

Effects of GnRH α active immunity on FSHR expression and uterine development in prepuberty and non cyclic ewes

Wei, S.^{1,2}; Gong, Z.^{3*}; Dong, J.²; Ouyang, X.²; Xie, K.⁴ and Wei, M.²

¹The Key Bio-Engineering and Technology Laboratory, National Nationality Commission, Northwest University For Nationalities, Lanzhou, Gansu Province, 730030, China; ²Department of Reproduction Medicine, Life Science and Engineering College, Northwest University for Nationalities, Lanzhou, Gansu Province, 730030, China; ³Department of Gynecology, Medicine College, Northwest University for Nationalities, Lanzhou, Gansu Province, 730030, China; ⁴Department of Histology, Basic Medicine College, Lanzhou University, Lanzhou, Gansu Province, 730030, China

*Correspondence: Z. Gong, Department of Gynecology, Medicine College, Northwest University for Nationalities, Lanzhou, Gansu Province, 730030, China. E-mail: yxgzd578@163.com

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Summary

This study explored the effects of GnRH agonist (GnRH α) immunity on expression of FSHR mRNA in pituitary and FSHR protein in uteri, and also confirmed its efficacy on uterine development. 42 prepuberty and non cyclic ewes were assigned to 6 experimental groups (EG) during anestrus season. Animals in EG-I, EG-II and EG-III were subcutaneously injected with 200, 300 and 400 μ g alarelin antigens (day 0 and 14). Animals in EG-IV and EG-V were subcutaneously injected with 200 and 300 μ g alarelin antigen (day 0, 7, 14 and 21). Uterine FSHR protein expression was detected using Western-blotting. RT-PCR was implemented to measure expression of pituitary FSHR mRNA on day 70 after the immunity. Compared to the CG, pituitary FSHR mRNA levels reduced by 58%, 88% and 91% ($P < 0.01$) in EG-I, EG-II and EG-III. FSHR mRNA in EG-IV and EG-V reduced by 64.29% ($P < 0.05$) and 10.11% compared to EG-I and EG-II. Expression levels of uterine FSHR protein in EG-III and EG-V were higher than that in CG. The uterine glands, mitochondria and mitochondrial crista decreased, and the glandular cavity reduced, also the microvilli shortened. Uterine horn weights in EG-I, EG-II and EG-III reduced by 4.66%, 10.20% and 16.63%, respectively. Uterine wall thickness (UWT) in EG-I, EG-II and EG-III reduced by 1.41%, 5.84% and 8.75% ($P < 0.05$). The endometrial epithelium thickness (EET) in EG-I, EG-II and EG-III decreased 2.70%, 8.87% and 19.15%. The positive correlations between uterine horn weights and UWT, EET and FSHR were calculated.

Key words: Gonadotropin-releasing hormone agonist, FSH receptor, Ultrastructure, Uterus, Ewe

Introduction

Gonadotropin-releasing hormone analogues (GnRH-A) has been shown to increase the release of LH and 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. Administration of a higher dose of GnRH-A inhibited gonad development, resulting in a contraceptive effect (Jiang *et al.*, 2010). It is not clear on the exact effects and the mechanism of GnRH α on reproductive performance in animals (Bertschinger *et al.*, 2006). Its mechanism is still unclear (Hosseini *et al.*, 2010).

GnRH exerts its actions through GnRH receptors (GnRHR). GnRH antibodies combine GnRHR following GnRH α immunity, which can reduce the amounts of GnRHR, and affects the functions of endogenous GnRH (Ni *et al.*, 2007; Bo *et al.*, 2011). Treatments of animals with GnRH or GnRH-A have effects on reproductive organs, which depend on the species, dose, route and the moment of administration (Davis *et al.*, 2003). GnRH antibody can specifically neutralize GnRH in animal bodies so that the synthesis and secretion of FSH and LH

will be influenced since GnRH reduces the amounts of gonad LH and FSH receptors (Wei *et al.*, 2011). GnRH immunity can reduce the expression of pituitary GnRH levels, cause changes in the reproductive hormones and behavior (Bertschinger *et al.*, 2006). Therefore, the suppression of GnRHR is important in regulating the gonadal development and reproductive functions.

