Equine chorionic gonadotropin influence on sheep oocyte *in vitro* maturation, apoptosis, and follicle-stimulating hormone receptor and luteinizing hormone receptor expression

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ABSTRACT. We assessed the effects of equine chorionic gonadotropin (eCG) on oocyte *in vitro* maturation (IVM), apoptosis, and follicle-stimulating hormone receptor (FSHR), luteinizing hormone receptor (LHR), and gonadotropin-releasing hormone receptor (GnRHR) expression and mRNA levels. Cumulus-oocyte complexes (COCs) were recovered from sheep ovaries and pooled in groups, before being cultured in IVM media containing varying eCG concentrations. Maturation and apoptosis rates were then calculated. Expression of FSHR, LHR, and GnRHR mRNA in oocytes was measured using quantitative reverse transcription polymerase chain reaction. Protein levels were ascertained by western blotting. Matured oocytes displayed and released an intact first polar body. Sheep oocyte maturation rates

gradually increased as eCG concentration was raised from 0 to 20 μg/mL. Apoptosis rates of eCG-treated oocytes were lower than those of the control group, and were lowest using 20 μg/mL eCG. FSHR, LHR, and GnRHR mRNA expression increased ($P < 0.01$, $P < 0.05$, and $P < 0.05$, respectively, compared to 0 μg/mL eCG) with eCG concentration, being highest following exposure to 20 μg/mL. FSHR and GnRHR protein levels were significantly higher in oocytes administered 20 μg/mL eCG compared with those matured in the absence of eCG. eCG dose positively correlated with FSHR, LHR, and GnRHR mRNA and protein expression. In conclusion, eCG enhances maturation and decreases apoptosis of oocytes undergoing IVM, and heightens FSHR, LHR, and GnRHR expression. Such increased expression may facilitate oocyte IVM. These findings contribute to our understanding of the mechanisms of underlying hormonal control of sheep oocyte IVM, advancing ovine reproductive methods.

Key words: *In vitro* maturation; Apoptosis; Receptor; Sheep; Equine chorionic gonadotropin; Cumulus-oocyte complexes

INTRODUCTION

In vitro maturation (IVM) of oocytes is often conducted in media supplemented with gonadotropins such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH) to induce cumulus cell expansion and nuclear maturation (Lee et al., 2007; Xiao et al., 2014). Multiple factors have been implicated in oocyte maturation. FSH, LH, and estradiol have become the most important hormones employed in oocyte IVM (Lu and Qi, 2013). For instance, addition of 10 μg/mL FSH and 10 μg/mL LH has been shown to increase the IVM rate of ovine oocytes (Hobbs et al., 2012). Equine chorionic gonadotropin [eCG, previously known as pregnant mare serum gonadotropin (PMSG)] demonstrates an efficacy similar to those of FSH and LH, and has a high affinity with the receptors (FSHR and LHR, respectively) of both of these hormones in the ovaries. eCG administration can improve reproductive performance and increase ovulation and pregnancy rates in noncyclic cows (Uslu et al., 2012; Hashim et al., 2013) and ewes (Wei et al., 2016a). However, the effect of supplementing IVM medium with eCG on IVM, apoptosis, and the expression and mRNA levels of reproductive hormone receptors in Lanzhou fat-tailed sheep remains unknown (Karami-Shabankareh et al., 2015).

For gonadotropins to act *in vitro*, FSHR and LHR genes must be expressed by cumulus cells (Xiao et al., 2014). Gonadotropin-releasing hormone receptors (GnRHRs) are expressed in ovaries (Wei et al., 2013), but their expression in sheep cumulus-oocyte complexes (COCs) has not been described in detail (Aziz et al., 2014; Karami-Shabankareh et al., 2015).

