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## Original Research Article

# Cetrorelix and Triptorelin active immunization influences follicle development and receptor expressions of ovaries in mice



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## ABSTRACT

The study investigated comparatively the effects of active immunization of Cetrorelix and Triptorelin on the follicle development, histological structures, and expressions of FSHR and LHR proteins in the ovary. One hundred and five female mice, 35 days old, and body weight of  $26.56 \pm 2.89$  g, were randomly assigned into Cetrorelix (CET), Triptorelin (TRI) and control groups (CG). Mice in CET-1, CET-2 and CET-3 ( $n = 15$ ) were subcutaneously injected with 0.1, 0.2 and 0.4 mL Cetrorelix antigen (100  $\mu$ g/mL) for seven days, respectively. Mice in TRI-1, TRI-2 and TRI-3 were injected with 0.1, 0.2 and 0.4 mL Triptorelin antigen (100  $\mu$ g/mL) for seven days. Mice in CG ( $n = 15$ ) were injected with 0.2 mL saline for seven days. Western blotting was utilized to determine the expressions of follicle stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) proteins. ELISA was used to measure the serum FSH and LH concentrations. The ovarian slices were observed under optical microscopy. The results showed that the ovarian weights of CET and TRI groups increased slightly with a maximum increment of 27.02% of CET-1 mice on day 35. Ovarian weights in TRI group increased dose-dependently with a maximum increment of 45.77% in TRI-3 on day 35. The numbers of the primordial follicle (POF), primary follicle (PF), secondary follicle (SF) and mature follicle (MF) of CET and TRI groups increased at different degrees when compared to CG. The granular layer in SF was arranged tightly and zona pellucida (ZP) was thickened. Follicles developed fully. Follicle longitudinal diameter (FLD), follicle transverse diameter (FTD) and follicle wall thickness (FWT) in CET and TRI groups increased as compared to CG. FLD, FTD and FWT of TRI-3 had a larger increment than CG and CET-1 on days 21 and 35. Expression levels of FSHR and LHR proteins in TRI group increased when compared to CG. Expression levels of FSHR and LHR proteins of CET group decreased. Serum FSH of TRI group was slightly higher than the CG and CET group on day 21. On day 35, serum FSH levels of CET-1 and TRI-1 were greater than CG and the rest of the subgroups. Serum FSH levels had no

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significant differences among all groups. Expression levels of LHR and FSHR proteins in CET group had obvious negative correlations to FLD, FTD and FWT. However, in the TRI group, these correlations were positive. In conclusion, Cetrorelix and Triptorelin immunization could improve ovarian growth, increase the follicle numbers, enhance dose-dependently the FLD, FTD and FWT, and eventually promote the ovary and follicle development. Cetrorelix decreased expression levels of FSHR and LHR proteins in the ovaries of mice. Triptorelin enhanced expression levels of FSHR and LHR proteins. Triptorelin treatment had more obvious effects than Cetrorelix treatment.

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## Introduction

Gonadotropin releasing hormone agonist (GnRHa) and GnRH antagonist (GnRHant) are widely used for stimulating ovulation and improving pregnancy rate (Bartolome et al., 2005; Sullivan et al., 2006; Marci et al., 2013). They have regulating effects on the ovaries by different mechanisms of action (Meirow et al., 2004; Danforth et al., 2005). GnRH analogues influence ovarian functions (Chryssa et al., 2012; Montorsi and Tomlinson, 2015). Although two drugs share common clinical indications, their mechanisms of action are completely different (Huirne and Lambalk, 2001). GnRHa depressed follicle growth and recruitment, decreased chemotoxic sensitivity, and conserved the ovarian reserve from chemokilling. GnRHant promoted follicle growth, sensitized follicles to chemotoxicity, and helped to exhaust the ovarian reserve (Janssens et al., 2000; Jeruss and Woodruff, 2009). However, a higher dose and long-term treatment showed anti-fertility effect (Jiang et al., 2010). The studies revealed that GnRHant inhibited LH surge and also attenuated the growth of follicles and endometrium (Tarlitzis and Kolibianakis, 2007). On the contrary, low dose of Cetrorelix (a GnRH antagonist) did not prevent a premature luteinizing hormone surge (Kerimoglu, 2014). Thus, the mechanism is still unclear (Hosseini et al., 2010; Montorsi and Tomlinson, 2015).

Acute administration of GnRHant suppresses gonadotropin and sexsteroid secretion rapidly and dose-dependently in animals and in humans (Al-Inany and Aboulghar, 2002; Coccia et al., 2004). Meanwhile, GnRHa and GnRHant had the different regulating effects on the expression of GnRHR-I in ovaries (Mo et al., 2010). Triptorelin (a GnRH agonist) could significantly downregulate GnRHR-I expression in the ovary of female rats, especially in late growing follicles. Cetrorelix slightly decreased ovarian expression of GnRHR-I mRNA and contrarily improved GnRHR-I expression in the growing follicles. Up to date, it is unknown whether GnRHa and GnRHant affect the expression levels of FSHR and LHR proteins of ovaries (Parlakgumus et al., 2015).

