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Original Research Article

Maturation rates of oocytes and levels of FSHR, LHR and GnRHR of COCs response to FSH concentrations in IVM media for sheep

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ABSTRACT

This study investigated the FSH influence on maturation rates of oocytes *in vitro* maturation (IVM), and expression levels of the follicle-stimulating hormone receptor (*FSHR*), luteinizing hormone receptor (*LHR*) and gonadotropin releasing hormone receptor (*GnRHR*) of cumulus-oocyte complexes (COCs) response to FSH treatment. 1686 COCs were harvested from 1063 ovaries of sheep. COCs were cultured 26 h at 38.5 °C and 5.0% CO_2 in IVM media supplemented with 0, 5, 10, 20 and 30 IU/ml FSH. They were allocated in to FSH-1 (basal line), FSH-2, FSH-3, FSH-4 and FSH-5 groups. The apoptosis of COCs was assessed by Tunel assay. Expression levels of mRNA and protein for *FSHR*, *LHR* and *GnRHR* in sheep COCs were detected using real time RT-PCR and Western blotting respectively. The results showed that the maturation rates of oocytes were improved gradually when FSH supplement increased from 0 to 10 μg/ml. FSH-3 group showed the highest maturation rate. Apoptosis rates of FSH-treated groups were less than that of FSH-1 group with a minimum of FSH-3 group. Expression levels of *FSHR* and *LHR* mRNAs in FSH-3 and FSH-4 were significantly higher than in FSH-1. Expression level of *GnRHR* mRNA in FSH-3 was higher than in FSH-1 ($P < 0.05$). Expression levels of *FSHR* proteins in FSH-3 and FSH-4 groups were higher than that of FSH-1 group. Expression levels of *GnRHR* proteins increased gradually with a maximal increment of FSH-5. Maturation rates of COCs had significant positive correlations with mRNA and protein levels of *FSHR*, *LHR* and *GnRHR*. In conclusion, FSH could accelerate the maturation rate of sheep oocytes and reduce their apoptosis rate, also increase the expression levels of *FSHR*, *LHR* and *GnRHR* mRNAs, and strengthen expressions of *FSHR* and *GnRHR* proteins. 10 IU/ml FSH additions were the optimal dose for IVM of sheep oocytes.

Introduction

In vitro maturation (IVM) of oocytes were often conducted in the media that were supplemented with follicle-stimulating hormone (FSH) and luteinizing hormone (LH) to induce cumulus cell expansion and nuclear maturation of oocytes (Junk et al., 2003; Lee et al., 2007; Xiao et al., 2014). Previous studies confirmed that hormones promoted IVM of sheep oocytes (Lu and Qi, 2013), but different hormones exerted different effects on oocytes in the same animal species (Yang et al., 2015; Zou et al., 2012). FSH and LH are known to be responsible for ovarian follicular development and steroidogenesis in the human ovary. Under the influence of FSH and LH, the primordial follicles from the ovary undergo the process of folliculogenesis in each cycle (Rajshri et al., 2013). Currently, FSH, LH and estradiol (E_2) are crucial hormones for IVM of oocytes (Lu and Yi, 2013; Xu et al., 2011). Adding suitable dose of FSH into IVM media may promote the maturation rate of oocytes and release of the first polar body (Zhao et al., 2013). Addition of 10 μg/ml FSH and 10 μg/ml LH could increase IVM rate of oocytes (Rebecca et al., 2012). The optimal concentration of FSH in IVM media was 5 μg/ml FSH, which resulted in the greatest cleavage (79.1%) and blastocyst rates (16.1%) of yak cumulus cells (Xiao et al., 2014). Up to date, it is undetermined that which FSH dose in media is the optimum of IVM of sheep oocytes.

For FSH and LH to act, the cognate FSH receptor (*FSHR*) and LH receptor (*LHR*) must be expressed in the oocytes (Xiao et al., 2014). Currently, little information has been recorded about FSH effects on expression levels of mRNAs and proteins *of FSHR, LHR* and

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gonadotropin releasing hormone receptor (*GnRHR*) in the COCs of human and animals (Hamed et al., 2015; Nor Azlina et al., 2014). The present study was designed to investigate the effects of adding different concentrations of FSH into the IVM media on oocytes maturation, also to explore its impact on the expression levels of mRNAs and proteins for *FSHR, LHR* and *GnRHR* in sheep COCs.