The Lanzhou fat-tailed sheep is a breed unique to the Lanzhou area of China (latitude 35°34'N to N 38°10'N, longitude 102°30'E to 104°37'E). It is characterized by a large tail, fast development, fleshiness, high fat content, and delicious meat. However, its embryonic developmental competence is low, with a lambing percentage of only approximately 117% (Zhang, 2010). Therefore, improving the reproductive performance of this breed to increase population sizes has become an urgent necessity. Currently, little information exists concerning oocyte IVM in relation to this sheep. In the current investigation, the effects of different eCG concentrations on

sheep oocyte IVM, apoptosis, and FSHR, LHR, and GnRHR expression and mRNA levels were determined, with the aim of facilitating the breeding of Lanzhou fat-tailed sheep.

MATERIAL AND METHODS

Preparation of maturation media

Basal culture medium (bMM) consisted of 9.5 g Medium 199 powder (Sigma, St. Louis, MO, USA), 2.2 g $NaHCO_3$, 25 mg sodium pyruvate, 4.8 g 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 50 IU ampicillin sodium, 50 μg streptomycin sulfate, and ultrapure water up to a final volume of 1000 mL. This medium was filtered through a 0.22-μm membrane and stored at 4°C. Maturation medium comprised bMM supplemented with 0.68 mM L-glutamine, 2.1 g $NaHCO_3$, 10% fetal calf serum (v/v; HyClone, Logan, UT, USA), and 5% fetal bovine serum (FBS; Minhai Company, Lanzhou, China).

Collection of sheep ovaries

Ovaries were collected between September and November of 2014 from 530 immature and noncyclic ewes (6-7 months old) immediately after their slaughter at the local shambles They were transported to the laboratory in Dulbecco's phosphate-buffered saline (DPBS) (Sigma Co. Ltd, Beijing, China), and placed at 30°-35°C within 3 h after collection. Use of these animals was approved by the Institutional Animal Ethics Committee of Northwest University for Nationalities, and all experiments were conducted according to the conventions of the Committee for the Purpose of Control and Supervision of Experiments on Animals of China.

Collection and classification of oocytes

Extraneous tissues and fat on ovary surfaces were removed using sterile scissors. The ovaries were placed on a Petri dish, to which 2 mL pre-equilibrated extraction fluid (PBS containing 3 mg/mL bovine serum albumin, incubated at 38°C overnight) was added, while they were gently held in place using sterile ophthalmic tweezers. Follicles on the ovarian surface were then scratched with a scalpel blade. COCs 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. COCs taken from all animals were collected together in one instrument tray. A total of 1086 COCs were used for subsequent experiments.

Sheep oocyte IVM

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 medium with 0, 5, 10, 20, or 30 μg/mL eCG (Ningbo Sansheng Hormone Factory, Ningbo, China) covered with 300 μL mineral oil. 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.

Evaluation of oocyte maturation

Following IVM, oocytes were denuded by 0.3% hyaluronidase digestion following three PBS rinses. The 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 deemed mature.

Detection of oocyte apoptosis

Morphological analysis of COC apoptosis

COCs having undergone IVM were mounted and observed under a microscope to identify apoptotic cells according to morphological criteria (Yang and Rajamahendran, 2000).

Detection of COC apoptosis by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

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 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.

Fluorescence-based quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Primer design

Primers specific to FSHR (GenBank accession No. NM_001009289), LHR (GenBank accession No. L36329.1), and GnRHR (GenBank accession No. NM_001009397.1) were designed using the Beacon Designer 7.0 software (Premier Biosoft International, Palo Alto, CA, USA) and the National Center for Biotechnology Information Primer-BLAST program (Table 1). The gene encoding ovine GAPDH (GenBank accession No. HM043737.1) was chosen as an internal reference for normalization of target gene expression. Melting curve analysis was used to test various primer concentrations (100, 200, 300, and 500 nM) and check for primer dimer formation. Only those primer concentrations resulting in dimer-free

reactions were used in the final analysis. Primers and probes were synthesized by Beijing AoKeDingSheng Biotechnology Co. Ltd. (Beijing, China).

Table 1. Primers used to amplify ovine genes encoding receptors of follicle-stimulating hormone (FSHR), luteinizing hormone (LHR), and gonadotropin-releasing hormone (GnRHR).

Target	Primer	Sequence (5'-3')	T_m (°C)	Product (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	

T_m = melting temperature.

