduced by 4.66%, 10.20% (P<0.05) and 16.63% (P<0.05), respectively. UWT in EG-Ⅰ, EG-Ⅱ and EG-Ⅲ reduced by 1.41%, 5.84% and 8.75% (P<0.05). EET in EG-Ⅰ, EG-Ⅱ and EG-Ⅲ decreased 2.70%, 8.87% and 19.15% (P<0.05). Compared to EG-Ⅰ and EG-Ⅱ, the bilateral uterine weights, UWT and EET in EG-Ⅳ and EG-Ⅴ reduced further. They were also less than that in CG (P<0.05). The results indicated that alarelin treatment can decrease uterine weights, EET and UWT, significantly inhibit the uterine development of ewes, in particular, the development of endometrial epithelial cells. The effects were dose-dependent.

Table 2: Measurements of uterine weights, UWT and EET (g, μm)

Group	Left uteri	Right uteri	UWT (μm)	EET (μm)
CG	4.51±0.76^{a}	4.59±0.65^{a}	571.2±36.9^{a}	77.8±9.7^{a}
EG-Ⅰ	4.30±0.41^{a}	4.35±0.96^{a}	563.1±30.9^{a}	75.7±6.0^{a}
EG-Ⅱ	4.05±0.33^{a}	4.07±0.97^{a}	537.8±36.2^{a}	70.9±9.5^{a}
EG-Ⅲ	3.76±0.92^{b}	3.71±0.46^{b}	521.2±32.0^{b}	52.9±7.5^{c}
EG-Ⅳ	4.09±0.83^{a}	4.05±0.68^{a}	528.1±47.8^{b}	48.5±8.4^{c}
EG-Ⅴ	3.60±0.89^{b}	3.49±0.95^{b}	517.1±72.4^{b}	47.4±7.9^{c}

Note: The slices were observed under optical microscope (×400). UWT—uterine wall thickness; EET—endometrial epithelium thickness.

Compared to control group, the same superscript letters in the same column mean that there was no significant difference (P>0.05). The different superscripts mean that there were significant differences between groups, of which adjacent superscripts (such as ab, bc) indicate the difference was significant (P＜0.05), while interval superscripts (such as ac, bd) show the difference was highly significant (P＜0.01).

4.5 Correlations between protein expressions and uterine development: Pearson's correlation analyses demonstrated that a significant positive correlation between left uterine weight and UWT (r=0. 939, P<0.01), EET (r=0. 819, P<0.05) and expression of FSHR mRNA (r=0. 822, P<0.05) were calculated (Table 3). The equivalent findings were calculated for the right uterine. Namely, the decrease of uterine weight is due to the reduction of UWT and EET. There was the negative correlation between uterine weights and FSHR protein in the uteri. The expression level of FSHR mRNA in the pituitary has a negative correlation and FSHR protein in the uteri.

Table 3: Pearson correlations between FSHR expression and uterine weight (g, μm)

Items	Left uteri	Right uteri	UWT	EET	FSHR Protein
Right uteri	0.998**				
UWT	0.939**	0.948**			
EET	0.819*	0.849*	0.918**		
FSHR Protein	-0.828*	-0.860*	-0.881*	-0.963**	
FSHR mRNA	0.822*	0.818*	0.873*	0.706	-0.438

Note: The images of tissue slices were photographed under optical microscope(×400). UWT—uterine wall thickness; EET—endometrial epithelium thickness.
Correlations were analysed between the means of left uteri weights (g), right uteri weights (g), UWT (μm), EET (μm), FSHR protein and FSHR mRNA.
*Correlation is significant at the 0.05 level (2-tailed).
**Correlation is significant at the 0.01 level (2-tailed).

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5 DISCUSSION

The study of GnRH agonist active immunization in sheep and goats has rarely been reported. The increased expression level of FSHR with higher doses of alarelin also has been seen in studies with ducks (Ni *et al.,* 2007) and ewes (Lopot *et al.,* 2008), but contradiction with the fish wrasse Pseudolabrus sieboldi (Hajime *et al.,* 2011). However, little is known of the expression level of FSHR mRNA in the pituitary (Schirman-Hildesheim *et al.,* 2005; Ni *et al.,* 2007). In the present experiment, the results showed a dose-dependent ability of alarelin to inhibit the expression of FSHR mRNA in the pituitary gland of ewes. The findings need further study.