Materials and methods

Preparation of maturation media

The basal maturation media (bMM) was consisted of 9.5 g M199 powder (Sigma, St. Louis, MO, USA), 2.2 g $NaHCO_3$, 25 mg sodium pyruvate, 4.8 g hydroxyethyl piperazine ethane sulfonic acid (HEPES), 50 μg ampicillin sodium, 50 μg streptomycin sulfate, added with ultrapure water to a final volume of 1000 ml, filtered through 0.22 μm filter paper for sterilization, and preserved at 4 °C. The maturation media containing bMM was supplemented with 0.68 mM L-glutamine, 25 mM $NaHCO_3$, 10% (v/v) fetal calf serum (FCS) (Hyclone, USA), 5% fetal bovine serum (FBS, Suigonan, Intervet, Wiesbaden, Germany). All experiments were performed in sterile conditions.

Collections of sheep ovaries

Collections and IVM of sheep oocytes were performed in accordance with the early methods (de Frutos et al., 2013; Paola et al., 2005). Briefly, 1063 ovaries were harvested from 536 immature and non-cycle ewes (6–7 months old) at local abattoir from September to November, and transported to the laboratory in Dulbecco's phosphate buffered saline (DPBS, Sigma Co. Ltd, Beijing, China), then maintained at 30–35 °C within 3 h after slaughter. Use of the animals was approved by the Institutional Animal Ethics Committee of Northwest University for Nationalities, and all the experiments were conducted as per the norms of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), China.

Collection and classification of oocytes

The extraneous tissues and fat on the ovary surfaces were removed using sterile scissors. The ovaries were placed on a petri dish, added with 2 ml of pre-equilibrated extraction fluid (PBS containing 3 mg/ml BSA, incubated at 38 °C overnight), and fixed gently using the sterile ophthalmic tweezers. Subsequently, follicles on the ovarian surface were scratched with a scalpel blade. A total of 1686 COCs were used for subsequent experiments.

Cumulus-oocyte complexes (COCs) from antral follicles (3.0–5.0 mm in diameter) were recovered by gently cutting follicles with a scalpel on a petri dish. COCs were collected from each animal and pooled in groups. They were washed twice in Medium 199 (Sigma) supplemented with 0.68 mM L-glutamine (Sigma), 1 mM pyruvate, 20 mM HEPES (Sigma), 100 U/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma), and 10% FBS (Invitrogen, Carlsbad, CA, USA). Oocytes with intact cumulus cells and a homogeneous cytoplasm were selected and classified into four grades (A, B, C, and D) according to their cumulus cell layers (Wood and Wildt, 1997). Only COCs with at least three complete cumulus cell layers were considered suitable for IVM.

Sheep oocyte IVM at different FSH concentrations

A microdrop culture system was utilized in this experiment. Collected COCs were rinsed three times with extraction fluid, and pre-equilibrated for 3 h before IVM culture. At least 30 COCs were randomly taken from the instrument tray and placed in one culture well (Nunc Inc., Naperville IL, USA) containing 600 μl maturation media covered with 300 μl mineral oils. COCs were then left to complete their maturation at 38.5 °C in an atmosphere of 5.0% carbon dioxide in humidified air for 26 h.

To assess the effects of different FSH concentrations in IVM media on COCs IVM and optimal FSH concentration, COCs were matured in IVM media that were supplemented with the different doses of FSH (Ningbo Sansheng Hormone Factory, Ningbo, China) at 0, 5, 10, 20 and 30 IU/ml into IVM Media, respectively. They were allocated to FSH-1 (basal line), FSH-2, FSH-3, FSH-4 and FSH-5 group, respectively.

Evaluation of oocyte maturation

Following IVM, oocytes were denuded by 0.3% hyaluronidase digestion following three PBS rinses. Mature denuded oocytes were then fixed and subjected to Giemsa staining to determine their progression to metaphase II. The harvested oocytes were observed under a microscope, and those displaying an intact first polar body were identified as matured oocytes. Experiments were replicated at least 3 times.

