Small Ruminant Research 105 (2012) 237–243

Contents lists available at SciVerse ScienceDirect

Small Ruminant Research

journal homepage: www.elsevier.com/locate/smallrumres

Effect of a GnRH agonist on the FSH receptors in prepubertal ewes

Suocheng Wei[a,*], Zhuandi Gong[b], Jiangling Dong[a], Xiahui Ouyang[a], Min Wei[a], Kun Xie[c], Qiongyi Li[a], Feng Zhang[a], Jianlong Sun[a]

[a] College of Life Science and Engineering, Northwest University for Nationalities, Lanzhou 730030, PR China
[b] Medicine College, Northwest University for Nationalities, Lanzhou 730030, PR China
[c] Basic Medicine College, Lanzhou University, Lanzhou 730030, PR China

ARTICLE INFO

Article history:
Received 29 October 2011
Received in revised form 14 February 2012
Accepted 20 February 2012
Available online 13 March 2012

Keywords:
GnRH agonist
FSH receptor
Gene expression
Immunohistochemistry
Ovary
Ewe

ABSTRACT

The present study was aimed at investigating the effects of the GnRH agonist, alarelin, on the expression of the follicle-stimulating hormone receptor (FSHR) in the pituitary gland. Also to evaluate the presence and immunolocalization of FSHR in the ovary, as well as to confirm the efficacy on the development of the follicles in the ovaries of ewes. Twenty-eight prepubertal ewes (5–6 months of age) were randomly assigned to four experimental groups (EG, $n = 7$ per group). The animals in experimental group EG-I, EG-II and EG-III were subcutaneously injected with 200 μg, 300 μg or 400 μg alarelin antigens twice (day 0 and 14), respectively. Animals in the control group (CG) were twice subcutaneously injected with 2.0 mL of a solvent (day 0 and 14). Samples of the pituitary gland and ovaries were collected aseptically on day 70 following treatment. Blood samples were taken from the jugular vein on day 0, 7, 14, 21, 28, 35, 50, 60 and 70 after the first alarelin antigen injection and ELISA used to measure the serum concentration of the GnRH antibody and FSH. Fluorescence quantitative RT-PCR was implemented to detect the gene expression of FSHR mRNA in the pituitary. Immunohistochemistry was performed to localize the FSHR in the ovary. GnRH antibody concentrations in the EG-I, EG-II and EG-III treatment groups increased gradually and were higher than that of the CG ($P < 0.05$) or control from day 14 to day 60. Pituitary FSHR mRNA levels were significantly reduced ($P < 0.05$) 1.38, 7.33 and 10.11 times in the EG-I, EG-II and EG-III groups, respectively. Immunohistochemistry identified that the immunostained cells of the FSHR were present in the ewes' ovaries, which predominantly concentrated in the cytomembrane, cytoplasm and nuclei of the follicle cells. The oocytes had different immunostaining intensities at different developing stages. The gray values of microscopy images in EG-I, EG-II and EG-III groups increased, when compared to the CG. Ovaries in alarelin treated ewes of groups EG-II and EG-III were significantly higher ($P < 0.05$), when compared to ewes in group EG-I and CG. The follicle vertical diameter (FVD), follicle transverse diameter (FTD), follicle-wall thickness (FWT), follicle externatheca thickness (FET) and follicle internatheca thickness (FIT) in the alarelin immunity ewes were greater than those in the CG. The serum FSH concentrations of the treated ewes remained higher than that in the CG ($P < 0.05$). In conclusion, active immunity with alarelin stimulated the production of the GnRH antibody, inhibited the expression of FSHR mRNA in the pituitary gland, improved the distribution of FSHR in the ovary, increased the FSH secretion and thereby promoted the development of the ovaries and follicles in the ewes. This has important potential for developing a novel technique in the superovulation and regulation of reproductive functions in ewes.

* Corresponding author. Tel.: +86 931 2937773.
E-mail address: weisc668@163.com (S. Wei).

0921-4488/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.smallrumres.2012.02.014

1. Introduction

Studies have shown the GnRH analogue to act on the gonadotrophin cells to influence the differential synthesis, storage and secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Crawford and McNeilly, 2002; Schneider et al., 2006). The administration of GnRH or its analogues (GnRH-A) have been shown to increase the release of LH and FSH in the pituitary gland, and therefore improve the pregnancy rate by 12–15% in sheep (Schneider et al., 2006). Injecting exogenous gonadotrophins in cows on day 15–16 during the estrous cycle has promoted the follicular development and superovulation (Padula and Macmillan, 2005). Sang (2005) reported a quicker oestrus and ovulation, a shorter uterine involution and calving interval in dairy cows after GnRH-A administration. Similarly, Dufour et al. (1999) demonstrated that GnRH agonist immunity increased the number of oocytes recovered in superovulated heifers and ewes. This increase was related to an induced increase in follicular development. However, heterogeneous effects are caused by larger doses. Administration of a large dose of GnRH-A inhibited gonad development, resulting in a contraceptive effect (Leung et al., 2003). GnRH agonist immunity in cows also resulted in the downregulation of GnRH responsiveness and a state of anoestrus (Leung et al., 2003). Thus, it is not clear on the exact effects and the mechanism of the GnRH analogue on reproductive performance in animals (Leung et al., 2003; Bertschinger et al., 2006).