Total RNA extraction

Total RNA was extracted from cumulus cells after IVM (during which, cells were exposed to IVM medium containing different eCG concentrations) using TRIzol reagent (Invitrogen, Beijing, China) according to the manufacturer's instructions, before being reverse transcribed (Wei et al., 2013, 2016a).

qRT-PCR

The expression of FSHR, LHR, and GnRHR mRNA was determined using qRT-PCR (Wei et al., 2013, 2014). Briefly, on 96-well plates, 25-μL reactions were carried out, each comprising 4 μL complementary DNA template (diluted 1:50), 1 μL each primer pair at 10 μM, 0.25 μL 10 μM TaqMan probe, and 12.5 μL 10X TaqMan Universal PCR Master Mix containing DNA polymerase, buffer, deoxynucleotides, and SYBR Green II (Promega, Beijing, China). Plates were sealed with optical adhesive film (Promega). After an initial denaturation step of 15 min at 95°C, 44 cycles of amplification were performed as follows: denaturation for 30 s at 95°C, annealing for 20 s at 60°C, and extension for 20 s at 72°C. GAPDH was used as an endogenous control, and the $2^{-\Delta\Delta Ct}$ method was employed to calculate relative mRNA expression. Samples were analyzed in triplicate.

Western blotting of FSHR, LHR, and GnRHR protein

To evaluate FSHR, LHR, and GnRHR expression levels in sheep COCs during IVM, western blotting was conducted as previously described (Wei et al., 2013). Briefly, COCs were lysed in lysis buffer, and the resulting proteins were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, before being transferred to polyvinylidene fluoride membranes. These membranes were then blocked for 2 h in a 5% non-fat milk solution containing 10 mM Tris (pH 7.5), 100 mM NaCl, and 0.1% Tween 20 (w/v). Membranes were incubated with rabbit anti-sheep GnRHR, FSHR, LHR (Sigma, diluted 1:200), and rabbit anti-sheep β-actin (1:1000) polyclonal antibodies at 4°C overnight, before being exposed to the appropriate secondary antibody (1:2000) for 1 h. Mouse anti-β-actin

monoclonal antibody (1:10,000) was used as a sample loading control. Blots were developed using a chemiluminescent reagent (SuperSignal West Pico; Thermo Scientific, Rockford, IL, USA). The integrated optical densities of bands in the scanned images were measured with the Quantity One software (Bio-Rad, Hercules, CA, USA). Relative protein expression was calculated as the ratio of the gray value of the target band (FSHR, LHR, or GnRHR) to that of the β-actin band. Samples were run in triplicate. The negative control was not incubated with the primary antibodies.

Pearson correlation analysis

Pearson correlation analysis was used to determine relationships between eCG dose, maturation rate, apoptosis rate, and expression and mRNA levels of FSHR, LHR, and GnRHR.

Statistical analysis

Data are reported as means ± standard errors of means. Statistical analysis was performed with SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). After square root transformation of the data, all variables complied with the assumptions of one-way analysis of variance (ANOVA). Post-ANOVA comparisons between groups were carried out using the contrast option under the general linear model procedure (Scheffé test). When significant differences were identified, supplementary Tukey's post-hoc tests were conducted to investigate pairwise differences. P values <0.05 were considered significant.

RESULTS

Sheep COC IVM rates

In order to evaluate the influence of eCG on IVM and apoptosis of sheep oocytes, COCs were cultured using the microdrop method. As shown in Table 2, sheep oocyte maturation rate gradually improved as eCG concentration increased from 0 (control group) to 20 μg/mL (group eCG-4), reaching a peak value of 51.7%, before declining at the highest eCG dose. Apoptosis rates of eCG-treated groups were lower than that of the control group (group eCG-1). Apoptosis was lowest in group eCG-4. Therefore, the optimal eCG concentration for sheep oocyte IVM was found to be 20 μg/mL. Excessive levels of eCG may inhibit IVM of these cells.

Table 2. Effects of equine chorionic gonadotropin (eCG) on *in vitro* maturation of sheep oocytes.