To estimate rates of apoptosis, COCs were analyzed according to treatment group using a TUNEL kit, following the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, COCs were fixed in 4% PBS-buffered paraformaldehyde for 20 min at 23–25 °C, before being washed three times with 0.1% polyvinyl alcohol (PVA) in PBS, and permeabilized with 0.5% Triton X-100 in PBS for 10 min. Positive-control COCs were treated with 50 U/ml RNAse-free DNAse in cacodylate buffer for 1 h at 37.5 °C. COCs were placed in 30-ml drops of TUNEL solution and incubated in the dark for 1 h at 37.5 °C. For the negative control group, the TUNEL reagent was omitted. Apoptosis is reported as the number of labeled cells expressed as a percentage of the total cell number.

Real time RT-PCR (qRT-PCR) of FSHR, LHR *and* GnRHR *mRNAs*

Primer design

In order to access the effect of addition of FSH in IVM media on FSHR, LHR and GnRHR mRNAs expression in COCs after IVM, primers specific for FSHR (GenBank accession number: NM-001009289.1), LHR (GenBank accession number: L36329.1) and GnRHR (GenBank accession number: NM-001009397.1) were designed with Beacon Designer 7.0 software (Premier Biosoft International, Palo Alto, CA, USA) according to manufacturers guidelines and Primer-BLAST on NCBI (Table 1). Ovine GAPDH gene (GenBank accession number: HM-043737.1) was selected as the reference gene for normalizing expression levels of target genes. The concentrations of the primers (100 nM, 200 nM, 300 nM and 500 nM) were evaluated, and formation of primer-dimers was evaluated using the melting curve analysis. Thus, only those concentrations of primers which showed dimmer-free reactions

Table 1
Primer sequences for RT-qPCR and PCR conditions.

Gene	Primer(5′-3′)	Sequence	Tm	bp
FSHR	Forward	TCTTTGCTTTTGCAGTTGCC	59.1	126
	Reverse	GCACAAGGAGGGACATAACATAG	58.4	
LHR	Forward	CCTGAAGAAGATGCACGATGACGCC	60.2	189
	Reverse	ACCCATTCCCTGTCTGCCAGTCT	59.3	
GnRHR	Forward	TTCGGAGTATTCAGCAACCAAC	59.2	161
	Reverse	CAGGAATGTTCTATCCCCCAGT	59.7	
GAPDH	Forward	CTTCAACAGCGACACTCACTCT	57.1	152
	Reverse	CCACCACCCTGTTGCTGTA	57	

were used for the final analysis. Primers were synthesized by Beijing AoKeDingSheng Biotechnology Co. Ltd., China.

Total RNA extraction

After IVM under different FSH concentrations in IVM media, total RNA of 30 COCs was extracted using the TRIzol reagent (Invitrogen, Beijing, China), according to the manufacturer's instructions, then reverse transcribed (Wei et al., 2013; Wei et al., 2016b). The extraction was repeated three times with 30 COCs for each replicate.

qRT-PCR detection of mRNAs

Expression level of *FSHR, LH* and *GnRHR* mRNA was determined using qRT-PCR (Wei et al., 2013; Wei et al., 2014), respectively. Briefly, each 25 μl reaction volume in a 96-well plate was comprised of 4 μl of a 50 × diluted cDNA templates, 1 μl of each primer pair at 10 μl and 12.5 μl of 10 × Taqman Universal PCR Master Mix containing DNA polymerase, 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. The relative amount of each mRNA was determined by the $2^{-\Delta\Delta CT}$ method and normalized to an endogenous reference gene, GAPDH. Each sample was executed in triplicate, and each experiment was replicated three times with 30 COCs for each replicate.