GnRH antibodies combine GnRHR following GnRH immunization, leading to gonadotropin releasing hormone receptor I (GnRHR-I) reduction (Ni et al., 2007; Bo et al., 2011) so that the synthesis and secretion of FSH and LH are influenced, resulting in the change of reproductive performance in animals since GnRH declines the amounts of gonad LH and FSH receptors (Bertschinger et al., 2006; Wei et al., 2011). In addition, active immunization with recombinant GnRH-I also reduced the

serum estradiol concentrations as compared with the controls (Liu et al., 2015). The study showed a dose-dependent protective effect of GnRH analogue on ovarian reserve against ovarian toxic chemotherapy, demonstrating an important role of GnRH analogues in fertility preservation (Eman et al., 2013). However, a recent study reported that neither GnRHa nor GnRHant can offer protection against cyclophosphamide-induced ovarian damage since GnRHant reduced the numbers of primordial follicles (Parlakgumus et al., 2015). Triptorelin and Cetrorelix have minor effects on steroidogenesis. Moreover, the primordial follicles in rats were reduced after 35 days of intraperitoneal injection 0.1 mg/kg Cetrorelix (Parlakgumus et al., 2015). The actual effects of both GnRHa and GnRHant on ovaries still need to be further investigated. Thus far, only a few studies have compared the efficacies of GnRHa and GnRHant for ovarian preservation (Danforth et al., 2005; Li et al., 2013). The quantitative effects of Cetrorelix and Triptorelin on ovarian development still remain undecided (Anjum et al., 2012; Wei et al., 2012). Based on our previous studies in immunoregulation of female rabbits and mice (Gong et al., 2011; Wei et al., 2010, 2011, 2013a), the present strictly controlled animal study was conducted to comparatively investigate the efficacy of active immunization against GnRHa (Triptorelin) and GnRHant (Cetrorelix) antigens on development and histological structure of the ovary and serum hormones in mice, and also to assess comparatively the effects of Triptorelin and Cetrorelix on the expressions of FSHR and LHR proteins in the ovaries and additionally, to explore thoroughly the GnRHa and GnRHant mechanisms in regulating ovarian functions.

Summarily, the aims of this experiment are to explore comparatively the effects of GnRHa (Triptorelin) and GnRHant (Cetrorelix) on follicle development and receptor expressions in ovaries, and further to investigate the efficacy on reproduction from the viewpoints of the histology, endocrinology at the levels of molecule, genes and proteins through strictly controlled animal tests by using immunological methods, so as to provide the scientific bases for treating immunologically ovarian diseases with GnRH analogues, and applying them in the assisted reproductive techniques of animals and human.

## Materials and methods

### Animals and ethics statement

The preparations of the Cetrorelix and Triptorelin (Taishi Bio-Technology Co. Ltd., Shanghai, China) antigen emulsion

(100 µg/mL) were performed according to our previous report (Wei and Zhang, 2008). The security and physical property of the Cetrorelix and Triptorelin antigen were tested according to the Veterinary Biological Product Quality Inspection. One hundred and five Kunming female mice (*Mus musculus*), 35 days old and body weight of  $26.56 \pm 2.89$  g, were purchased from Experiment Animal Center, Lanzhou University [License No. SCXK (Gansu) 2005-0007]. All mice were randomly assigned into Cetrorelix (CET), Triptorelin (TRI) and control groups (CG). CET group and TRI group were again classified into three subgroups, namely CET-1, CET-2, CET-3 and TRI-1, TRI-2, TRI-3 ( $n = 15$ ), respectively. Referring to the early reports (Wei et al., 2012, 2013a), the mice in CET-1, CET-2 and CET-3 were subcutaneously injected with 0.1, 0.2 and 0.4 mL (100 µg/mL) Cetrorelix antigen, respectively, once a day for seven consecutive days, to enhance immune response. Mice in TRI-1, TRI-2 and TRI-3 were subcutaneously injected with 0.1, 0.2 and 0.4 mL (100 µg/mL) Triptorelin antigen, once a day for seven consecutive days. Mice in the control group (CG,  $n = 15$ ) were injected subcutaneously with 0.2 mL saline for seven days, once a day. Injections were performed in the morning (at 8–9 AM) each day (Table 1). All mice were accurately weighed each day using an electronic balance, and raised in the group and kept in mice cages equipped with automatic water dispensers under the same conditions in the room maintained at 22–24 °C and 30–50% relative humidity. The light cycle in the room provided 12 h light/day. Mice received a commercial diet (Lanzhou Taihua Feed Co. Ltd, China) depending on their physiological condition. Water was provided ad libitum. The experiment was initiated following a 10-day adjustment period. All procedures referring to animal treatment were approved by the Experiment Animal Care and Use Committee of Gansu province, the People's Republic of China. All mice were treated in the humanitarianism and ethical rules.