Early studies indicated that GnRH α treatment could significantly reduce the uterine growth (Sun *et al.*, 2007). However, the reduction of uteri was not associated to the administrating time of GnRH α (Chia *et al.*, 2006). Alarelin, a nonapeptide GnRH α , was applied in gynecological clinics and animal practice for adjusting reproduction. Alarelin active immunity inhibited uterine growth and development, especially the EEC and uterine gland (Wei *et al.*, 2012). However, the quantitative effects of GnRH α immunity in the uterine development of ewes at different doses and durations remain undecided (Chia *et al.*, 2006; Wei *et al.*, 2011).

Alarelin active immunity can suppress the expression of GnRHR, FSHR and LHR mRNAs in the pituitary gland of mice (Wei *et al.*, 2012). Little is known regarding the uterine localization of FSHR in the sheep (Leonardo *et al.*, 2006). It is uncertain as to whether a GnRH α influences the expression level of FSHR mRNA and FSHR protein in the uterus of ewes (Crawford *et al.*,

2009).

The present study aimed to explore the effects of the GnRHa immunity on the expression of FSHR mRNA in the pituitary and FSHR protein in the uteri, investigate the relation between the expression levels of FSHR mRNA and FSHR protein, confirm the efficacy of the uterine histological structure and development of uteri in ewes, and to expound the mechanisms of the GnRHa in modulating the uterine development.

Materials and Methods

Animals and experimental design

Forty-two prepuberty and non cyclic ewes (*Ovis aries*), 5 to 6 months of age 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 6 experimental groups (EG, n=7 per group). The puberty of this breed was about 7 to 8 months of age. The experiments were carried out from October 2010 to January 2011. Animals in experimental group EG-I, EG-II and EG-III were subcutaneously injected with 200 µg, 300 µg and 400 µg alarelin antigens prepared in our laboratory (Wei and Zhang, 2008), twice (day 0 and 14) to enhance the immune response, respectively. Preparation of the alarelin antigen emulsion was performed according to the previous report (Wei and Zhang, 2008). Animals in EG-IV and EG-V were subcutaneously injected with 200 µg and 300 µg alarelin antigens, four times (day 0, 7, 14 and 21) to enhance the immune response, respectively. Animals in the control group (CG) were subcutaneously injected with 2.0 mL of a solvent, twice (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 following the Regulations for the Administration of Affairs Concerning Experimental Animals in China.

Collection of samples

Blood samples were collected from the jugular vein at day 0, 7, 14, 21, 28, 35, 45, 60 and 70. Serum was separated through centrifugation $3000 \times g$ 20 min and stored at -20°C until processed. Animals were anesthetized by injecting 0.2 mg/kg xylazine intramuscularly and sacrificed by exsanguination from the carotid at day 70. Pituitary gland and uterine horns were harvested aseptically from each ewe. Each sample from each ewe was immediately weighed using an electronic balance, and the uterine indexes were

calculated. Uterine horn index in each ewe was equal to the average weight of both right and left uterine horns divided by body weight at day 70. The pituitary gland and uterine horns in each ewe were divided into three parts separately. Two parts were fixed in 10% formaldehyde (Yuexin Co., Guangdong, China) and 3% glutaric dialdehyde (Xinweihua Co., Jiangsu, China), respectively. The third part was stored in liquid nitrogen.

Western-blotting analysis of FSHR protein in uteri

The Western-blotting was performed. Briefly, the uterine samples were lysed in lysis buffer. 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 secondary antibody (1:500). Anti- β -actin mouse monoclonal antibody was diluted in 1:10000 for sample loading control. The blots were further developed using the chemiluminescence 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 Co., 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.

Fluorescence quantitative RT-PCR (FQ-PCR)

The primers of glyceraldehyde-3-phosphate dehydrogenase (GAPDH, GenBank accession No.: HM-043737.1) and FSHR (GenBank accession No.: NM-001009289) were designed. Primers were synthesized by Takara Bio, Dalian, China (Table 1).

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. cDNA was synthesized with the superscriptTM III first-strand synthesis system for the reverse transcription PCR (RT-PCR) (Invitrogen, Beijing, China). The resulting single stranded cDNA products were quantified using a Nanodrop spectrophotometer (Zhiyan Co., Shanghai, China), and then diluted 50-fold with deionized water prior to being used as templates for the fluorescence quantitative PCR (FQ-PCR), using SYBR Green I as fluorescent.

The expression levels of FSHR mRNA were determined by using FQ-PCR. Gene amplifications were performed using a SLAN thermocycler (Hongshi,

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

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

Shanghai, China). 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. GAPDH was used as an endogenous control. The $2^{-\Delta\Delta C_t}$ method was used to calculate the relative values of mRNA abundances, compared to the CG.