Hormone	Group	Dose	Cultured oocytes	Matured oocytes	Maturation rate (%)	Apoptotic oocytes	Apoptosis rate (%)
eCG (μg/mL)	eCG-1	0	52	19	36.5	15	28.9[a]
	eCG-2	5	59	27	45.8[a]	15	25.4
	eCG-3	10	56	26	46.4*	12	21.4
	eCG-4	20	58	30	51.7**[b]	11	19.0[b]
	eCG-5	30	60	27	45.0	16	26.7
FSH (IU/mL)	FSH	10	68	33	48.5[b]	14	20.6[b]

Different superscript letters indicate a significant difference between groups. eCG-1 was included as the blank control group. Oocytes treated with 10 IU/mL follicle-stimulating hormone (FSH) comprised the positive control group. *P < 0.05 and **P < 0.01 compared to group eCG-1 (blank control).

Morphological analysis of apoptosis

Based on microscopy observations of oocyte morphology, features of apoptosis were evident in the cumulus masses of COCs after 26 h of IVM. These apoptotic characteristics consisted of the following: cell shrinkage or cytoplasmic condensation (Figure 1), membrane blebbing, marginated chromatin, pyknotic appearance, multiple nuclear fragments, and formation of apoptotic bodies. The number and distribution of these apoptotic cells and subcellular structures varied among the COCs. As apoptosis proceeded, oocyte morphology exhibited shrinkage (an early sign of apoptosis), then DNA fragmentation or apoptotic body formation.

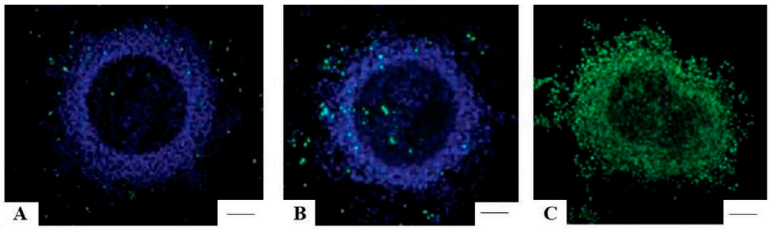

Figure 1. Representative images of post-*in vitro* maturation sheep cumulus-oocyte complexes (COCs) subjected to terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) analysis to detect apoptosis. COCs in the control group (**A**) and eCG-treated group (**B**). **C.** Green staining indicates fragmented DNA in cells undergoing apoptosis, whereas intact cell nuclei are stained blue. Scale bar = 25 μm.

TUNEL assays and apoptosis rates

Oocyte quality has been identified as a crucial factor influencing the outcome of oocyte maturation and subsequent developmental competence. The incidence of apoptosis in COCs was determined by TUNEL assay, and the findings are presented in Table 2 and Figure 2. In response to eCG, oocyte apoptosis rates demonstrated a trend opposite to that observed for maturation rates. Apoptosis rates were gradually reduced as eCG dose increased from 0 to 20 μg/mL. Apoptosis level was lowest in group eCG-4, compared to which, the control (eCG-1) group showed a significantly higher rate (19.0 *vs* 28.9%, respectively). Apoptosis was slightly more frequent in group eCG-5 than eCG-4. Our results revealed 20 μg/mL to be the optimal eCG concentration for sheep oocyte IVM.

FSHR, LHR, and GnRHR mRNA expression in COCs

To assess the impact of eCG on expression and mRNA levels of FSHR, LHR, and GnRHR in oocytes, qRT-PCR was employed. These transcripts were detected in sheep COCs after IVM, and their levels were augmented as the concentration of eCG in the IVM medium was increased (Figure 3). The greatest expression of FSHR, LHR, and GnRHR mRNA was

detected in group eCG-4 [$P < 0.01$, $P < 0.05$, and $P < 0.05$, respectively, compared to the control group (eCG-1)]. These findings demonstrated that eCG can enhance the expression of FSHR, LHR, and GnRHR mRNA in sheep COCs.