Western blotting of FSHR, LHR *and* GNRHR *proteins*

To evaluate the expression levels of *FSHR, LHR* and *GnRHR* proteins in sheep COCs, Western blotting was implemented referring to the preceding description (Wei et al., 2013). Briefly, COCs were lysed in lysis buffers. Proteins were loaded on 10% SDS-PAGE, then transferred to polyvinylidene fluoride (PVDF) membranes and blocked in 5% non-fat milk in 10 mmol/l Tris, pH 7.5, 100 mmol/l NaCl, 0.1% (w/v) Tween 20 for 2 h. Rabbit anti-sheep GnRHR, FSHR and LHR polyclonal antibodies (Sigma, 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 (1:2000). Anti-β-actin mouse monoclonal antibody was diluted at 1:10000 for sample loading control. Blots were further developed using a chemiluminescence reagent (SuperSignal West Pico, Rockford, IL, USA). The integral optical density (IOD) of the scanned band images was achieved by using Quantity One software (Bio-Rad Company, Hercules, CA, USA). The relative concentrations of *FSHR, LHR* and *GnRHR* proteins were presented as the ratio between gray values of *FSHR, LHR* and *GnRHR* divided by that of β-actin. A negative control was performed without primary antibody. Assays were executed in triplicate.

Pearson's correlations analysis

Pearson's correlations were analyzed between the FSH doses and expression levels of FSHR, LHR and GnRHR mRNAs and proteins.

Data statistical analyses

Statistical analysis was done using SPSS v. 18.0 (SPSS Inc., Chicago, IL, USA). Data is presented as means ± SEM. All variables of five groups complied with the assumptions for a one-way ANOVA. 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

The optimal concentration of FSH in IVM medium

FSH effects on maturation rates and apoptosis rates of sheep oocytes were shown in Table 2. Maturation rates of oocytes in FSH-treated groups were increased in comparison with FSH-1 group (basal line). Maturation rates of FSH-3 and FSH-4 were significantly greater than that of FSH-1 ($P < 0.05$). The highest maturation rate was detected in FSH-3 group. The findings demonstrated that the optimal concentration of FSH in IVM medium was 10 IU/ml.

TUNEL assays and apoptosis rates

The incidence of apoptosis was demonstrated by TUNEL assay in COCs. As shown in Table 2 and Fig. 1, apoptosis rates of sheep COCs changed in contrast with the maturation rates. In comparison to FSH-1 group (basal line), apoptosis rates were reduced when FSH concentrations in IVM media increased from 0 to 10 IU/ml. Apoptosis rates in FSH-3 and FSH-4 groups were significantly less than FSH-1 group ($P < 0.01$ or $P < 0.05$). Additionally, apoptosis rate in FSH-3 was significantly less than FSH-2 and FSH-5 groups ($P < 0.05$).

The results indicated that the lower dose of FSH could promote the maturation and reduce apoptosis rate of sheep oocytes. 10 IU/ml FSH additions were the optimal concentration for IVM of sheep COCs. At this condition, the maturation rate of COCs was the highest with a minimal apoptosis rate.

Effect of addition of FSH in IVM media on FSHR, LHR *and* GnRHR *mRNA expression in COCs*

FSHR, LHR and *GnRHR* mRNAs were detected in sheep COCs after IVM. *FSHR, LHR* and *GnRHR* mRNA expressions of COCs in media supplemented with different concentrations of FSH were shown in Fig. 2. Expression levels of *FSHR* and *LHR* mRNAs were enhanced after FSH was added to IVM media. Expression levels of *FSHR* and

Table 2
Effects of FSH (IU/ml) on *in vitro* maturation and apoptosis of oocytes (Mean ± SEM).

Group	FSH dose (IU/ml)	Cultured oocytes	Matured oocytes	Maturation rate %	Apoptotic oocytes	Apoptosis rate %
FSH-1	0	62 ± 8	24 ± 3	38.7 ± 5.6	18 ± 2	29.0 ± 6.3
FSH-2	5	69 ± 7	30 ± 4	43.5 ± 7.5	17 ± 3	24.6 ± 7.2[b]
FSH-3	10	68 ± 6	33 ± 4	48.5 ± 6.2[*]	14 ± 2	20.6 ± 6.7[**a]
FSH-4	20	67 ± 9	31 ± 3	46.3 ± 6.8[*]	15 ± 2	22.4 ± 5.9[*]
FSH-5	30	65 ± 7	29 ± 3	44.6 ± 7.6	16 ± 3	24.5 ± 6.7[b]

Note: FSH-1, FSH-2, FSH-3, FSH-4 and FSH-5 represent addition of 0, 5, 10, 20 and 30 IU/ml of FSH into IVM media.
Data from each group are calculated as the average value of three experiments.
The different superscripts [a,b] point out a significant difference between experimental groups ($P < 0.05$).
[*] $P < 0.05$ when compared to FSH-1 group (basal line); [**] $P < 0.01$ when compared to FSH-1 group (basal line).