### Measurements and sample collections

After 5 mice from each subgroup were anesthetized by injecting 0.1 mg/kg xylazine intramuscularly, they were sacrificed by cervical dislocation on days 0, 21 and 35, respectively. Left and right ovaries of each mouse were dissected aseptically. Each ovary was immediately weighed on an electronic scale respectively. The size of each ovary was measured with a vernier caliper. The average value was calculated from the left and right ovaries for each mouse.

Blood samples were taken aseptically using vacutainers (Zhejiang Gongdong Medical Technology Co Ltd, Zhejiang, China) at the same time on days 0, 21 and 35, respectively. The

samples were allowed to coagulate during 2 h at room temperature, and then centrifuged ( $3000 \times g$ , 20 min); the serum was stored at  $-20$  °C until analysis.

### Histological observations and image measurement of ovaries

The ovary tissues fixed in 10% formaldehyde were sliced (5 µm), and stained with hematoxylin and eosin (H&E). The sections were observed under the light microscope (Leica, Japan). Microscopic images of the ovaries were photographed. Six sites in each section (5 sections in every subgroup, totaling 150 sites for each group) were measured. Data of follicle longitudinal diameter (FLD), follicle transverse diameter (FTD) and follicle wall thickness (FWT) were measured using Images Advanced 3.2 and Image Pro Plus 2.0 (MOTIC Company, Hong Kong, China).

### Western blotting analysis of FSHR and LHR proteins in ovaries

To evaluate the expression levels of LHR and FSHR proteins in the ovary following Cetrorelix and Triptorelin active immunization, Western blotting was implemented referring to the previous description (Wei et al., 2013b). The relative contents of LHR and FSHR proteins were presented as the ratio between gray values of LHR and FSHR divided by that of  $\beta$ -actin. The samples were run in triplicate. A negative control was performed without primary antibody.

### Pearson's correlations analysis

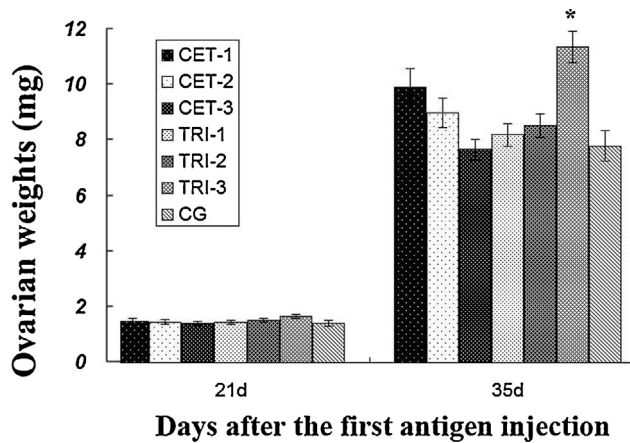
Pearson's correlations were analyzed between the averages 157 of ovarian weights (g), FLD (µm), FTD (µm), FWT FTD (µm), expressions of FSHR and LHR proteins as well as the serum concentrations of FSH and LH.

### Statistical analyses

Statistical analysis was done using SPSS v. 18.0 (SPSS Inc., Chicago, IL, USA). For each subgroup, the averages of all parameters described above were calculated based on the data of 5 mice in each subgroup, respectively. Data are presented as means  $\pm$  SEM. All variables of three groups complied with the assumptions for a one-way ANOVA. Pearson's correlation analysis was utilized to analyze the correlations among the ovarian parameters. When significant differences were identified, supplementary Tukey's post hoc tests were performed to investigate pairwise differences. Data were evaluated at the significance level  $2\alpha = 0.05$ .