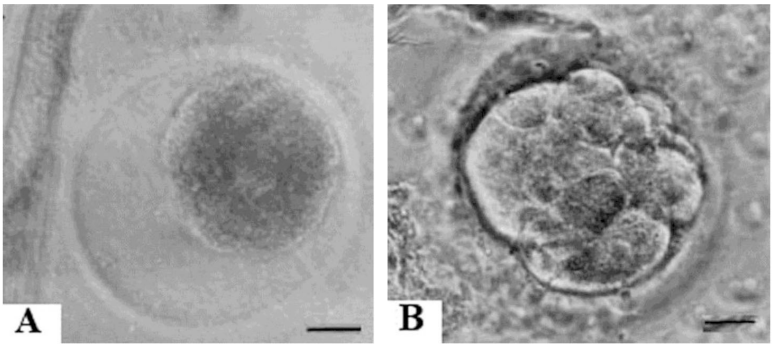

Figure 2. Cytoplasmic condensation (**A**) and fragmentation (**B**).

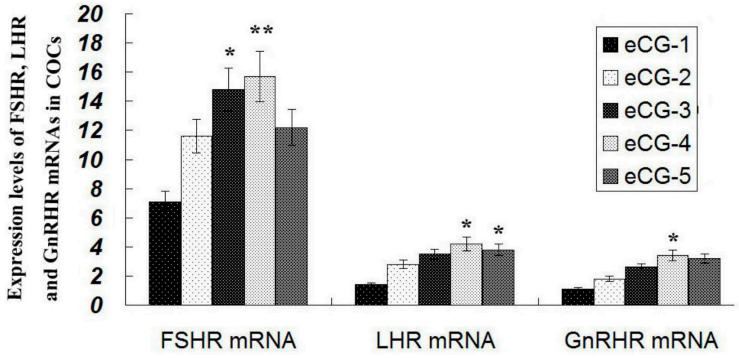

Figure 3. Expression of FSHR, LHR and GnRHR mRNA. Expression levels of each transcript increased as eCG concentration was raised. The highest FSHR, LHR, and GnRHR mRNA expression was observed in group eCG-4. *$P < 0.05$ and **$P < 0.01$ compared to the control group (eCG-1).

Western blotting of FSHR, LHR, and GnRHR proteins

Expression of FSHR, LHR, and GnRHR proteins in sheep COCs was detected by western blotting. As shown in Figure 4, levels of these three receptors increased gradually, being highest when 20 μg/mL eCG was added to the IVM medium. Expression of FSHR and GnRHR proteins in group eCG-4 was significantly higher than that in the control group (eCG-1; $P < 0.05$). However, LHR protein level did not significantly differ between the groups. This demonstrated that eCG treatment can increase FSHR and GnRHR levels, with a concentration of 20 μg/mL exerting the greatest effect. Addition of eCG to IVM medium heightened expression of FSHR and GnRHR proteins in sheep oocytes, but had no effect on LHR protein levels.

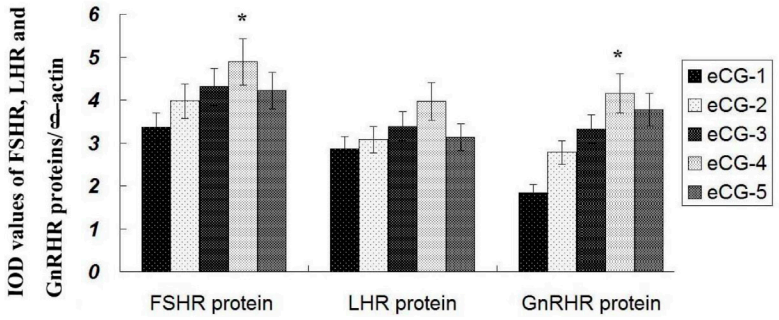

Figure 4. Expressions of FSHR, LHR and GnRHR proteins. *P < 0.05 when compared to control group (eCG-1 group). IOD = integrated optical density, eCG = equine chorionic gonadotropin.