Histological observation and image measurement of uteri

Tissue samples were embedded, sliced (5 μ m) and stained with haematoxylin 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 and Image Pro Plus 2.0 (MOTIC Co., Hong Kong, China). The correlations between the averages of left uterus weights (g), right uterus weights (g), UWT (μ m), EET (μ m), and expression of FSHR were analyzed using the Pearson's model of SPSS 18.0, respectively.

Statistical analysis

The results were presented as the means \pm 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.

Results

Expression of FSHR mRNA in the pituitary

The expression levels of pituitary FSHR mRNA in 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. 1). Meanwhile, FSHR mRNA expressions in EG-IV and EG-V were reduced by 64.29% ($P < 0.05$) and 10.11% ($P < 0.01$), compared to the EG-I and EG-II.

The expression levels of FSHR mRNA in EG-I were 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 negative dose-dependent ability of alarelin to inhibit the expression of FSHR mRNA in the pituitary gland of ewes.

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 immunity. The expression levels of FSHR protein in five EGs tended to increase along with the

alarelin immunity dosages compared to CG. Meanwhile, expression levels of FSHR protein in EGs were higher than that of CG ($P < 0.05$ or $P < 0.01$). The expression levels of FSHR protein in EG-III and EG-V were higher than ($P < 0.01$) that in CG (Fig. 2). This illustrated that alarelin active immunity can promote the expression of FSHR proteins in uteri of ewes.

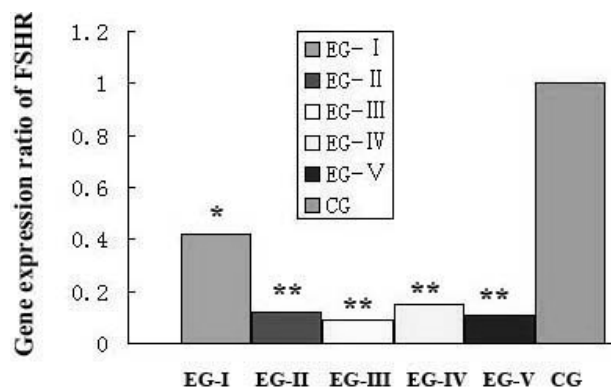


Fig. 1: Expression levels of FSHR mRNA in the pituitary gland of ewes. * indicates the difference was significant ($P < 0.05$) when compared to CG. ** indicates the difference was highly significant ($P < 0.01$) when compared to CG

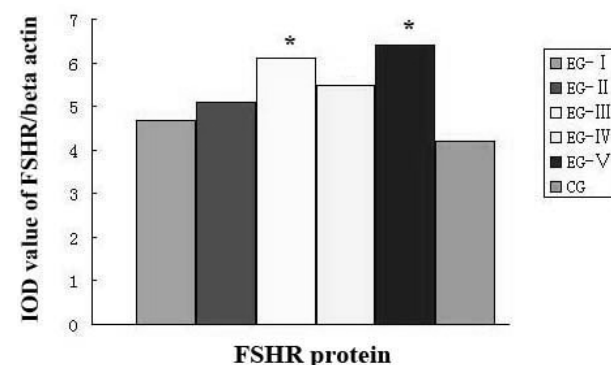


Fig. 2: Expression levels of FSHR protein in the uteri of ewes following immunity with alarelin. * indicates the difference was significant ($P < 0.05$) when compared to the control group (CG)

Ultrastructural changes of the uteri of ewes

CG
The epithelial cells were secretory 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 (Fig. 3A).

EG-I

Compared to CG, the organelles, secretory granules and glycogen granules were observed as well. The Golgi body developed insufficiently. The microvilli shortened and chromatins became sparse (Fig. 3B).