**Table 1 – Dosages and frequencies for injecting Cetrorelix and triptorelin antigen in mice.**

Group	Subgroup	Antigen	Dosage	Frequency	Location
CET group	CET-1	Cetrorelix antigen	0.1 mL (10 µg)	At 8–9 AM on day 1–7, once a day for seven times	Subcutaneous injection at the neck
	CET-2	Cetrorelix antigen	0.2 mL (20 µg)		
	CET-3	Cetrorelix antigen	0.4 mL (40 µg)		
TRI group	TRI-1	Triptorelin antigen	0.1 mL (10 µg)		
	TRI-2	Triptorelin antigen	0.2 mL (20 µg)		
	TRI-3	Triptorelin antigen	0.4 mL (40 µg)		
Control	CG	Normal saline	0.2 mL		



**Fig. 1 – Changes in ovarian weight of mice following Cetorelix and Triptorelin immunization. The findings indicated that Cetorelix immunization treatment could promote the ovarian growth of mice. \*Significant as compared with controls (CG).**

## Results

### Measurements of ovarian weights in mice

Compared to the control group, the ovarian weights of CET and TRI groups increased slightly on days 21 and 35 (Fig. 1). Increment degree of ovarian weights in CET group decreased

along with the injection dose of CET antigen. The maximum increment of 27.02% was determined in CET-1 mice on day 35. On the contrary, increment degree in TRI group increased dose-dependently with a maximum increment of 45.77% in TRI-3 subgroup on day 35. The findings indicated that Cetorelix and Triptorelin could improve ovarian growth of mice.

### Histology structures in ovaries of mice

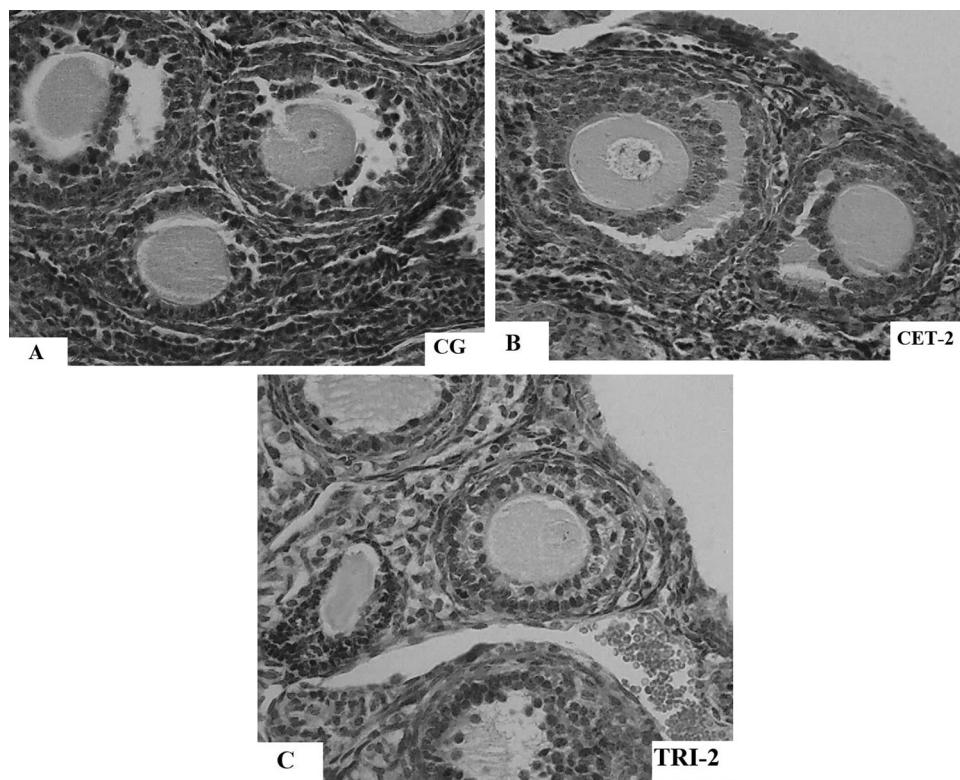
**CG:** The structures of the ovaries and follicles were complete (Fig. 2A). The size of the primordial follicles (POF) and primary follicles (PF) was small. A few mature follicles (MF) existed.

**CET-1:** There were plentiful POF, PF and MF in comparison with CG. Many POF were in the active stage. Loose granular layer distributed in the secondary follicles (SF). The zona pellucida (ZP) was clear.

**CET-2:** POF and PF developed quickly and became abundant (Fig. 2B). Rich SF and MF were distributed. POF in active phase increased in comparison to CET-1. There were few apoptosis follicles.

**CET-3:** Histological changes in CET-3 were highly similar to CET-2. POF was scarcer than in CET-2 (Fig. 2B). A lot of SF and mature MF were observed. They became larger in comparison with that of CG. The granular layer in SF distributed tightly. The zona pellucida (ZP) became thick. Follicles developed well.

**TRI-1:** PF and SF numbers increased as compared to CG. The granular layer fell into follicle fluid. A few SF contain the apoptosed granular cells.