Pearson correlation analysis

Pearson correlation analysis revealed that eCG dose positively correlated with mRNA levels and expression of FSHR, LHR, and GnRHR (Table 3). FSHR, LHR, and GnRHR mRNA levels positively correlated with expression of the corresponding proteins. These results demonstrated that increased expression of FSHR, LHR, and GnRHR can promote sheep oocyte IVM.

Table 3. Pearson correlation analysis of oocyte parameters.

Parameter	eCG dose	Maturation rate	Apoptosis rate	FSHR mRNA	LHR mRNA	GnRHR mRNA	FSHR protein	LHR protein
Maturation rate	0.3560							
Apoptosis rate	-0.1660	-0.884*						
FSHR mRNA	0.5410	0.884*	-0.887*					
LHR mRNA	0.8100	0.7660	-0.6660	0.927*				
GnRH mRNA	0.897*	0.6300	-0.5590	0.8460	0.972**			
FSHR protein	0.6550	0.7130	-0.7450	0.954*	0.950*	0.913*		
LHR protein	0.4390	0.5480	-0.7430	0.8640	0.7850	0.7640	0.935*	
GnRH protein	0.8530	0.6720	-0.5990	0.889*	0.990**	0.990**	0.951*	0.8050

eCG = equine chorionic gonadotropin, FSHR = follicle-stimulating hormone receptor, LHR = luteinizing hormone receptor, GnRHR = gonadotropin-releasing hormone receptor. *P < 0.05; **P < 0.01.

DISCUSSION

From a morphological perspective, oocyte IVM consists of a series of physiological changes, including nuclear and cytoplasmic maturation. Extrusion of the first polar body is a key indicator of oocyte IVM and nuclear maturity. A previous study reported that maturation of the cytoplasm and nucleus in cumulus cells is synchronous *in vivo*. However, maturity of the nucleus is achieved prior to that of the cytoplasm *in vitro*. Cumulus cell expansion is a sign of cytoplasmic maturation.

FSH, LH and estradiol are necessary for oocyte maturation, and previous studies have confirmed that they can be used to promote oocyte IVM (Lu and Qi, 2013), although different hormones from the same animal exert divergent effects in this respect (Yang et al., 2015). Few studies have been conducted concerning *in vitro* culture of Lanzhou fat-tailed sheep oocytes. In the present study, the effects of various eCG concentrations on sheep oocyte IVM were explored.

eCG can promote nuclear maturation and expansion (Meng et al., 2001). At concentrations of 10, 50, and 100 IU/mL, it has been shown to improve canine oocyte cumulus expansion, but not significantly increase nuclear maturation rate (Songsasen et al., 2003). This is because eCG is an inhibitor of meiosis, and thus, its actual efficacy during oocyte IVM still needs to be examined in detail. Previous research has indicated that application of 20 μg/mL eCG results in the highest sheep oocyte maturation rate, compared with a concentration of 30 μg/mL and control animals. However, it has also been reported that use of 10 IU/mL eCG in IVM medium results in optimal maturation and blastocyst rates in sheep (Hu, 2011). Therefore, the ideal eCG concentration for sheep oocyte IVM remains to be determined. The data presented here indicate that eCG promotes the development of sheep oocytes, with a maximum maturation rate and minimum apoptosis rate at 20 μg/mL. Our results are consistent with those of earlier investigations (Songsasen et al., 2003; Yang et al., 2015). However, because of the small sample sizes of each group, these findings need to be verified in future study.

Apoptosis is a physiological process of programmed cell death, and may be an effective marker of oocyte quality and developmental competency. Oocytes matured *in vivo* degenerate if not fertilized. In the ovary, oocytes showing early signs of atresia have high developmental potential (Aziz et al., 2014). Therefore, most oocytes collected from slaughtered animals by slicing will have arisen from atretic follicles.

Morphological signs of apoptosis were rarely seen at the beginning of IVM, whereas they were evident in the cumulus masses of COCs after 24-28 h. Our study showed that the apoptotic features appearing after 26 h of IVM included cell shrinkage or cytoplasmic condensation, membrane blebbing, pyknotic appearance, multiple nuclear fragments, and the formation of apoptotic bodies. The number and distribution of these apoptotic cells and subcellular structures differed among the COCs examined. Apoptosis proceeded from oocyte shrinkage to apoptotic body formation. These observations are similar to those made in earlier reports (Elmogy and Takeda, 2009).