The expression level of FSHR proteins in the uteri of ewes was studied in the experiment. It is evident from the present study that alarelin active immunity can promote expressions of FSHR proteins in the uteri. The findings are consistent with the work of Lopot *et al.* (2008). The hypothesis that GnRH agonist immunity may affect the expression of hormone receptor proteins in the gonads of ewes was supported. The reason alarelin behaves as an agonist on ovarian gonadotropin receptors is still unclear. Little effect has been elucidated on its molecular mechanism (Rupesh *et al.*, 2005, Leonardo *et al.*, 2006). FSHR has been found to be expressed in multiple tissues, including the uterus and ovary (Murase *et al.,* 2005; Durlej *et al.,* 2011; Sharma *et al.,* 2011). However, it is unknown the FSHR distribution in uteri of sheep. Whether active immunization of a GnRH agonist influences the distribution and localization of FSHR in the uteri and ovaries of ewes is still unclear. The present study demonstrates that the FSHR immunostains localized predominantly in the cytoplasm and nuclei of the UEC and GEC of ewes. Different doses of alarelin immunization showed different staining intensities in FSHR-immunostained cells. The maximum FSHR-immunostains were observed in EG-III and EG-Ⅴ. Alarelin active immunization may enhance distribution and expression of FSHR in uteri of ewes. This is similar to previous results in rabbits (Wei *et al.,* 2011a, 2011b). GnRHa may significantly impact uterine growth and development (Liu and Zhang, 2002; Chia *et al.,* 2006; Sun et al., 2007). The uterine volume decreased following subcutaneous injection of 3.75 mg of GnRHa (Friedman *et al.,* 1991). Early experiments showed the uterine cavity and glandular lumen volumes of mice narrowed and the uterine wall became thinner dramatically, particularly the uterine endometrium (Shunichiro *et al.,* 2006; Li and Xu, 2008). However, there was no significant difference between the efficacies of different doses and durations of GnRHa (Jasonni *et al.,* 2001). Therefore, the reported results varied greatly. The quantitative effects of GnRHa (especially alarelin acetate) on uterine development remain still unknown (Bruno, 2009). The present study demonstrated alarelin immunization may reduce the number of the uterine glands, mitochondria and mitochondrial crista, and shrinks the glandular cavity, also shortens the microvilli, reduce the EET and UWT obviously, so as to suppress the microstructure and ultrastructure, leading to the inhibition of the uterine development of ewes. Therefore, uterine weights in EGs decreased compared to CG. These findings are consistent with reports of Chia *et al.* (2006), Sun *et al.* (*2007*), Jiang *et al.* (2010) and Wei et al. (2010). The results need to be further verified through experiments and clinical applications. The present study showed the positive correlations existed between uterine weights and UWT, EET and FSHR mRNA in the pituitary gland. Namely, the decrease of uterine weight is due to the reduction of UWT and EET. However, there was the negative correlation between uterine weights and FSHR protein in the uteri. Also, the expression level of FSHR mRNA in the pituitary has a negative correlation and FSHR protein in the uteri. So far, similar reports have not been published. Thus, the actual statistical correlations between these parameters need further researches. Our results open a novel thought and method for studying quantitatively the effects of GnRH agonist on the reproductive functions in animals.

6 CONCLUSION

Alarelin antigen active immunization can promote the expression of FSHR protein in the uteri, increase the localization of FSHR in the uteri, and inhibit the expression of FSHR mRNA in the pituitary gland. It can also lessen EET and UWT, decrease the uterine weights, similarly affect the microstructure and ultrastructure of the uterus and suppress the uterine development dose-dependently. This may be used to treat the uterine diseases (such as tumors,

endometriosis and infertility) and improve reproduction functions in ewes.

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