**Fig. 2 – Microstructure changes in the ovaries under optic microscope in mice (400×) The Cetorelix and Triptorelin active immunization can promote the ovary and follicle development and maturation. A, B and C represent CG, CET and TRI groups, respectively.**



**Table 2 – Follicle indexes of mice ( $\bar{X} \pm SD, \mu\text{m}$ ).**

Group	FLD		FTD		FWT	
	21d	35d	21d	35d	21d	35d
CET-1	419.6 $\pm$ 89.2	452.4 $\pm$ 59.5	310.5 $\pm$ 90.0	313.4 $\pm$ 52.1	70.2 $\pm$ 26.5	95.9 $\pm$ 19.6
CET-2	429.7 $\pm$ 51.4	478.6 $\pm$ 70.1	329.3 $\pm$ 83.6	325.5 $\pm$ 21.9	75.2 $\pm$ 23.9	98.5 $\pm$ 12.0
CET-3	446.3 $\pm$ 69.3 <sup>*</sup>	480.1 $\pm$ 60.6	335.4 $\pm$ 79.4	329.6 $\pm$ 58.2	86.3 $\pm$ 30.5	100.2 $\pm$ 20.0
TRI-1	437.3 $\pm$ 66.5	453.2 $\pm$ 81.4	331.1 $\pm$ 32.6	322.4 $\pm$ 68.1	87.0 $\pm$ 12.1 <sup>*</sup>	101.8 $\pm$ 11.8
TRI-2	488.9 $\pm$ 61.2 <sup>*</sup>	499.0 $\pm$ 83.9	364.9 $\pm$ 60.1 <sup>*</sup>	360.2 $\pm$ 107.1	96.3 $\pm$ 48.5 <sup>*</sup>	105.0 $\pm$ 19.0
TRI-3	526.3 $\pm$ 66.5 <sup>*</sup>	540.6 $\pm$ 163.9 <sup>*</sup>	381.1 $\pm$ 32.6 <sup>*</sup>	398.7 $\pm$ 103.9 <sup>*</sup>	106.0 $\pm$ 12.1 <sup>*</sup>	112.5 $\pm$ 34.2 <sup>*</sup>
CG	418.5 $\pm$ 70.6	443.6 $\pm$ 69.5	303.8 $\pm$ 81.7	307.8 $\pm$ 87.6	66.4 $\pm$ 14.5 <sup>*</sup>	90.4 $\pm$ 16.0

Note: FLD, follicle longitudinal diameter; FTD, follicle transverse diameter; FWT, follicle wall thickness.  
<sup>\*</sup> Significant as compared with controls (CG).

**TRI-2:** A larger numbers of SF and MF existed in comparison with TRI-1. The granular layer in SF arranged tightly. ZP was thickened.

**TRI-3:** SF and MF in the ovarian cortex increased in comparison with TRI-2, many of which were in active stage. Follicles developed well (Fig. 2C).

The results demonstrated that both Cetrorelix and Triptorelin active immunization can promote the ovary and follicle development and maturation. The larger the dose is, the higher is the promotion. The Triptorelin treatment had more brilliant effects than Cetrorelix.

#### UWT and EET in mice

As shown in Table 2, the follicle longitudinal diameter (FLD), follicle transverse diameter (FTD) and follicle wall thickness (FWT) in CET and TRI groups increased as compared to CG. FLD of TRI-2 and TRI-3 subgroups were larger than that of CG on days 21 and 35, with the increment of 12.49% and 21.87% on day 35, respectively. They were also higher than that of CET-1 and TRI-1. FTD of TRI-3 was larger than CG on days 21 and 35, with an increase of 29.53% at day 35. FWT of TRI-3 was larger than that of CG on days 21 and 35, with the increments of 59.64% and 24.45%, respectively. The values were likewise greater than that of CET-1.

The findings demonstrated that Cetrorelix and Triptorelin immunization could enhance dose-dependently the FLD, FTD and FWT. In other words, GnRHa and GnRHant promoted follicular development. The effects of Triptorelin were more noticeable than Cetrorelix.

#### Expressions of FSHR and LHR proteins in ovaries

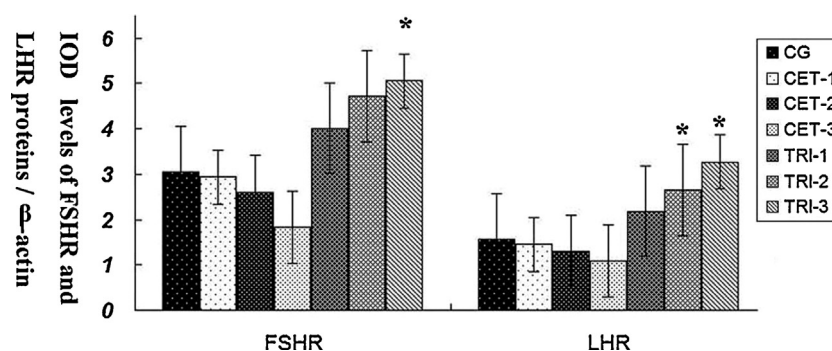
As shown in Fig. 3, the expression levels of FSHR and LHR proteins of CET group decreased along with the antigen dose. The reduction of CET-3 was the maximum. Expression levels of FSHR and LHR proteins in TRI group increased when compared to CG. The increment of TRI-3 was the maximum. The results showed a dose-dependent ability of Triptorelin treatment to improve the expression of FSHR and LHR proteins in the ovaries of mice.