EG-II

The chromatins became noticeably sparse. The

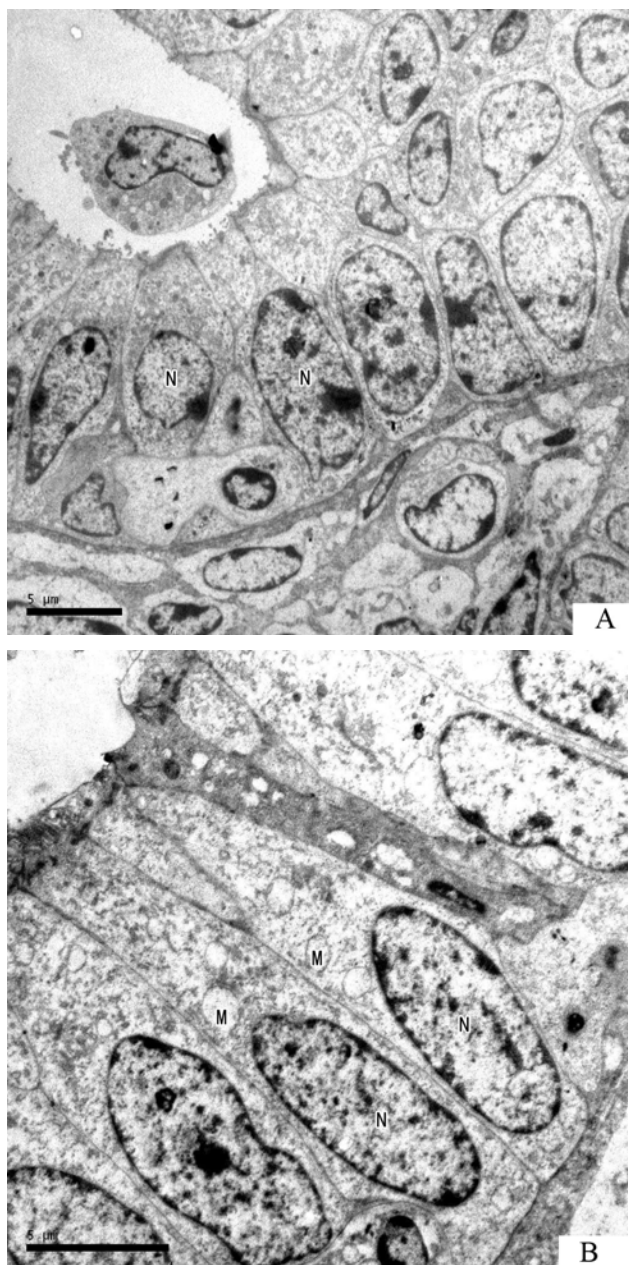


Fig. 3: Ultrastructure of uteri under electron microscope in ewes ($\times 6000$). A and B represent CG groups and EG-I, respectively. N: Nucleus, and M: Mitochondrion

secretory granules and glycogen granules scattered. The lysosomes containing many vacuoles were distributed sporadically. The mitochondria, mitochondrial crista and rough endoplasmic reticulum (RER) became less. A few karyothecas invaginated slightly and microvilli shortened.

EG-III

The columnar epithelial cells were arranged irregularly and organelles became fewer. The mitochondria and mitochondrial cristae reduced obviously and microvilli shortened. The RER decreased. The secretory granules and glycogen granules almost disappeared. The karyotheca structure was clear.

EG-IV

Compared to EG-I, fewer secretory granules and scarcer chromatin were observed. Several vacuoles existed in cytoplasm. The mitochondria and mitochondrial cristae reduced and microvilli shortened further.

EG-V

Compared to EG-II, the chromatin became sparser. The lysosomes containing numerous vacuoles and were distributed sporadically. The mitochondria and RERs reduced further. The mitochondrial crista almost disappeared. The microvilli shortened still more.

The findings showed that alarelin antigen immunity can lead to uterine ultrastructural changes obviously, decrease the quantities of mitochondria, mitochondrial crista and microvilli, also shorten the microvilli, therefore the uterine development of ewes was inhibited.

Measurements of uterine horn weights, UWT and EET in ewes

In comparison with the CG, the bilateral uterine horn weights, EET and UWT in all EGs reduced dramatically along with the alarelin doses and frequencies (Table 2). Left uterine horn 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 horn weights, UWT and EET in EG-IV and EG-V decreased further. They were also less than that in CG ($P < 0.05$). The results indicated that alarelin treatment can decrease uterine horn weights, EET and UWT, significantly inhibit the uterine development of ewes, in particular, the development of endometrial epithelial cells. The effects were dose-dependent.

Correlations between protein expressions and uterine development

Pearson's correlation analyses demonstrating the significant positive correlations between left uterine horn 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). Equivalent findings were calculated for the right uterine horn weight. Namely, the decrease of uterine horn weight is due to the reduction of UWT and EET. There was negative correlation between uterine horn weights and FSHR protein in the uteri. The expression levels of FSHR mRNA in the pituitary have a negative correlation to FSHR protein in the uteri.