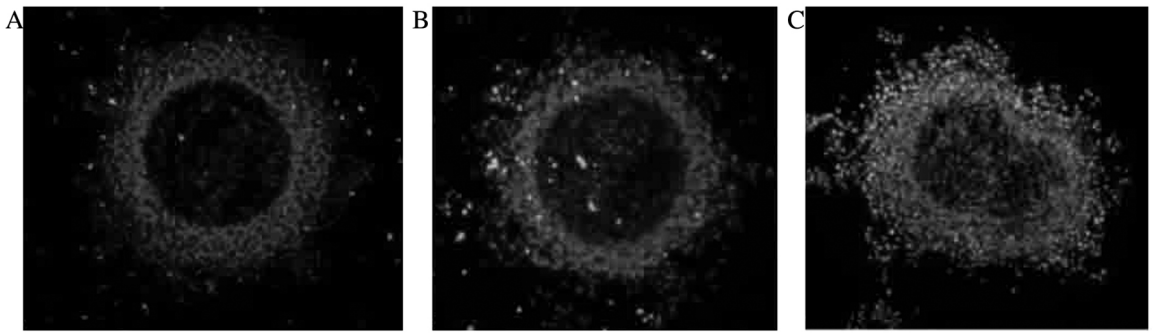

Fig. 1. Representative images of sheep COCs after IVM subjected to TUNEL analysis (A) COCs of the control group; (B) FSH-3 group; (C) Positive control for TUNEL analysis. Green staining indicates fragmented DNA in cells undergoing apoptosis, whereas intact cell nuclei are stained blue.

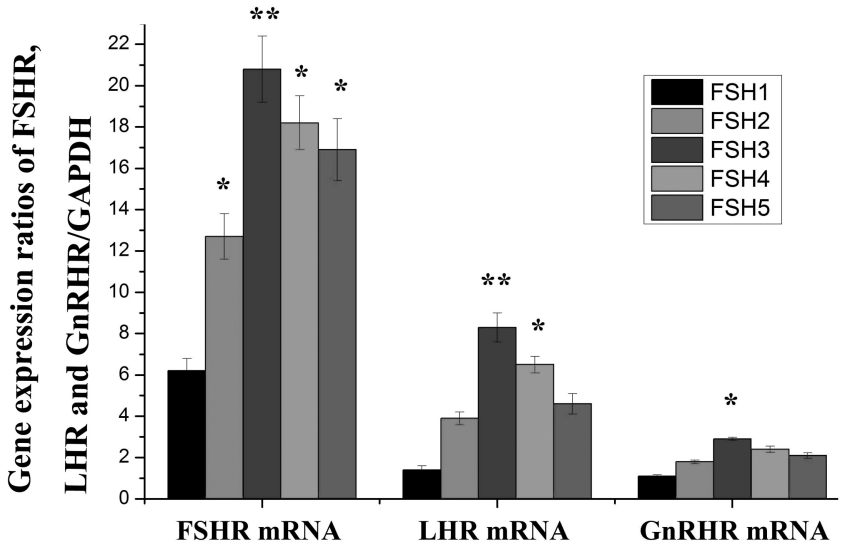

Fig. 2. Expression levels of *FSHR, LHR* and *GnRHR* mRNAs Expression levels of *FSHR* and *LHR* mRNAs were enhanced after FSH was added to IVM media. Expression levels of *FSHR* and *LHR* mRNAs in FSH-3 and FSH-4 groups were higher than that in FSH-1 (basal line) with the greatest increment in FSH-3 group. Expression level of *GnRHR* mRNA in FSH-3 was higher than that in FSH-1. $^{*}P < 0.05$ when compared to control group (FSH-1 group); $^{**}P < 0.01$ when compared to control group (FSH-1 group).