#### Serum FSH and LH concentrations

**Serum LH concentrations:** as shown in Fig. 4, serum LH concentrations in CET and TRI groups decreased on days 21 and 35 as compared to CG, with maximum reduction of CET-3 and TRI-3, respectively. There were no significant differences among other subgroups.

**Serum FSH concentrations:** as shown in Fig. 5, on day 21 serum FSH concentrations in CET groups decreased slightly in comparison to CG. Serum FSH of TRI group was slightly higher than the CG and CET group. On day 35, serum FSH levels of CET-1 and TRI-1 were greater than CG and the rest subgroups. Serum FSH levels of CET-2 and TRI-2 were less than CG and the rest of the subgroups. However, there was no significant difference among all groups.

The findings demonstrated that Cetrorelix and Triptorelin immunization had no obvious effects on LH and FSH secretions.



**Fig. 3 – Expression levels of FSHR and LHR proteins in ovary compared with CG, FSHR and LHR protein levels of the TRI group increased. FSHR and LHR protein levels of CET group reduced.**

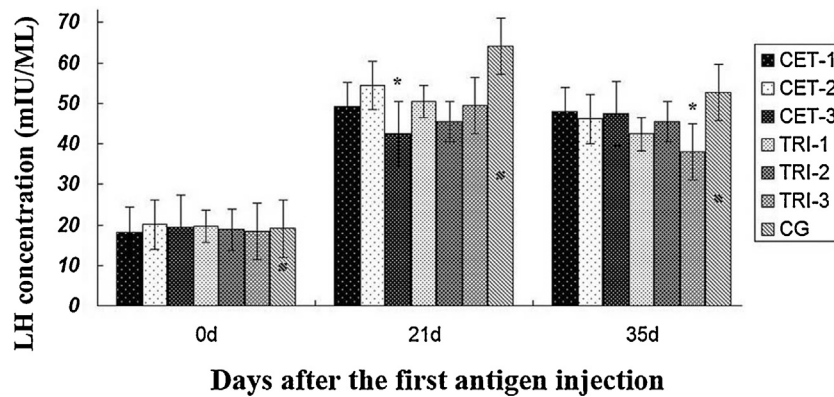


Fig. 4 – Serum LH concentrations in mice following immunization injection. \*Significant as compared with controls (CG).

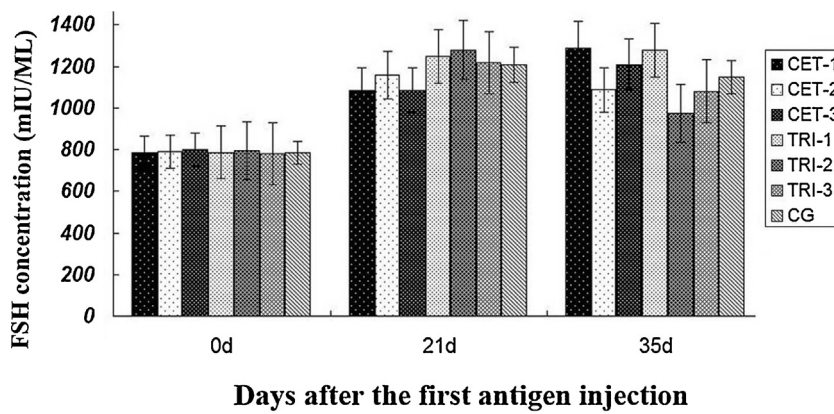


Fig. 5 – Serum FSH concentrations in mice following immunization injection.

#### Correlations between ovarian indexes on day 35

Pearson's correlation analyses indicated that the negative correlations between ovarian weight and expression levels of LHR and FSHR proteins as well as serum FSH and LH contents were found in both CET and TRI groups (Table 3). Similar results were likewise calculated for FLD and FTD.

In CET group, expression levels of LHR and FSHR proteins had obvious negative correlations to FLD, FTD and FWT. However, in the TRI group, expression levels of LHR and FSHR proteins had significant positive correlations to FLD, FTD and FWT, which demonstrated that the expression levels of LHR and FSHR proteins increased dose-dependently following tiptorelin immunization.