Measurements of uterine horn weights, UWT and EET in ewes

In comparison with CG, the bilateral uterine horn weights, EET and UWT in all EGs reduced dramatically along with the alarelin doses and frequencies (Table 2). Left uterine horn weights in EG-I, EG-II and EG-III reduced by 4.66%, 10.20% and 16.63% ($P < 0.05$),

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

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

Note: The slices were observed under optical microscope ($\times 400$). UWT: Uterine wall thickness, and 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 ($P < 0.01$)

Table 3: Pearson correlations between FSHR expression and uterine horn 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 ($\times 400$). UWT: Uterine wall thickness, and EET: Endometrial epithelium thickness. Correlations were analyzed 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)

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 horn weights, UWT and EET in EG-IV and EG-V decreased further. They were also less than that in CG ($P < 0.05$). The results indicated that alarelin treatment can decrease uterine horn weights, EET and UWT, significantly inhibit the uterine development of ewes, the development of endometrial epithelial cells.

Correlations between protein expressions and uterine development

Pearson's correlation analyses demonstrated significant positive correlations between left uterine horn weight and UWT ($P < 0.01$), EET ($P < 0.05$) and expression of FSHR mRNA ($P < 0.05$) were calculated (Table 3). The equivalent findings were calculated for the right uterine horn weight. Namely, the decrease of uterine horn weight is due to the reduction of UWT and EET. The expression levels of FSHR mRNA in the pituitary have a negative correlation to FSHR protein in the uteri.

Discussion

The increased expression level of ovarian FSHR with higher doses of alarelin has been seen in the studies with ducks (Ni *et al.*, 2007) and pituitary FSHR mRNA of ewes (Lopot *et al.*, 2008) but contradict wrasse *Pseudolabrus sieboldi* (Hajime *et al.*, 2011). Little is known of the expression level of FSHR mRNA in the pituitary (Ni *et al.*, 2007). In the present experiment, the expressions of FSHR mRNA in the pituitary and FSHR proteins in uteri of ewes were examined and their

correlations were investigated. The results showed a negative dose-dependent ability of alarelin to inhibit the expression of FSHR mRNA in pituitary gland of ewes. The findings need further study.

GnRHa may significantly impact uterine growth and development (Sun *et al.*, 2007). Early experiments showed the uterine cavity and glandular lumen of mice narrowed and the uterine wall became thinner, particularly the uterine endometrium (Shunichiro *et al.*, 2006). However, there was no significant difference between the efficacies of different doses and durations of GnRHa (Jasonni *et al.*, 2001). The reported results varied greatly. The quantitative effects of GnRHa on uterine development remain unknown (Bruno, 2009). The present study demonstrated alarelin immunity may decrease the number of uterine glands, mitochondria and mitochondrial crista, and shrink the glandular cavity, shorten the microvilli, reduce the EET and UWT obviously, leading to the inhibition of the uterine development of ewes. Therefore, uterine horn weights in EGs decreased compared to CG. These findings are consistent with the reports of Sun *et al.* (2007) and Wei *et al.* (2010). The results require further study.

The present study showed there were positive correlations between uterine horn weights and UWT, EET and FSHR mRNA in the pituitary gland. The expression levels of FSHR mRNA in pituitary had negative correlation and FSHR protein in uteri. So far, similar reports have not been published. Thus, the actual statistical correlations between these parameters require further research. Our results open a novel thought and method for studying quantitatively the effects of GnRHa on reproductive functions in animals and human.

Alarelin active immunity can promote the expression of FSHR protein in uteri, inhibit expression of FSHR

mRNA in pituitary. It can also lessen EET and UWT, affect uterine ultrastructure, which suppress the uterine development dose-dependently. This is significant for the therapy of uterine diseases and for the improvement of reproductive functions in ewes.