LHR mRNAs in FSH-3 and FSH-4 groups were higher than that in FSH-1 (basal line) ($P < 0.01$ or $P < 0.05$) with a maximal increment in FSH-3 group. Expression level of *GnRHR* mRNA in FSH-3 was higher than that in FSH-1 ($P < 0.05$). The results demonstrated that supplement of FSH to IVM media could significantly promote the expression levels of *FSHR*, *LHR and GnRHR* mRNAs.

Detection of FSHR, LHR *and* GnRHR *proteins in COCs*

The results of Western blotting showed that expression levels of *FSHR, LHR* and *GnRHR* proteins were increased in sheep COCs after FSH supplementation to IVM media (Fig. 3). Significant increments in *FSHR* proteins expressions of FSH-3 and FSH-4 groups were found as compared with FSH-1 group (basal line) ($P < 0.05$). Expression levels of *GnRHR* proteins increased gradually with a maximal increment of FSH-5 in comparison with FSH-1 group. However, there was no significant difference on expression levels of *LHR* proteins between all groups. The findings demonstrated that addition of FSH into IVM media could strengthen expressions of *FSHR* and *GnRHR* proteins.

Pearson correlation assay between receptor expression and FSH doses

As indicated in Table 3, Pearson's correlation analyses demonstrated that FSH doses had a positive correlation with proteins expression of GnRHR. Maturation rate of COCs had significant positive correlations with mRNA and protein levels of three receptors. The mRNAs levels of FSHR and LHR had significant positive correlations with their proteins, respectively ($P < 0.05$, $P < 0.01$). Expression levels of GnRHR mRNAs and proteins had significant correlations with expressions of mRNAs and proteins of FSHR and LHR ($P < 0.05$ or $P < 0.01$). The findings demonstrated that the increases of expression levels of FSHR, LHR and GnRHR could promote IVM of sheep oocytes.

Discussions

FSH effects on IVM of sheep oocytes

The oocyte competence for fertilization and ability to support embryonic development was influenced by additions of FSH and LH during the maturation process (Xiao et al., 2014). The quality of oocytes has been reported as a key factor influencing the outcome of oocyte maturation and subsequent development. The extrusion of the first polar body is a principal sign of oocyte IVM and nucleus maturation. The previous studies have confirmed that FSH promoted IVM of sheep oocytes (Lu and Qi, 2013). The supplementation of IVM media with 1.5 μg/ml rbFSH yielded a high degree of cumulus expansion of sheep oocytes (de Frutos et al., 2013). The mechanism is that FSH stimulates the transition