## Discussion

#### Effects of Cetorelix and Triptorelin immunization on ovarian development

Early many randomized controlled trials (RCTs) had compared the GnRHa long luteal protocol to the GnRHant protocol both in single (Roulier et al., 2003) and in multiple doses (Albano et al., 2000). The studies indicated that GnRHant promotes the follicle development (Jeruss and Woodruff, 2009) and ovarian

functions (Chryssa et al., 2012; Montorsi and Tomlinson, 2015). GnRHa has inhibitory effects. Such GnRHa was applied to protect the ovarian reserve (Janssens et al., 2000; Jeruss and Woodruff, 2009). This extra-pituitary action of GnRH-I was due to anti-differentiation of granulose cells and depression of steroid synthesis in the ovary (Trimino et al., 1993). The weights of the reproductive organs were greatly reduced after

Table 3 – Pearson's correlations of ovarian indexes of CET and TRI groups on day 35.

Items	Ovary weight	FLD	FTD	FWT	FSHR protein
<b>CET group</b>					
FLD	0.385				
FTD	0.301	0.991*			
FWT	0.448	0.927	0.946		
FSHR protein	0.134	−0.831	−0.893	−0.816	
LHR protein	−0.09	−0.925	−0.966*	−0.919	0.975*
<b>TRI group</b>					
FLD	0.174				
FTD	0.120	0.998*			
FWT	−0.252	0.903	0.927		
FSHR protein	−0.232	0.912	0.930	0.978*	
LHR protein	−0.088	0.964*	0.978*	0.985*	0.977*

\* Data were evaluated at the significance level  $2\alpha = 0.05$ .

30 days of 100 µg/day Cetorelix administration (Horvath et al., 2002). GnRH analogue had a dose-dependent protective effect on the ovarian reserve against ovarian toxic chemotherapy (Eman et al., 2013). Dogs immunized with recombinant GnRH-I showed clear signs of atrophy and significant reduction in the weights and sizes of the ovaries. Neither early antral nor antral follicles were found in the immunized group (Liu et al., 2015). In the present study, the ovarian weights of CET and TRI groups increased slightly on days 21 and 35 when compared to the control group. Increment degrees of ovarian weights in CET group decreased along with the injection dose of CET antigen. Contrarily, increments in TRI group increased dose-dependently. Whether it is associated with the estrus stage and species of experimental animals need to be further investigated. Moreover, its actual clinical efficacy still needs to be verified in the future.

### Effects of on histology structure of ovaries

Few studies reported the histomorphological changes of the ovaries when GnRH or GnRH analogues were used in women and female animals. GnRH facilitates the development of the gonads and follicles and ovulation in rabbits, sheep and pigs after the animals are injected intravenously with GnRH (Zhang, 2007; Wei et al., 2011). Quantitative research on GnRH<sub>a</sub> and GnRH<sub>ant</sub> effects on the development of follicles and ovaries has rarely been reported (Li et al., 2013; Marci et al., 2013; Kerimoglu, 2014).

The findings of the present study indicated that Cetorelix and Triptorelin immunization may dose-dependently promote the ovary and follicle development. Primordial follicles (POF) and primary follicles (PF) developed quickly in Cetorelix- and Triptorelin-immunized mice. Secondary follicles (SF) and mature follicles (MF) became more abundant. They increased numbers of POF, PF and SF, facilitated follicles maturation, thickened zona pellucida (ZP), and also reduced the apoptosis follicles. These lead to increases of FLD, FTD and FWT of CET and TRI mice. Triptorelin is more potent than Cetorelix. It is similar with our early report in the rabbits (Gong et al., 2011) and ewes (Wei et al., 2013a). However, the results were inconsistent with reports of Li and Xu (2008). Whether the results are associated with the species and estrus stages of the experimental animal as well as dosages of GnRH<sub>a</sub> need to be further studied. So far, little information was reported regarding the changes of ovarian histostuctures following Cetorelix and Triptorelin administrations in human and animals (Chryssa et al., 2012; Liu et al., 2015; Montorsi and Tomlinson, 2015). Our findings need to be testified in other animals.

### Effects on expression levels of FSHR and LHR proteins in ovaries

GnRH exerts its actions through specific GnRH receptors (GnRHR), which not only exist in the pituitary gland, but also in healthy tissue of male and female reproductive organs (Seitz et al., 2014). Contrary to GnRH<sub>a</sub>, GnRH<sub>ant</sub> is a competitive inhibitor of GnRH binding to GnRHR (Coccia et al., 2004) and has different receptor occupancy from GnRH<sub>a</sub> with high affinity. Thereby, GnRH<sub>ant</sub> directly suppresses the endogenous GnRH by preventing its binding to GnRHR-I (Halmos and Schally, 2002). However, up to date, little result has been reported regarding the effects of Cetorelix and Triptorelin

immunization on the expressions of FSHR and LHR proteins in the ovary tissues (Soltysik et al., 2014). It is unknown whether Cetorelix and Triptorelin immunization influences the expressions of FSHR and LHR proteins in the ovaries of female animals and humans.