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References

- Bertschinger, HT; Jago, I and Nothing, J** (2006). Repeated use of the GnRH analogue deslorelin to down-regulate reproduction in male cheetahs (*Acinonyx jubatus*). *Theriogenology*, 66: 1762-1767.
- Bo, Yu; Jane, R and Gregory, C** (2011). The role of peripheral gonadotropin-releasing hormone receptors in female reproduction. *Fertil. Steril.*, 95: 465-473.
- Bruno, L** (2009). GnRH analogues and cancer: plus a review of the treatment of uterine leiomyomata. *The Proceedings of the 2nd International Symposium on GnRH Analogue (Gnrh Analogues and Cancer)*. Ver. 4, Hardcover. CRC Press. PP: 138-169.
- Chia, CC; Huang, SC and Chen, SS** (2006). Ultrasonographic evaluation of the change in uterine fibroids induced by treatment with a GnRH analog. *Taiwan J. Obstet. Gynecol.*, 45: 124-128.
- Crawford, JL; Heath, DA; Haydon, LJ; Thomson, BP and Eckery, DC** (2009). Gene expression and secretion of LH and FSH in relation to gene expression of GnRH receptors in the brushtail possum (*Trichosurus vulpecula*) demonstrates highly conserved mechanisms. *Reproduction*, 137: 129-140.
- Davis, TL; Mussard, ML; Jimenez-Severiano, H; Enright, WJ and Kinder, JE** (2003). Chronic treatment with an agonist of gonadotropin-releasing hormone enhances luteal function in cattle. *Biol. Reprod.*, 69: 398-403.
- Hajime, K; Susumu, I; Kohei, O; Toshiaki, H; Akihiko, Y and Michiya, M** (2011). Molecular cloning of two gonadotropin receptors and their distinct mRNA expression profiles in daily oogenesis of the wrasse *Pseudolabrus sieboldi*. *Gen. Com. Endocrinol.*, 172: 268-276.
- Hosseini, MA; Aleyasin, A; Saeedi, H and Mahdavi, A** (2010). Comparison of gonadotropin-releasing hormone agonists and antagonists in assisted reproduction cycles of polycystic ovarian syndrome patients. *J. Obstet. Gynaecol. Res.*, 36: 605-610.
- Jasonni, VM; Danna, R and Mancuso, A** (2001). Randomized double blind study evaluating the efficacy on uterine fibroids shrinkage and on intraoperative blood loss of different length of leuprolide acetate treatment before myomectomy. *Acta Obstet. Gynecol. Scand.*, 80: 956-958.
- Jiang, C; Zheng, R and Zhang, Y** (2010). Study on mini-dose depot gonadotropin-releasing hormone agonist for short protocol. *Chin. J. Family Plan.* 7: 423-426.
- Leonardo, GG; Natalia, PM and Adelino, VM** (2006). Evolution of GnRH ligands and receptors in gnathostomata. *Biochem.*, 144: 272-283.
- Lopot, M; Ciechanowska, M and Malewski, T** (2008). Changes in the GnRH mRNA and GnRH receptor (GnRH-R) mRNA levels in the hypothalamus-anterior pituitary unit of anestrus ewes after infusion of GnRH into the third cerebral ventricle. *Reprod. Biol.*, 8: 149-161.
- Ni, YD; Zhou, YC and Lu, LZ** (2007). Developmental changes of FSHR, LHR, ER- β and GnRH-I expression in the ovary of prepubertal ducks (*Anas platyrhynchos*). *Anim. Reprod. Sci.*, 100: 318-328.
- Pengda, S; Xiaopeng, Y; Yichen, Y; Fang, H and Shujie, S** (2007). Efficacy analysis on different dose of GnRH α treating adenomyosis complicated with sterility. *Mod. Diag. Treat.*, 18: 25-26.
- Sang, R** (2005). Application of GnRH and its analogue to animal reproduction. *Chin. J. Anim. Sci.*, 4: 55-58.
- Schneider, F; Tomek, W and Grundker, C** (2006). Gonadotropin-releasing hormone (GnRH) and its natural analogues: a review. *Theriogenology*, 66: 691-709.
- Shunichiro, T; Kentaro, T; Izumi, I; Kazuro, S; Kohji, M and Yoichi, N** (2006). MRI evaluation of the uterine structure after myomectomy. *Gynecol. Obstet. Invest.*, 61: 106-110.
- Wei, S; Bai, J; Gong, Z; Ma, W and Wei, M** (2011). GnRH agonist active immunization influences ovarian development and GnRH receptor mRNA expression levels of pituitary in Japanese white rabbits (*Oryctolagus cuniculus*). *Livest. Sci.*, 139: 222-229.
- Wei, S; Gong, Z; Wei, M; Xie, K and Li, J** (2012). GnRH α active immunization influences gonadotropin receptor expression in pituitary gland, uterine development and secretion of peripheral reproduction hormones in female mice. *Clin. Exp. Med. J.*, 6: 251-262.
- Wei, S; Gong, Z; Wei, M and Zhao, X** (2010). GnRH-A immunization and the changes of reproductive hormone concentration in female rabbits. *Acta Lab. Anim. Sci. Sinica*. 18: 247-250.
- Wei, S and Zhang, J** (2008). Preparation of GnRH-A antigen and researches on the efficacies of active immunization castration with different dosage. *J. NW. Univ. Nation.*, 19: 49-53.