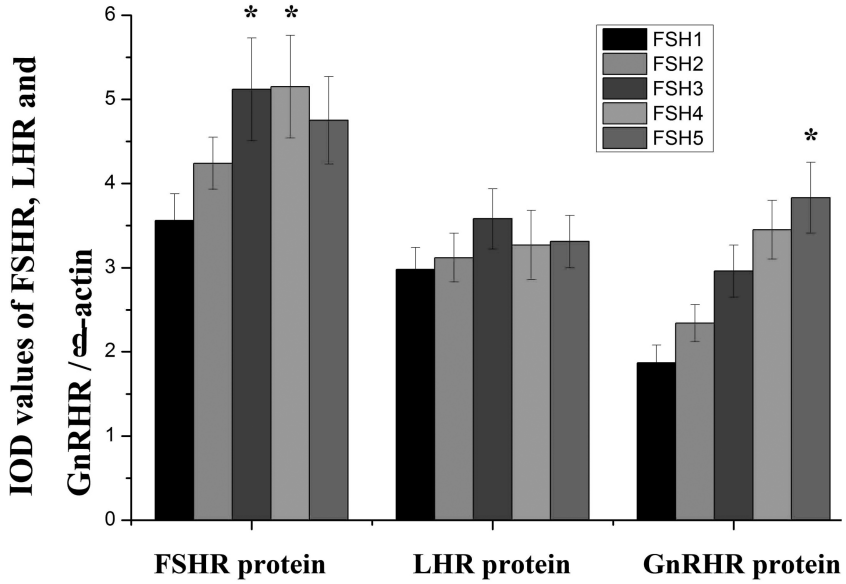

Fig. 3. Expression levels of *FSHR, LHR* and *GnRHR* proteins Expression levels of *FSHR* and *GnRHR* proteins were enhanced in sheep COCs when FSH supplemented to IVM media. Expression levels of *FSHR* proteins in FSH-3 and FSH-4 groups were higher than that in FSH-1 group. Expression levels of *GnRHR* proteins increased gradually with a maximal increment of FSH-5. However, there was no significant difference on expression levels of LHR proteins between all groups. $^{*}P < 0.05$ when compared to control group (FSH-1 group); $^{**}P < 0.01$ when compared to control group (FSH-1 group).

Table 3
Pearson correlation coefficients for gene expression/or mRNA expression.

Indexes	Dose	M rate	FSHR m	LHR m	GnRHR m	FSHR p	LHR p
M rate	0.485						
FSHR m	0.619	0.986**					
LHR m	0.391	0.984**	0.960**				
GnRHR m	0.466	0.994**	0.982**	0.994**			
FSHR P	0.657	0.960**	0.982**	0.940*	0.953*		
LHR p	0.435	0.927*	0.928*	0.937*	0.956*	0.86	
GnRHR p	0.972**	0.671	0.781	0.595	0.656	0.815	0.609

Notes: M rate- maturation rate; FSHR m, LHR m and GnRHR m represent mRNA of FSHR,
LHR and GnRHR, respectively; FSHR p, LHR p and GnRHR p represent protein of FSHR,
LHR and GnRHR respectively. 30 COCs were used for gene expression for each replicate in each treatment groups.
* $P < 0.05$ when compared to FSH-1 group (basal line); ** $P < 0.01$ when compared to FSH-1 group (basal line).

from a preantral to antral follicle and the proliferation, cumulus cell expansion and nucleus maturation (Junk et al., 2003). It also promotes secretion of follicle fluid and follicle development of cat. (Li and Albertini, 2013). In the absence of FSH, granulose cells form an unusually dense network of the transzonal projection (TZP) that terminates at the oocyte surface. When rescued with FSH-treatment, TZP retraction occurs coincident with the acquisition of meiotic competence (Li and Albertini, 2013). These results indicate that FSH induced sheep oocyte maturation (de Frutos et al., 2013). However, optimal FSH concentration for IVM of sheep oocytes is undetermined.

In the present study, maturation rates of oocytes were improved gradually when FSH supplement in IVM increased from 0 to 10 μg/ml. Apoptosis rates of FSH-treated groups were less than that of FSH-1 group (basal line). 10 IU/ml FSH additions were the optimal concentration for IVM of sheep oocytes. Our findings were consistent with the early reports (de Frutos et al., 2013; Yang et al., 2015; Zhao et al., 2013).

Apoptosis of COCs

Apoptosis of oocytes could be a valuable marker for oocyte quality and development competency. Previous studies reported that the oocytes apoptosis affected embryo quality, because of the presence of molecules that regulate the apoptosis mechanism in the maternal mRNA stored in the oocytes (Metcalfe et al., 2004). The numbers and distributions of the apoptosis cells and subcellular structures were variable in the COCs. COCs comprises an intimate relation between a cumulus cell syncytium in conjunction with the oocyte involving large gap junctions. When stimulated by follicle-stimulating hormone, these gap junctions are open and allow even larger molecules to move freely between the cumulus cells and the oocyte. Signaling molecules for the regulation of the apoptosis process can easily be distributed among cells in the COC (Paola et al., 2005).

In the present study, apoptosis rates of FSH-treated groups were lower than that of the basal line. Apoptosis rate of FSH-3 group was the least in all groups. IVM media in the presence of 5–10 IU/ml FSH could promote the maturation and reduce apoptosis rate of sheep oocytes. The results were in agreement with the previous report of cows (Paola et al., 2005; Qu et al., 2008).