In this study, the expression levels of FSHR and LHR protein levels in CET group decreased dose-dependently with a maximum reduction of CET-3 subgroup. But expression levels of FSHR and LHR proteins of TRI group increased remarkably with a maximum increment of TRI-3. The results demonstrated that Triptorelin treatment enhanced the expression of FSHR and LHR proteins in ovaries of mice. It was consistent with studies in ducks (Ni et al., 2007) and ewes (Lopot et al., 2008; Wei et al., 2013b) following alarelin (a GnRH agonist) immunization. The probable reasons may be that Cetorelix treatment inhibited expressions of pituitary GnRHR mRNA and protein and protein in the rat (Horvath et al., 2002; Mo et al., 2010). Conversely Triptorelin increased it (Magdolna and Andrew, 2001). Meanwhile, Cetorelix (a GnRH antagonist) competitively inhibited GnRH binding to GnRHR (Coccia et al., 2004). The actual mechanism has to be explained with deep comprehensive researches.

### Effects on FSH and LH secretion

FSH and LH are synthesized and stored in the gonadotropin cells under the regulation of multiple mechanisms of GnRH and its agonists (Crawford et al., 2009). GnRH and its analogues decreased LH level of the pituitary gland, caused changes in gonadotropin secretion, eventually leading to a reduction in reproductive performance (Rupesh and Jodi, 2005) since GnRH<sub>a</sub> and GnRH<sub>ant</sub> are possible to prevent untimely LH surge and allow the appropriate development of the leading follicle by suppressing hypophyseal activity (Marci et al., 2013). Intramuscular injection of Cetorelix (100 µg/day) reduced serum LH in female rats (Horvath et al., 2002). FSH and LH levels were obviously reduced after rams and ewes were immunized against GnRH (Clarke et al., 1998). Therefore GnRH immunization resulted in the changes in reproductive hormones and behaviors (Bertschinger et al., 2006).

The present experiment showed that serum LH concentrations in CET and TRI groups decreased slightly on days 21 and 35 when compared to CG. Serum FSH concentrations in CET groups decreased slightly, but TRI group slightly increased on day 35. This is in agreement with reports of rat (Clarke et al., 1998), pigs (Zamaratskaia et al., 2007) and ewes (Khan et al., 2007; Wei et al., 2013a). GnRH<sub>a</sub> immunization enhanced the synthesis and secretion of FSH, which was probably due to an increased feedback from Triptorelin. However, comparative studies of GnRH<sub>a</sub> and GnRH<sub>ant</sub> active immunization were rarely reported (Kåss et al., 2014), and the actual effects of Cetorelix and Triptorelin on the synthesis and secretion of reproductive hormones need to be further investigated.

### Correlations analyses

Pearson's correlation analyses of the data in the present study indicated that the negative correlations between ovarian weight and expression levels of LHR, FSHR proteins as well as serum FSH and LH contents were found in CET and TRI groups. Similar

results were also found for FLD and FTD. Expression levels of LHR and FSHR proteins in TRI group had significant positive correlations to FLD, FTD and FWT. However, in CET group there existed positive correlations between the expression levels of LHR and FSHR proteins to FLD, FTD and FWT. Currently, similar reports have been scarce. These correlation findings need to be explored further in future studies.

## Conclusion

Cetrorelix and Triptorelin immunization could improve ovarian growth, increase the follicle numbers (including POF, PF, SF and MF), enlarge dose-dependently the follicle longitudinal diameter (FLD), follicle transverse diameter (FTD) and follicle wall thickness (FWT), and eventually promote follicle development. Cetrorelix decreased expression levels of FSHR and LHR proteins in the ovaries of mice. Triptorelin enhanced expression levels of FSHR and LHR proteins. Triptorelin treatment had more potent than Cetrorelix. These findings open a novel thought and method for quantitatively studying the effects of Cetrorelix and Triptorelin on the ovarian functions. The findings are also helpful for scientifically and efficiently applying them in the practice of treating ovarian diseases in animals and humans.

## Authors' contributions

Professor Wei Suocheng was responsible for the experimental designs and writing manuscript. Professor Gong Zhuandi raised the experimental animals and took the samples. Professor Ma Zhongren detected the receptor gene expressions. Professor An Lifeng observed the histology structure and determined the ovarian parameters. Miss Zhang Fengwei did the Western blotting assay. Miss Liu Jiankun did the data statistics analyses. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

## Conflict of interest

None of the authors has any potential conflict of interest related to this manuscript.

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