Previous studies have reported that apoptosis associated with oocytes may affect embryo quality because of the presence of transcripts encoding regulators of this mechanism in the maternal mRNA stored in such cells (Metcalfe et al., 2004). In the present study, addition of eCG to IVM medium not only enhanced expression of FSHR, LHR, and GnRHR mRNA and protein in sheep COCs, but also lowered oocyte apoptosis rates.

FSH acts through FSHR, which is primarily expressed by cumulus and mural granulosa cells in sheep (Tisdall et al., 1995). Cumulus cells have large numbers of FSHRs, but few or no LHRs (Peng et al., 1991; Xiao et al., 2014). FSHR, but not LHR mRNA, is expressed in the cumulus and granulosa cells of cows (van Tol et al., 1996). In addition, our previous study demonstrated that FSHR is expressed in the pituitary gland and ovaries of sheep (Wei et al., 2012, 2014). Alarelin immunization stimulates GnRH antibody production, suppresses expression of GnRHR protein, enhances FSHR and LHR protein levels in ovaries, and promotes FSH secretion, thereby accelerating the development of ovaries and follicles in ewes (Wei et al., 2013). FSHR variants interfere with the translation and dimerization of wild-type FSHRs in humans (Gerasimova et al., 2010).

Administration of eCG has been found to improve reproductive performance and increase ovulation and pregnancy rates in noncyclic cows (Uslu et al., 2012; Hashim et al., 2013; Wei et al., 2016a). Moreover, eCG promotes follicular growth resulting in the increase of FSHR expression (Wang et al., 2013). Therefore, eCG treatment is generally utilized in veterinary medicine to control the reproductive activity of female animals (De Rensis and López-Gatius, 2014; Wei et al., 2016a).

Our previous investigation demonstrated that synthesis and secretion of LH and FSH are increased in mice following intramuscular injection of 10, 20, or 40 IU eCG (previously known as PMSG) (Wei et al., 2015). In the current study, we showed that expression of FSHR, LHR, and GnRHR mRNA and protein increased as the concentration of eCG in IVM medium was raised. The highest expression levels were observed in group eCG-4. These findings demonstrate that eCG can enhance expression of these receptors at the mRNA and protein level in sheep COCs, and are consistent with previous reports (Uslu et al., 2012; Hashim et al., 2013; Xiao et al., 2014).

Early reports described a positive association between doses of prostaglandin analogs and the percentage of ewes in estrus. Our previous study indicated that eCG dose demonstrates a significant positive correlation with estrus and lambing rates in Lanzhou fat-tailed sheep (Wei et al., 2016a). However, to date, no study describing the quantitative relationship between eCG and oocyte IVM in humans or animals has been performed.

The Pearson correlation analysis conducted in the present investigation demonstrated that eCG dose was positively correlated with mRNA and protein levels of FSHR, LHR, and GnRHR. FSHR, LHR, and GnRHR mRNA levels positively correlated with expression of the corresponding proteins in sheep oocytes. This shows that increased levels of these receptors can promote sheep oocyte IVM. These results are similar to our earlier findings using mice (Wei et al., 2016b), and need to be further verified in other animals.

In conclusion, eCG can increase the maturation rate of oocytes undergoing IVM, decrease their apoptosis rate, and enhance the mRNA levels and expression of FSHR, LHR, and GnRHR in sheep COCs. The optimal eCG concentration for sheep oocyte IVM was found to be 20 μg/mL. Augmenting the expression of FSHR, LHR, and GnRHR may promote IVM of such cells. The results of the current study contribute to a better understanding of the mechanism behind hormonal control of sheep oocyte IVM and the development of ovine reproductive methods. This will ultimately enable us to overcome many problems related to abnormal reproductive status, and to treat follicular and ovarian diseases of sheep.

Conflicts of interest

The authors declare no conflict of interest.

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