Effects of FSH supplement on expression levels of mRNAs and proteins of hormone receptors

In order for FSH to exert its effects, the specific receptors must be located in follicle cells. The binding of FSH to FSHR activates intracytoplasmic signaling pathways leading to steroidogenesis (Rajshri et al., 2013), namely these steroids in turn regulate FSH action from the anterior pituitary through exerting negative feedback effect, such to regulate proliferation and differentiation of granulose cells of the ovary (Robker and Richards, 1998).

The cumulus cells had a high number of FSHR (Peng et al., 1991; Xiao et al., 2014). Our previous studies demonstrated *FSHR* was expressed in the sheep ovaries (Wei et al., 2012; Wei et al., 2014). In this study, *FSHR, LHR* and *GnRHR* mRNAs were also detected in sheep COCs when IVM media were supplemented with FSH.

FSH-treatment upregulated the expression levels *of FSHR* and *LHR* mRNAs with the greatest increment in the presence of 10 IU/ml FSH. However, FSH had no obvious influence on expression of GnRHR mRNA. The outcomes were in agreement with an earlier report (Sullivan et al., 2013).

The present study demonstrated that *FSHR, LHR* and *GnRHR* proteins distributed in COCs of sheep. Addition of FSH into IVM media could enhance the expression of *FSHR* and *GnRHR* proteins in COCs, resulting in increase of maturation rate of COCs. However, supplementation of FSH to IVM media had no obvious effect on expression levels of *LHR* proteins. *GnRHR* is a G protein-coupled receptor that is highly expressed in pituitary gonadotropes. The functions of *GnRHR* are mainly to mediate the activities of GnRH for promoting secretion of FSH and LH in ovarian development. Our initial studies demonstrated that *GnRHR* is also expressed in the ovaries and follicular tissues of ewes (Wei et al., 2013). In this study, *GnRHR* levels in sheep COCs were detected so as to evaluate the correlation *GnRHR* with oocyte IVM, and further to explore whether changes in oocyte *GnRHR* influence *FSHR* and/or *LHR* levels. Pearson's analyses verified that the expression levels of *GnRHR* mRNAs and proteins in sheep COCs had significant correlations with expressions of mRNAs and proteins of *FSHR* and *LHR*. The preliminary mechanism is probably that *GnRHR* increase the activities of hypothalamus GnRH, resulting in the promotion of FSH secretion in the pituitary (Wei et al., 2013). These findings need to be further testified in the future.

Correlations between FSH and oocytes IVM and receptor levels

Our previous studies indicated that FSH doses had significant positive correlations to estrus rate and lambing rate in Lanzhou fat-tailed sheep (Wei et al., 2016a). However, up until date, there is no literature describing the quantitative relationship between FSH administration in media and oocytes IVM in humans and animals. The present study demonstrated that maturation rate of COCs had highly positive correlations with mRNA and protein levels of *FSHR, LHR* and *GnRHR*. Based on these data, it is reasonable to demonstrate that the increases of *FSHR, LHR* and *GnRHR* levels could promote IVM of sheep oocytes. The results were consistent with our initial reports of mice (Wei et al., 2016a). These results are still to be testified in other animals.

Conclusions

FSH could increase the maturation rate of sheep oocytes, decrease apoptosis percentage of oocytes, and enhance expression levels of *FSHR*, *LHR* and *GnRHR* mRNAs, additionally strengthen expressions of *FSHR* and *GnRHR* proteins. 10 μg/ml FSH was optimal concentration in IVM media of sheep oocytes. These findings are useful to effectively *in vitro* culture oocytes of sheep. They have important practical significance. Meanwhile, the data shown in the present study had a better understanding of the mechanism of FSH regulation of IVM of oocytes in humans and animals.

Conflict of interests

All authors do not have any financial or personnel relationship with organizations or people that could influence or bias the study.

Authors' contributions

Professor Wei Suocheng was responsible for the experimental designs and writing the manuscript. Professor Gong Zhuandi raised the experimental animals and took the samples. Dr. Sheng Li detected the receptor gene expression levels. Miss Deng Yingying did the statistics analyses. Miss Liang Haoqin did apoptosis assays. Miss Lai Luju carried out Western blotting. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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