GnRH and its receptor (GnRHR) have been expressed in the ovary, testis, placenta, adrenal and mammary gland (Jennes et al., 1988; Millar et al., 1999). Distribution and the localization of GnRH and GnRHR have been identified in several animals such as the chicken, horse, cattle, monkey and pig, using immunohistochemistry (Xia and Huang, 2001). Little is known regarding the localization of FSH and FSHR in sheep (Schirman-Hildesheim et al., 2005; Leonardo et al., 2006). It is unclear whether a GnRH agonist influences the mRNA expression level of FSHR in the pituitary gland and the localization of FSHR in the ovaries – as well as the synthesis and secretion of the reproductive hormones in ewes (Crawford et al., 2009). On the basis of previous studies (Wei et al., 2010, 2011a), the present study aimed to explore the effects of the GnRH agonist, alarelin on the expression of the FSH receptor (FSHR) in the pituitary gland and immunolocalization of FSHR in the ovaries. Also to confirm the efficacy on the development of ovaries and follicles in ewes and to interpret the mechanisms of the GnRH agonist in regulating reproduction functions, while providing a novel technique for the superovulation and regulation of reproductive functions in ewes.

2. Materials and methods

2.1. Preparation of the GnRH agonist (alarelin) antigen

Dimethylaminopropyl-3-ethylcarbodiimide hydrochloride (EDC·HCL, Shanghai Yeyuan Biochemical Sci-Tech Company, Shanghai, China) was used as a coupling reagent. 20 mg alarelin acetate (Zillion Company, China) and 20 mg bovine serum albumin (BSA, Sigma, USA) were dissolved in 7 mL and 3 mL double distilled water (ddH_2O), respectively. Then mixed thoroughly to form a alarelin–BSA compound. 500 mg EDC·HCL was dissolved in 7 mL ddH_2O added to the above compound. The compound was dialyzed with distilled water for 48 h in a dialysis bag, and ddH_2O added to the compound, so as to make an alarelin concentration of 200 μg/mL. This was emulsified with Freund's incomplete adjuvant (Sigma, USA) in the equivalent proportion (1:1 ratio), to prepare the alarelin antigen emulsion (Wei and Zhang, 2008). The alarelin content of the emulsion was 100 μg/mL. The security and physical properties of the antigen were tested according to the Veterinary Biological Product Quality Inspection. The solvent was also prepared (containing EDC·HCL, BSA and Freund's incomplete adjuvant, excluding alarelin acetate) using the same method as previously described.

2.2. Animals and experimental design

Twenty-eight prepubertal ewes (*Ovis aries*), 5–6 months of age and with a body weight of 24.21 ± 2.51 kg, were randomly assigned to 4 experimental groups (EG, $n = 7$ per group). The animals in experimental group EG-I, EG-II and EG-III were subcutaneously injected with 200 μg, 300 μg or 400 μg alarelin antigen, twice (on day 0 and 14) to enhance the immune response, respectively. Animals in the control group (CG) were twice subcutaneously injected with 2.0 mL of a solvent (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.

2.3. Collection of samples

Blood samples were collected from the jugular vein on day 0, 7, 14, 21, 28, 35, 45, 60 and 70 following alarelin treatment. Serum was separated through centrifugation and stored at −20 °C until processed. Animals were anaesthetized by injecting 0.2 mg/kg xylazine intramuscularly and sacrificed by exsanguination from the carotid, on day 70. The pituitary gland, bilateral ovaries and uterine horns were harvested aseptically from each ewe. Each tissue was divided 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.

After the bilateral ovaries were harvested aseptically, the gonads of each ewe were immediately weighed using an electronic balance, and the ovarian index calculated. The ovarian index in each ewe was determined as the average weight of the right and left ovary, divided by her body weight on day 70.

2.4. Fluorescence quantitative RT-PCR (FQ-PCR)

2.4.1. Primer design

The primers and TaqMan probes of GAPDH (GenBank accession number: HM-043737.1) and FSHR (GenBank accession number: NM-001009289) were designed using Primer Premier 5.0 software. The TaqMan probe was labeled at the 5′ end, with the fluorescent label FAM and at the 3′ end with the fluorescent quencher BHQ1. The concentrations of the 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).

2.4.2. 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 a 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